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METHODS FOR DETECTING ABNORMAL ADAPTATION TO PROTEIN RESTRICTION IN HUMANS WITH SPECIAL REFERENCE TO INSULIN-DEPENDENT DIABETES MELLITUS

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ABSTRACT

Postprandial urea production in subjects with insulin dependent diabetes mellitus (IDDM) on conventional insulin therapy is normal when the previous diet is high in protein, but there is an incomplete adaptive reduction in urea production following protein restriction. To evaluate the nutritional implications of restricted protein intake in human diabetes mellitus, it is first necessary to establish a reliable method to measure changes in urea production and amino acid catabolism in response to changes in dietary protein intake. We therefore tested 1) the accuracy of the urea production rate (Ra) to depict changes in urea production, 2) whether sulfate production can be accurately depicted using tracer or nontracer approaches, after establishing the use of electrospray tandem mass spectrometry to measure sulfate concentrations and $^{34}SO_4$ enrichments following administration of the stable isotope tracer sodium [^{34}S]sulfate, 3) the reproducibility of urea and sulfate measurements following a test meal low in protein (0.25 g/kg) in subjects previously adapted to high (1.5 g/kg.d) and low (0.3 g/kg.d) protein intakes, and compared the metabolic fate of [^{15}N]alanine added to the test meal with that of [^{15}N]*Spirulina platensis*, a ^{15}N-labeled intact protein, and 4) whether we could identify the differences in postprandial urea and sulfate productions between normal subjects and persons with IDDM receiving conventional insulin therapy previously adapted to high protein intake, when the test meal was limiting in protein. Under basal conditions, steady state urea Ra is an accurate measure of urea production. Following changes in urea production, both the tracer and nontracer methods seriously

underestimated total urea Ra. The tracer method overestimated sulfate production by 20%, but the nontracer method provided an accurate measure of sulfate production and, hence, sulfur amino acid catabolism. Postprandial changes in urea and sulfate productions following normal adaptation to protein restriction are reproducible on repeat testing over a collection period of 6 h. [^{15}N]alanine in a test meal provides information about adaptive changes in dietary amino acid catabolism equivalent to provision of a ^{15}N-labeled intact protein tracer. Using a test meal limiting in protein, we conclude that whole body protein economy in persons with IDDM previously adapted to high protein intake is similar to that of normals. We have validated the use of reliable and practical methods to depict short term postprandial changes in whole body amino acid catabolism to be used in the clinical setting.

RÉSUMÉ

La production d'urée suivant un repas chez les personnes souffrant de diabète de type 1 et suivant l'insulinothérapie classique est normale quand la diète est riche en protéines, mais la réduction en production d'urée suivant l'adaptation à un apport protéique faible est incomplète. Afin d'évaluer les implications nutritionelles d'un apport protéique faible chez les personnes souffrant de diabète, il est premièrement nécessaire d'établir une méthode sûre pour mesurer les changements dans la production d'urée et dans le catabolisme des acides aminés en réponse aux variations de rations de protéines diététiques. On a donc étudié 1) la justesse du taux de production d'urée (Ra) à prédire les variations en production d'urée, 2) si la production de sulfate peut être determinée de façon précise en utilisant les méthodes de traceurs ou de non-traceurs, après avoir établit que le spectrometrie de masse "tandem electrospray" peut mesurer les concentrations de sulfate et les enrichissements de $^{34}SO_4$ suivant l'administration de traceur d'isotope stable le [^{34}S]sulfate de sodium, 3) la reproductibilité des mesures d'urée et de sulfate après un repas-test faible en protéines (0.25 g de protéine/kg poids corporel) chez des sujets qui ont été préalablement adaptés à un apport protéique élevé (1.5 g de protéine/kg poids corporel) et faible (0.3 g de protéine/kg poids corporel), et nous avons comparé le parcours métabolique de l'[^{15}N]alanine ajoutée au repas-test avec celui du [^{15}N]*Spirulina platensis*, une protéine intacte marquée en ^{15}N, et 4) si nous pouvons identifier les différences en production d'urée et de sulfate suivant un repas entre les sujets normaux et les personnes souffrant de diabète de type 1 suivant

l'insulinothérapie classique précédemment adaptés à un apport élevé en protéines quand le repas-test était faible en protéines. Dans des conditions stables, le Ra d'urée est une mesure précise de production d'urée. Suivant des variations en production d'urée, les deux méthodes, de traceurs et de non-traceurs, ont sérieusement sous-estimé le Ra d'urée total. La méthode de traceurs a surestimé la production de sulfate de 20%, mais la méthode de non-traceurs a fourni une mesure précise de production de sulfate et, donc, du catabolisme des acides aminés sulfurés. Les variations en production d'urée et de sulfate après un repas à la suite d'une adaptation normale à un apport protéique faible sont reproduites à maintes reprises pour une période de collecte de 6 hres. L'addition de l'[^{15}N]alanine dans le repas-test fournit des informations à l'égard des variations adaptives du catabolisme des acides aminés diététiques équivalentes à celles fournies par l'ajout d'une protéine intacte marquée en ^{15}N. En utilisant un repas-test faible en protéines, nous concluons que l'économie de protéines corporelles chez les personnes souffrant de diabète de type 1 précédemment adaptées à un apport élevé en protéines est semblable à celle des sujets normaux. Nous avons validé des méthodes sûres et pratiques qu'on peut utiliser dans un cadre clinique pour évaluer les variations à court terme dans le catabolisme des acides aminés corporels à la suite d'un repas.

In the name of Allah, Most Gracious, Most Merciful

"What is with you must vanish: what is with Allah will endure. And We will certainly bestow, on those who patiently persevere, their reward according to the best of their actions.

Whoever works righteousness, man or woman, and has Faith, verily, to him will We give a new Life, and life that is good and pure, and We will bestow on such their reward according to the best of their actions."

The translation of the meaning of surah 16:96-97 of the Holy Qur'an.

I dedicate this thesis to...

my mother, Bahie Khodir, my pillar and backbone

and...

Mazen Jamal Hamadeh, an interesting concept!

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ORIGINAL SCHOLARSHIP AND ADVANCEMENT OF KNOWLEDGE

This thesis investigates the use of sulfate production, as an alternative or adjunct to urea production, as an accurate measure of whole-body protein catabolism in subjects adapted to a high or low protein intake. In addition, we validated the use of the stable isotope $^{34}SO_4$ to measure sulfate space and determined it to be 80% that of the corrected bromide space, both estimates of the extracellular fluid volume(ECF). Moreover, we determined that adding tracer amounts of [^{15}N]alanine provides information about adaptive changes in dietary amino acid catabolism. The results from this thesis work have contributed to knowledge in the fields of amino acid and protein metabolism and clinical research by:

i) demonstrating that urea production, a measure of whole-body amino acid catabolism, cannot be accurately measured by the isotopic methodology and the nontracer technique when changes in urea production occur for periods as long as 8 h.

ii) validating the use of the nontracer method to accurately measure changes in sulfate production.

iii) showing that sulfate excretion is a convenient and noninvasive measure of sulfate production following a mixed test meal in normal subjects.

iv) demonstrating that sulfate production rapidly adapts to changes in sulfur amino acid intake, is a measure of whole-body sulfur amino acid catabolism and postprandial protein oxidation, and is an adjunct to urea in elucidating specific amino acid retention and postprandial whole-body protein conservation.

v) demonstrating that the extent of the transfer of ^{15}N added to a mixed test meal as [^{15}N]alanine to urea is an accurate measure of postprandial dietary protein conservation.

vi) demonstrating the use of electrospray tandem mass spectrometry to measure both sulfate concentrations and isotopic enrichments in serum and urine.

vii) using the stable isotope sodium [^{34}S]sulfate to measure the sulfate space and comparing the results with the simultaneously determined corrected bromide space (CBS), and determining the CBS to provide a more accurate measure of the extracellular fluid space (ECF).

viii) confirming previous results from this laboratory that persons with insulin-dependent diabetes mellitus exhibit normal body protein economy when adapted to standard high protein intake using the new independent sulfate methodology.

ix) establishing accurate and less invasive protocols that could be carried out in future clinical studies:

- 6-h collection periods give accurate results

- the ECF could be estimated as 0.20 of body weight for the purpose of measuring sulfate production

- correction for changes in ECF sulfate is not needed to measure sulfate production following consumption of mixed test meals in normal subjects

- test meals containing 0.25 g protein/kg could detect changes in postprandial dietary protein conservation as well as test meals with higher protein content but could be more useful in detecting subtle changes in metabolic dysregulation

- adaptation following short-term (4 d) protein restriction (0.3 g protein/kg body weight.d), though not complete, is significant.

CONTRIBUTION OF CO-AUTHORS TO MANUSCRIPTS

This thesis consists of six manuscripts. For manuscripts 1 and 3-6 (chapters 3 and 5-8) and under the supervision of Dr. L. John Hoffer, the candidate was responsible for planning and designing the studies, conducting pilot studies, recruiting the subjects, carrying out the clinical studies on both healthy and diabetic subjects, analyzing the samples in the laboratory, gathering and organizing the data, and performing calculations and statistical analyses. The candidate was responsible for writing manuscripts 1 and 3-6 under the close guidance of Dr. Hoffer.

For manuscript 2 (chapter 4), the candidate was responsible for carrying out the clinical studies, performing statistical analysis, and was involved in purification techniques, some of the injections on ESI-MS/MS, and running samples on the IEC-CD. Under the supervision of Dr. Mamer and in collaboration with Dr. Hoffer and Line Robitaille, Dr. Boismenu developed the new ESI-MS/MS methodology to measure $^{34}SO_4$. Line Robitaille was involved in all the laboratory techniques and the methodology for the $^{34}SO_4$ analysis. Dr. Boismenu, Dr. Mamer, Dr. Hoffer, Line Robitaille and the candidate were involved in writing up the manuscript. Dr. Hongsprabhas helped with the clinical studies and laboratory techniques. All the above was conducted under the close guidance and supervision of Dr. Hoffer, who was the originator of the project.

For manuscript 5 (chapter 7), Dr. Alicia Schiffrin helped with recruiting diabetic subjects and designed the insulin and glucose monitoring strategies.

RESEARCH PUBLICATIONS IN REFEREED JOURNALS

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LIST OF ABBREVIATIONS

[^{15}N]ALA	[^{15}N]alanine
[^{15}N]SPI	[^{15}N]*Spirulina platensis*
ANOVA	Analysis of variance
AUC	Area-under-the-curve
BMI	Body mass index
CBS	Corrected bromide space
DM	Diabetes mellitus
ECF	Extracellular fluid volume
ESI-MS/MS	Electrospray tandem mass spectrometry
FFM	Fat-free mass
GCMS	Gas chromatogrpahy-mass spectrometry
HP	High protein
IDDM	Insulin-dependent diabetes mellitus
IEC-CD	Ion exchange chromatography with conductivity detection
IRMS	Isotope ratio-mass spectrometry
i.v.	Intravenous
ln	Natural logarithm
LP	Low protein
NPU	Net protein utilization

Ra	Rate of appearance
SAA	Sulfur amino acids
SD	Standard deviation
SEM	Standard error of the mean
SIM	Selected ion monitoring
SS	Sulfate space
TBDMS	Tert-butyldimethylsilyl
TBW	Total body water
TTR	Tracer to tracee ratio
Wt	Body weight

CHAPTER I

Introduction

PROJECT SUMMARY AND RATIONALE

Diabetes mellitus (DM) is a common disease in Canada and the major cause of chronic renal failure. Dietary protein restriction could reduce or delay the progression of renal failure, however persons with insulin dependent diabetes mellitus (IDDM) would be at risk of developing protein malnutrition if they were unable to reduce their rate of amino acid catabolism to the level necessary to maintain protein balance (Hoffer, 1993; Hoffer 1998). This is an important consideration, since protein turnover and amino acid catabolism are increased when persons with IDDM lack sufficient insulin and remain abnormal even during standard insulin treatment (Hoffer et al, 1997). It is therefore important to determine under which conditions of diabetic control DM persons can or cannot adapt normally to decreased protein intake.

It is largely during the fed state period that the body allocates amino acids either to be used for protein synthesis or catabolized. Consequently, it would be useful to establish accurate and reliable methods to measure postprandial changes in urea production and amino acid catabolism simple enough to be applied in the clinical setting. We therefore undertook studies to develop short-term simple but accurate protocols for measuring postprandial amino acid catabolism suitable for clinical investigation.

Urea appearance rates (Ra) have been used to assess short-term amino acid catabolism (Vilstrup, 1980). However, there is a lack of consensus amongst researchers about the accuracy of using tracer methodology to accurately depict short-term changes in urea kinetics (Jahoor and Wolfe, 1987; Matthews and Downey, 1984). We therefore investigated the accuracy of estimating known short-term steady-state urea Ra and the ability of the tracer method to depict acute changes in urea production by the tracer

(Tserng and Kalhan, 1983) and nonisotopic methods under conditions reproducing postprandial changes in urea production after one meal or nibbling for 8 hours.

We were also interested in using sulfate as a potential adjunct to urea in depicting whole body postprandial amino acid catabolism. Sulfate excretion closely matches urea excretion when measured over 24-h periods (Jourdan et al, 1980; Lakshmanan et al, 1976; Zlotkin and Anderson, 1982). Its short term in vivo kinetics could prove to be simpler and easier to trace in the short term than those of urea (Bauer, 1976; Cheema-Dhadli and Halperin, 1993). To establish and validate a method to calculate postprandial sulfate production as a measure of SAA catabolism, we investigated the accuracy of measuring sulfate production under basal conditions using both the tracer and nonisotopic methods and of estimating short-term changes in sulfate production using the nonisotopic method following intravenous administration of sulfate, oral methionine, and intravenous administration of a mixture of essential amino acids containing methionine.

In earlier research from this laboratory, a 0.5 g/kg protein test meal was used to demonstrate a reduction in postprandial cumulative urea production following adaptation to protein restriction in normal subjects (Hoffer et al, 1997). A tracer dose of [^{15}N]alanine was included in test meals and the extent of transfer of ^{15}N into urea was used as a measure of postprandial first pass amino acid oxidation. Though this high protein test meal was able to detect changes in the efficiency to retain dietary protein in the fed state following adaptation to dietary protein restriction, its sensitivity for detecting subtle deficits in the efficiency of amino acid uptake into protein could be questioned, since this test meal provided a surfeit amount of protein. We therefore evaluated whether a test meal limiting in dietary protein (0.25 g protein/kg) could impose

a requirement on the body to efficiently conserve the amino acids in the test meal. Although rapidly transaminated (Battezzati et al, 1999), [^{15}N]alanine may not represent the extent to which absorbed dietary amino acids in general are immediately retained for protein synthesis or transaminated and oxidized, and their N converted to urea. In order to validate the use of [^{15}N]alanine as a measure of first pass dietary amino acid metabolism, we compared the results of feeding our subjects a mixed test meal containing either [^{15}N]alanine or [^{15}N]*Spirulina platensis*, a ^{15}N-labeled intact protein.

Finally, we investigated whether a test meal limiting in protein (0.25 g/kg body weight) would identify the differences in protein catabolism between normal and IDDM subjects previously adapted to high protein intake using urea production, sulfate production, and the transfer of ^{15}N added to the test meal as [^{15}N]alanine to urea.

THESIS OBJECTIVES

The overall objective was to establish and validate the use of reliable methods to depict fed-state short-term changes in whole body amino acid catabolism under different dietary protein intakes, and to use the validated reliable methods to compare postprandial amino acid catabolism in IDDM and normal subjects. The specific objectives were:

a) To investigate the accuracy of estimating short-term acute changes in urea production by both the tracer and nonisotopic methods by means of intravenous urea injections that mimic postprandial urea production patterns.

b) To develop a method using electrospray tandem mass spectrometry to measure the concentration and enrichment of the stable isotope $^{34}SO_4$ in biological fluids.

c) To determine the use of stable isotope sodium [^{34}S]sulfate to measure sulfate space and compare it to the simultaneously measured corrected bromide space, both measures of the extracellular fluid volume.

d) To validate the use of sulfate production, measured by both the tracer and nonisotopic methods, in depicting short-term changes in sulfate production and whole body SAA catabolism.

e) To validate the use of sulfate production, as a potential adjunct to urea, in depicting whole body postprandial amino acid catabolism.

f) To investigate whether the extent of the transfer of ^{15}N added to a mixed test meal as [^{15}N]alanine to urea is an accurate measure of postprandial dietary protein conservation following protein restriction in normal subjects.

g) To investigate the use of a test meal low in protein (0.25 g protein/kg.body weight) to detect changes in postprandial dietary protein conservation following protein restriction in normal subjects.

h) To test whether a test meal limiting in protein (0.25 g/kg body weight) would identify the differences in protein catabolism between normal and IDDM subjects previously adapted to a high protein intake using urea production, sulfate production, and the transfer of ^{15}N added to the test meal as [^{15}N]alanine to urea.

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CHAPTER II

Review of the Literature

and Thesis Overview

PROTEIN METABOLISM IN NORMAL ADULTS AND PERSONS WITH INSULIN DEPENDENT DIABETES MELLITUS

Normal adaptation to dietary protein manipulations occur through changes in whole-body and peripheral protein and amino acid metabolism (Brodsky and Devlin, 1996; Hoffer et al, 1997; Motil et al, 1996; Motil et al, 1981; Tom et al, 1995). Following protein restriction, whole body protein turnover and breakdown and whole body amino acid oxidation decrease (Motil et al, 1981). However, the effect of protein restriction on whole body protein synthesis rate is more variable (Brodsky and Devlin, 1996; Motil et al, 1981). The end result of these adaptations to dietary protein restriction is a decrease in urinary total and urea N excretion and reestablishment of neutral N balance (Brodsky and Devlin, 1996; Tom et al, 1995).

In contrast, persons with insulin dependent diabetes mellitus (IDDM) in poor glycemic control have an increased rate of protein synthesis and breakdown and amino acid oxidation in the postabsorptive state (Nair et al, 1983). However, protein breakdown is increased more than protein synthesis resulting in net protein loss. Insulin replacement decreases all three measures (Nair et al, 1995).

Dietary protein restriction could reduce or delay the progression of renal failure in diabetic subjects (Barsotti et al, 1988; Ciavarella et al, 1987; Dullaart et al, 1993; Walker et al, 1989; Zeller et al, 1991), especially if the decrease in amino acid oxidation and postprandial protein degradation and the neutral nitrogen balance achieved with dietary protein restriction were sustained over the long term as seen in nondiabetic subjects (Tom et al, 1995). Following adaptation to protein restriction, persons with IDDM on intensive insulin treatment show normal decrease in whole body protein breakdown and amino

acid oxidation in the postabsorptive and fed states (Brodsky and Devlin, 1996; Hoffer et al, 1997; Larivière et al, 1992).

However, small variations from strict intensive insulin treatment, hence deviations from strict euglycemia, are correlated with increased daily urinary obligatory N during dietary protein restriction (Larivière et al, 1992). Moreover, IDDM subjects on conventional insulin treatment show impaired postprandial adaptation to protein restriction (Hoffer et al, 1997; Brodsky et al, 1992). Consequently, IDDM patients would be at risk of developing protein malnutrition if dietary protein is restricted and if they were unable to reduce their rate of amino acid catabolism to the level necessary to maintain protein balance (Brodsky et al, 1992; Hoffer, 1993; Hoffer 1998).

Under North American dietary conditions of ample protein intake, persons with IDDM consume enough protein to offset the increase in net whole body amino acid oxidation and the decrease in the efficiency to retain dietary amino acids. However, under conditions of dietary protein restriction, persons with IDDM would be at risk of protein deficiency if they are unable to successfully adapt to conditions conducive to increasing the efficiency to retain dietary amino acids. Therefore, it is possible that the minimum dietary protein requirement in IDDM is higher than normal. Consequently, the recommended protein intake, which is based on the minimum requirement with correction for between-subject variability, would be higher for persons with IDDM than for non-diabetic subjects.

The aim of this thesis was to develop and validate sensitive and robust methods that can detect abnormal efficiency to retain dietary amino acids normally masked by high protein intake, and that are convenient and simple enough to be used in the clinical

setting. To do this, we, first, developed and studied clinical tools for measuring postprandial whole body amino acid catabolism in normal subjects prior to and following adaptation to protein restriction, and, second, we tested whether a test meal low in protein (0.25 g protein/kg body weight) can detect successful adaptation to protein restriction in normal subjects. Also, we measured postprandial response following a test meal low in protein in persons with IDDM on conventional insulin therapy previously adapted to high protein intake and compared the results with those for normal subjects.

FED STATE

It is largely during the fed state period that the body allocates amino acids to the different metabolic pathways (Berthold et al, 1995; Biolo et al, 1992; Cayol et al, 1996; Cortiella et al, 1988; Hoerr et al, 1991; Hoerr et al, 1993; Motil et al, 1981; Stoll et al, 1998a; Stoll et al, 1998b). They are either used for protein synthesis or catabolized to urea, sulfate, carbon dioxide, and the carbon skeleton of the amino acids converted either to glucose or fatty acids (Crim and Munro, 1994). Indeed, a significant amount (25-76%) of dietary essential amino acids are metabolized during first pass through the splanchnic bed, with amino acids of dietary origin being preferentially used for protein synthesis in the fed state (Berthold et al, 1995; Biolo et al, 1992; Cayol et al, 1996; Cortiella et al, 1988; Hoerr et al, 1991; Hoerr et al, 1993; Stoll et al, 1998a; Stoll et al, 1998b). Postabsorptively, the body calls upon these stores for maintenance and growth. It is thus appropriate to carry out studies in the fed state in order to investigate the efficiency of the body to retain amino acids.

To evaluate the nutritional implications of protein restriction in human diabetes

mellitus, we need to establish accurate and reliable methods to measure postprandial changes in amino acid catabolism in humans that are simple enough to be applied in the clinical setting.

There is no fully satisfactory method for measuring amino acid catabolism in the non-steady state that follows normal protein consumption. Both urea production and tracer-determined amino acid oxidation are commonly used to measure whole-body amino acid catabolism over short periods of time (Beaumier et al, 1995; Carraro et al, 1993; El-Khoury et al, 1994; Ensinger et al, 1994; Quevedo et al, 1994; Wolfe et al, 1987; Young et al, 2000). However, the amino acid oxidation technique requires an accurate measurement of expired tracer carbon dioxide, and tracer-based methods for measuring short-term changes in urea production may not be reliably depicted (Jahoor and Wolfe, 1987; Matthews and Downey, 1984). In this thesis, we consider sulfate production as a potentially useful adjunct to urea production and essential amino acid oxidation when measuring whole-body amino acid catabolism.

UREA PRODUCTION

Urea is the predominant nitrogenous product in urine, accounting for more than 80% of urinary N on a normal diet (Cheema-Dhadli and Halperin, 1993). Amino acid catabolism is the main determinant of the rate of urea synthesis in the normal liver (Halperin et al, 1986). Thus, urea production and amino acid oxidation fluctuate concomitantly (El-Khoury et al, 1994; Giesecke et al, 1989; Motil et al, 1996; Motil et al, 1994; Steffee et al, 1981; Young et al, 2000). Oral and intravenous administration of protein or the urea precursor alanine cause a dose-dependent increase in urea production

(Kay et al, 1986; Rafoth and Onstad, 1975). Not surprisingly then, urea appearance rates are measured to assess amino acid catabolism (Vilstrup, 1980).

Urea is distributed in total body water (TBW), which constitutes on average 60% of total body, including intestinal water, where it is subject to the action of bacterial urease and the subsequent partial recycling of the resulting ammonia for new urea synthesis (Jackson et al, 1984; Jones et al, 1968; Long et al, 1978; Walser and Bodenlos, 1959). Because of this, along with losses from perspiration and saliva, urinary excretion of urea corrected for changes in TBW (the nonisotopic method) underestimates true production by 15-30% (El-Khoury et al, 1996; El-Khoury et al, 1994; Jackson et al, 1984; Jones et al, 1969; Jones et al, 1968; Long et al, 1978; Walser and Bodenlos, 1959). As well, plasma urea concentration tends to change under basal and fed conditions, and since body water is a large pool, small analytical errors in the plasma urea measurement are magnified when multiplied by TBW. This could lead to large errors in apparent urea retention or excretion. To overcome this shortcoming, tracer methods were developed to accurately measure true urea production in humans and animals (Jahoor and Wolfe, 1987; Matthews and Downey, 1984; Rypins et al, 1980).

Several researchers attempted to validate the primed continuous tracer infusion method of calculating urea turnover rates (Jahoor and Wolfe, 1987; Matthews and Downey, 1984; Wolfe, 1981). Matthews and Downey (1984) supported long-term (20-60 h) urea tracer infusions and single urea tracer doses for measuring urea turnover rates. Though the administration of the proper priming dose would ensure accurate results, under- or over-priming could give an 'apparent' isotopic steady state while, in fact, the levels of plasma tracer enrichment would still be changing, albeit very slowly. They

slowly. They further argued that this gradual change in enrichment could not be distinguished from the normal variance of the gas chromatography mass spectrometry measurement over a short-duration infusion (2-4 h). Compounded by the effect a large urea pool size and a slow pool turnover rate, approximately 9%/hour, could have in moderating any change in urea enrichment, they could not trust the use of primed continuous short duration infusions for detecting short-term changes in urea kinetics.

Jahoor and Wolfe (1987), on the other hand, contended that with a proper prime/continuous infusion ratio a true isotopic steady state was reached in all subjects in 2-4 h and was maintained throughout the duration of the continuous infusion. They also claimed to have reached a new isotopic steady state 2 h after a concomitant continuous non-primed infusion of alanine.

Due to this lack of consensus about the accuracy of the tracer method to measure short-term changes in urea production (Jahoor and Wolfe, 1987; Matthews and Downey, 1984), it is important to assess the validity of the steady-state urea turnover model using the primed continuous infusion method under basal conditions and to investigate the accuracy of estimating short-term changes in urea production, as might occur in the normal postprandial situation, by both the tracer dilution and nonisotopic methods.

SULFATE PRODUCTION

Sulfate is the major end-product of the sulfur amino acids (SAA), methionine, cysteine and cystine. Other products of SAA oxidation are mercaptolactate, mercaptoacetate, thiocyanate, thiosulfate and taurine, but they make only a small contribution to the total (Laidlaw et al, 1988; Martensson, 1982; Martensson and

Hermansson, 1984; Stipanuk, 1999).

Mean postabsorptive serum sulfate concentrations are 290-360 μmol/L in the adult, with no gender differences (Cheema-Dhadli and Halperin, 1993; Cole and Scriver, 1980; Hoffman et al, 1990). Serum sulfate concentrations increase following protein consumption (Cheema-Dhadli and Halperin, 1993; Cole et al, 1991) and decrease following administration of drugs (Hoffman et al, 1990; Morris and Levy, 1983). Sulfate is conjugated with endogenously produced compounds, xenobiotics, and drugs to render them more water soluble for excretion in urine or bile, PAPS (adenosine 3'-phosphate-5'-phosphosulfate) being the active sulfate donor. This is evident from the decrease in serum sulfate concentrations and the increase in sulfo-conjugated products upon oral administration of acetaminophen, tyramine, or salicylamide (Greiling and Schuler, 1963; Hendrix-Treacy et al, 1986; Hoffman et al, 1990; Levy and Matsuzawa, 1967; Smith and Mitchell, 1974).

Sherman and Hawk (1900) showed that postprandial sulfate and nitrogen excretions follow a similar pattern, increasing following the midday and evening meals and decreasing overnight. However, Cheema-Dhadli and Halperin (1993) concluded that, relative to N, S from dietary SAA is retained during feeding hours (day time) and excreted as sulfate overnight. Also, as suggested by the N/S molar ratio, S of dietary source is more retained than N in humans and dogs when habitual protein levels are reduced and upon repletion following N depletion (Bressani et al, 1965; Cheema-Dhadli and Halperin, 1993; Fay and Mendel, 1926; Wilson, 1925). This is most likely due to the retention of dietary S in intracellular non-protein compounds, such as glutathione, an important cysteine reservoir (Cho et al, 1984; Cho et al, 1981; Fukagawa et al, 1996;

Garcia and Stipanuk, 1992; Higashi et al, 1977; Tateishi et al, 1981; Tateishi et al, 1977).

In humans, 24-h urinary sulfate excretion closely matches dietary SAA intake (r = 0.66-0.99; Sabry et al, 1965; Zlotkin and Anderson, 1982), N excretion (r = 0.71-0.98; Jourdan et al, 1980; Lakshmanan et al, 1976; Wright et al, 1960; Zlotkin and Anderson, 1982), and urea-N excretion (r = 0.99; Jourdan et al, 1980). Furthermore, other researchers have found a good correlation between urinary sulfate and dietary protein (Miller and Mumford, 1964) and N intake (Jourdan et al, 1980; Wright et al, 1960). However, it has not been determined whether sulfate production can be measured accurately over periods of only a few hours, such as those following a typical meal. Short-term sulfate kinetics might be more accurately determined than those of urea (Bauer, 1976; Cheema-Dhadli and Halperin, 1993). Sulfate is excreted through the renal route, but unlike urea, which distributes throughout total body water, it distributes in a smaller body compartment, the extracellular fluid volume (ECF), and is not subject to important losses in the gut (Bauer, 1976; Chakmakjian and Bethune, 1966; Cocchetto and Levy, 1981; Omvik et al, 1979; Ryan et al, 1956; Walser and Bodenlos, 1959). Therefore, sulfate production is a potentially useful adjunct to urea production and essential amino acid oxidation when measuring whole-body amino acid catabolism.

TEST MEAL

Normal adaptation to dietary protein restriction requires an appropriate reduction in dietary and endogenous amino acid oxidation and urea production. Healthy subjects adapted to a high protein intake show a significant reduction in postprandial cumulative urea production and an improved metabolic nitrogen balance following adaptation to

protein restriction (Hoffer et al, 1997). This suggests an increase in whole-body protein conservation. However, persons with IDDM on conventional insulin therapy showed an impaired adaptation following protein restriction (Hoffer et al, 1997).

The test meal used in these studies was high in protein (0.50 g protein/kg body weight), hence not limiting. This high protein test meal detected changes in the efficiency to retain dietary protein in the fed state following adaptation to dietary protein restriction, however it could fail to detect subtle differences in the fine control of dietary protein found in persons with diabetes mellitus consuming a typical North American diet high in protein. This could be accomplished using a test meal more limiting in dietary protein (0.25 g protein/kg body weight), which will impose a requirement on the body to efficiently conserve the amino acids in the test meal. To do this, we investigated whole-body protein economy by measuring postprandial cumulative urea and sulfate production, metabolic N and S balance, net protein utilization and SAA retention following consumption of a low protein test meal in healthy subjects prior to and following adaptation to protein restriction.

Hoffer et al (1997) included tracer [^{15}N]alanine in test meals and used the extent of transfer of ^{15}N into urea as a measure of postprandial first pass amino acid oxidation. Following adaptation to protein restriction, healthy and IDDM subjects showed a significant reduction in the transfer of ^{15}N added to the test meal as [^{15}N]alanine into urea. Recent data in piglets strongly suggest that first-pass amino acid metabolism is an important determinant of fed-state protein synthesis (Stoll et al, 1998a; Stoll et al, 1998b). In humans, dietary alanine is rapidly transaminated (Battezzati et al, 1999), however tracer [^{15}N]alanine is not protein bound and its metabolism may not represent

the extent to which dietary amino acids in general, as constituents of whole dietary proteins, are immediately retained for protein synthesis or transaminated and oxidized, and their N converted to urea, upon their first passage through the splanchnic bed. The use of tracer [^{15}N]alanine in test meals to measure first pass dietary amino acid metabolism needs to be validated. This could be accomplished by comparing the transfer of ^{15}N from dietary [^{15}N]alanine into urea with that of [^{15}N]*Spirulina platensis*, a ^{15}N-labeled intact protein, and the extent reduction in this transfer between the two treatments following adaptation to protein restriction.

Postprandial urea production, metabolic N balance, and net protein utilization (NPU) in persons with IDDM receiving conventional insulin therapy are normal when the previous diet is high in protein (Hoffer et al, 1997). The test meal used was high in protein (0.50 g/kg body weight), hence it provided a surfeit amount of protein. However, conventionally treated IDDM subjects consuming high protein diets could show a reduced ability to conserve dietary amino acids if the test meal was low in protein. In this thesis, we investigate whether a test meal limiting in protein (0.25 g/kg body weight) would identify the differences in protein catabolism between normal and IDDM subjects previously adapted to high protein intake using urea production, sulfate production, metabolic N and S balance, and the transfer of ^{15}N added to the test meal as [^{15}N]alanine into urea.

THESIS OVERVIEW

Dietary protein restriction could reduce or delay the progression of renal failure in diabetes, but tracer studies carried out in recent years have shown that protein turnover

and amino acid catabolism remain abnormal even during standard insulin treatment of insulin dependent diabetes mellitus (IDDM) (Hoffer et al, 1997). IDDM patients would be at risk of developing protein malnutrition if dietary protein is restricted and if they were unable to reduce their rate of amino acid catabolism to the level necessary to maintain protein balance (Brodsky et al, 1992; Hoffer, 1993; Hoffer 1998).

During the fed state period, the body allocates the absorbed amino acids to be either used for protein synthesis or catabolized. It is thus appropriate to carry out studies in the fed state in order to investigate the efficiency of the body to retain amino acids. However, we need first to establish accurate and reliable methods to measure postprandial changes in amino acid catabolism in humans.

Urea production rate (Ra) is commonly used to measure short-term whole-body amino acid catabolism (Beaumier et al, 1995; Carraro et al, 1993; El-Khoury et al, 1996; El-Khoury et al, 1994; Ensinger et al, 1994; Quevedo et al, 1994; Wolfe et al, 1982; Wolfe et al, 1987). However, urinary urea excretion corrected for changes in urea concentration in TBW underestimates true urea production and the tracer-based methods may not reliably depict short-term changes in urea kinetics (Jahoor and Wolfe, 1987; Matthews and Downey, 1984). The purpose of the first study (chapter 3) was to investigate the accuracy of estimating known short-term steady-state urea Ra and the ability of the tracer method to depict acute changes in urea production by both the tracer (Tsering and Kalhan, 1983) and nonisotopic methods under conditions reproducing postprandial changes in urea production after one meal (Pulse) or nibbling for 8 hours (Step). The tracer method involves primed continuous infusion of [^{13}C]urea at a known rate for 12 h and measuring plasma tracer dilution upon the infusion of unlabeled urea or

alanine. The nonisotopic method derives the rate of urea appearance from measuring urinary urea excretion rate corrected for changes in total body water urea.

We also attempted to establish and validate a method to calculate postprandial sulfate production as a measure of sulfur amino acid (SAA) catabolism (chapter 6). Nonisotopic sulfate production is measured by calculating urinary excretion corrected for changes in sulfate concentrations in ECF, which is best approximated by the sulfate space (SS) as measured by $^{35}SO_4$ isotope dilution (Bauer 1976; Bauer et al. 1975; Kragelund and Dyrbye 1967; Lacroix et al. 1965; Malpartida and Moncloa 1967; Omvik et al. 1979; Pierson et al. 1982; Ryan et al. 1956; Walser et al. 1953). However, the radioactivity and short half-life of ^{35}S limit its usefulness. In chapters 4 and 5, we describe a method to measure serum sulfate concentrations and $^{34}SO_4$ enrichments using electrospray tandem mass spectrometry (ESI-MS/MS) and the use of the orally administered stable isotope sodium [^{34}S]sulfate to determine the SS. We also investigated the accuracy of measuring sulfate production under basal conditions using both the nonisotopic and tracer methods following primed continuous oral administration of the tracer sodium [^{34}S]sulfate, and of estimating short-term changes in sulfate production using the nonisotopic method following intravenous administration of sulfate, oral methionine, and intravenous administration of a mixture of essential amino acids including methionine (chapter 6).

Previously, Hoffer et al (1997) used a 0.5 g/kg protein test meal to show a reduction in postprandial cumulative urea production following adaptation to protein restriction in normal subjects. However, postprandial urea production in IDDM subjects receiving conventional insulin therapy was normal when the previous diet was high in protein, but there was an incomplete adaptive reduction in urea production following

protein restriction. The test meal used was high in protein (0.50 g/kg body weight), hence not limiting. Though this high protein test meal was able to detect changes in the efficiency to retain dietary protein in the fed state following adaptation to dietary protein restriction, it could fail to detect subtle deficits in the efficiency to retain dietary protein between normal and IDDM subjects under typical North American dietary conditions. This could be accomplished using a test meal limiting in dietary protein (0.25 g protein/kg), which will impose a requirement on the body to efficiently conserve the amino acids in the test meal (chapters 7 and 8).

Moreover, Hoffer et al (1997) used the extent of transfer of ^{15}N added to the test meal as [^{15}N]alanine into urea as a measure of postprandial first pass amino acid oxidation. However, the metabolic fate of [^{15}N]alanine may not represent the extent to which dietary amino acids consumed are immediately retained for protein synthesis or transaminated and oxidized. Therefore, in order to validate the use of [^{15}N]alanine as a measure of first pass dietary amino acid metabolism, we compared the results of feeding our subjects a mixed test meal containing either [^{15}N]alanine or [^{15}N]*Spirulina platensis*, a ^{15}N-labeled intact protein (chapter 8).

Finally, we used a test meal limiting in protein (0.25 g/kg body weight) to test whether it would depict the differences in protein catabolism between normal and IDDM subjects previously adapted to high protein intake using urea production, sulfate production, and the transfer of ^{15}N added to the test meal as [^{15}N]alanine to urea (chapters 7 and 8).

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CHAPTER III

Tracer Methods Underestimate Short-term Variations in Urea Production in Humans

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Tracer Methods Underestimate Short-term Variations in Urea Production in Humans

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ABSTRACT

Urea production rate (Ra) is commonly measured using a primed continuous tracer urea infusion, but the accuracy of this method has not been clearly established in humans. We used intravenous infusions of unlabeled urea to assess the accuracy of this technique in normal, postabsorptive men under four different conditions: (1) tracer [^{13}C]urea was infused under basal conditions for 12 h (control); (2) tracer [^{13}C]urea was infused for 12 h, and unlabeled urea was infused from hours 4 to12 at a rate twofold greater than the endogenous Ra ("step" infusion); (3) tracer [^{13}C]urea was infused for 12 h, and unlabeled urea was infused from hours 4 to 8 ("pulse" infusion); and (4) tracer [^{13}C]urea was infused for 9 h, and unlabeled alanine was infused at a rate of 120 mg/kg.h (1.35 mmol/kg.h) from hours 4 to 9. Urea Ra was calculated using the isotopic steady-state equation (tracer infusion rate/tracer-to-tracee ratio), Steele's non-steady-state equation, and urinary urea excretion corrected for changes in total body urea. For each subject, endogenous urea Ra was measured at hour 4 of the basal condition, and the sum of this rate plus exogenous urea input was considered as "true urea input". Under control conditions, urea Ra at hour 4 was similar to that measured at hour 12. After 8-h step and 4-h pulse unlabeled urea infusions, Steele's non-steady-state equation underestimated true urea input by 22% (step) and 33% (pulse); whereas the nonisotopic method underestimated true urea by 28% (step) and 10% (pulse). Similar conclusions derived from the alanine infusion. These results indicate that, although Steele's non-steady-state equation and the nontracer method more accurately predict total urea Ra than the steady-state equation, they nevertheless seriously underestimate total urea Ra for as long as 8 h after a change in true urea Ra.

INTRODUCTION

Urea accounts for more than 80% of urinary N on a normal diet (Cheema-Dhadli and Halperin, 1993). The main determinant of the rate of urea synthesis in the normal liver is immediate amino acid catabolism (Halperin et al, 1986). Thus, urea production and amino acid oxidation fluctuate concomitantly (El-Khoury et al, 1994; Steffee et al, 1982), and orally and intravenously administered loads of protein or the urea precursor alanine cause a dose-dependent increase in urea production (Kay et al, 1986; Rafoth and Onstad, 1975). In addition to its urinary excretion, urea is distributed throughout total body water (TBW), including intestinal water, where it is subject to the action of bacterial urease. Because of this, urinary urea excretion, even when corrected for changes in body urea content, underestimates the actual urea synthetic rate by 20-30% according to most investigators (El-Khoury et al, 1994; Jones et al, 1968; Long et al, 1978; Walser and Bodenlos, 1959). This observation has led to the development of tracer methods to accurately measure true urea production in humans and animals (Jahoor and Wolfe, 1987; Matthews and Downey, 1984; Rypins et al, 1980).

Several researchers have evaluated the use of primed continuous tracer infusions for calculating urea turnover (Jahoor and Wolfe, 1987; Matthews and Downey, 1984; Wolfe, 1981). Jahoor and Wolfe (1987) reported that, after a proper priming dose, isotopic steady state is reached in 2-3 h. Others have pointed out, however, that under- or over-priming this slowly turning-over substrate pool could give rise to an "apparent" isotopic steady state when, in fact, the levels of plasma tracer enrichment would still be changing, albeit slowly (Matthews and Downey, 1984).

The present research was designed, first, to reassess the validity of the steady-

state urea turnover model using the primed continuous infusion method under basal conditions and, second, to investigate the accuracy of estimating short-term changes in urea production, as might occur in the normal postprandial situation, by both tracer dilution and the older nonisotopic method based on urinary urea excretion with a correction for changes in total body urea. To do this, we administered a primed continuous infusion of [^{13}C]urea and measured plasma tracer dilution during the infusion of unlabeled urea or of alanine, a precursor for urea synthesis.

METHODS

Subjects and Protocols

Eleven healthy men with normal blood biochemistries and taking no medications participated in four study protocols, each involving six separate infusions (Table 1). No subject was studied more than two times, and, when this occurred, at least one week separated infusion studies. All volunteers gave written consent for the study, which was approved by the Department of Medicine Human Ethics Committee of McGill University.

The subjects were admitted to the clinical research unit at 0900 h after an overnight fast. On this day, a low-protein breakfast (0930 h), lunch (1300 h), dinner (1800 h) and snack (2030 h) at maintenance energy were consumed to reduce and stabilize urea production by minimizing exogenous amino acid oxidation. Caloric intake was adjusted for individual basal energy expenditure as calculated using the Harris-Benedict equation multiplied by a factor of 1.7 to account for activity (Goran et al, 1993).

Table 1. Subject characteristics.

	Infusion			
	Control	Step	Pulse	Alanine
Age (y)	28.0 ± 2.5	27.7 ± 3.3	26.2 ± 3.5	33.3 ± 2.5
Weight (kg)	76.4 ± 2.8	73.8 ± 3.2	71.9 ± 3.0	72.5 ± 2.2
Height (cm)	181 ± 3	181 ± 2	180 ± 3	180 ± 2
BMI (kg/m^2)	23.4 ± 0.7	22.5 ± 0.9	22.0 ± 0.6	22.4 ± 0.7
FFM (kg)	61.7 ± 2.2	59.5 ± 1.5	58.4 ± 1.5	59.1 ± 1.5

Data are mean ± SEM, n = 6. BMI, body mass index; FFM, fat-free mass. Data between infusions were not significantly different, one-way ANOVA $P < 0.05$.

Proper hydration was maintained throughout the study period. The food consisted of protein-free muffins, low-protein wafers, butter and apple juice. Consumption of other foods and beverages was not permitted. Total energy intake was 42 kcal (176 kJ)/kg body weight (0.5% protein, 43% fat, and 56.5% carbohydrate). Infusion studies were carried out on the second morning with subjects in the postabsorptive state. Body weight was recorded, and TBW measured by bioimpedance analysis (BIA-101A; RJL Systems, Mt. Clemens, MI) (Kushner and Schoeller, 1986). No food was consumed for the duration of these studies.

In the *first protocol* (control), only tracer urea was infused for 12 h to measure endogenous urea Ra and confirm whether urea Ra, as measured during hour 4 of a primed continuous tracer infusion, accurately indicates urea Ra over the subsequent 8 h.

In the *second protocol* ("step" infusion), endogenous urea Ra was measured over the first 4 h, after which unlabeled urea was infused at a rate approximately two times the endogenous Ra for 8 h.

In the *third protocol* ("pulse" infusion), endogenous urea Ra was measured over the first 4 h, after which unlabeled urea was infused for 4 h. Only the tracer was infused from hours 8 to 12.

In the *fourth protocol*, endogenous urea Ra was measured over the first 4 h. This was followed by a 5-h infusion of unlabeled alanine while tracer administration continued.

In each of the four protocols, a primed-continuous infusion of [^{13}C]urea (Masstrace, Woburn, MA) was administered in which the priming dose was equal to the amount of tracer infused in 9.3 hours (Jahoor and Wolfe, 1987). This priming dose was

infused over 20 min to avoid tracer loss in the urine and was immediately followed by the continuous infusion of the tracer at 42 mg [^{13}C]urea/h (688 μmol/h) for either 12 (protocols 1-3) or 9 (protocol 4) h. Both the priming dose and the continuous infusion were administered using a Harvard Syringe Infusion Pump 681 E (Harvard Apparatus, Milford, MA). For the step and pulse infusions, unlabeled urea was infused using a Baxter Flo-Gard volumetric infusion pump adjusted to administer 20 mg of urea/kg.h (333 μmol/kg.h), approximately two times the endogenous Ra, from a stock solution with a concentration of 36 mg/ml (600 mmol/L).

For the *control study*, blood samples were drawn from an arterialized vein before the priming dose was given, after the priming dose, and every half hour thereafter. For the step infusion, blood was sampled at 10-min intervals during the first hour of unlabeled urea infusion and at 15-min intervals during the second hour. For the pulse infusion, blood was sampled at 10-min intervals during the first hour and at 15-min intervals during the second hour after the start and cessation of the unlabeled urea infusion.

For the *alanine infusion*, a priming dose of 120 mg/kg (1.35 mmol/kg) was administered over 15 min followed by continuous infusion of 120 mg/kg.h (1.35 mmol/kg.h) for 5 h commencing 4 h after the start of the [^{13}C]urea tracer continuous infusion, as described by Wolfe, Jahoor and Shaw (1987). Blood was sampled at 10-min intervals during the first hour and at 15 min intervals during the second hour after the start of the alanine infusion.

In each experiment, the amount of unlabeled urea and alanine infused was verified by weighing the infusate container bags before and after each infusion. All

infusates were aseptically prepared by the hospital pharmacy, and sterility and absence of pyrogens were confirmed.

To prevent starvation-induced amino acid catabolism and increased urea synthesis, 5% dextrose in 0.45% (77mmol/L) NaCl was infused at a rate of 80 mL/h (equivalent to 16 kcal/h) during the control, step and pulse infusion studies. The subjects also drank 150 mL of water hourly to maintain an adequate (227 ± 39 ml/h) but not excessive urine flow, which might alter urea clearance. Urine was collected hourly.

Analytical methods

Heparinized blood samples were centrifuged at 1400 *g* for 15 min at 4°C, and plasma stored at -30°C until analysis. To measure urea and amino acid concentration and enrichment, plasma (0.1 ml) was mixed with 20 μg of [^{13}C,$^{15}N_2$]urea (99% ^{13}C, 99% ^{15}N; MSD Isotopes, Montreal, QC, Canada) and 2.5 μg of norleucine and L-[3,3,3-$^{2}H_3$]alanine (99.4% ^{2}H; MSD Isotopes) internal standards, acidified with 1.5 mL of 1 M acetic acid, and applied to 1-mL columns of cation exchange resin (Dowex 50W-X8, 100-200 mesh hydrogen form; Bio-Rad Laboratories, Richmond, CA). Urea and amino acids were eluted into 3.7-mL flat-bottomed vials equipped with Teflon-lined caps (E. I. du Pont de Nemours, Wilmington, DE) with three sequential 1-mL additions of 3 M NH_4OH. The NH_4OH fraction was evaporated under a gentle stream of N_2. Tert-butyldimethylsilyl (TBDMS) derivatives of urea and amino acid were prepared as described by Patterson et al (1993). Urinary urea enrichment was measured similarly by mixing 0.01 ml of urine with 20 μg of [^{13}C,$^{15}N_2$]urea internal standard, bypassing the resin-treatment step.

Gas chromatography (GC)-mass spectrometry (MS) analyses were performed

using an HP-5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) directly coupled to an HP-5988A quadrupole mass spectrometer. Samples were introduced by splitless injection (1.0 μL) from an HP-7673 autoinjector onto a fused silica DB-1 capillary column (30 x 0.25 mm, 0.25-μm film thickness; J&W Scientific, Folsom, CA) under the following GC conditions: initial column temperature, 110°C (maintained for 2 min); program rate, 6°C/min; final column temperature, 200°C; helium carrier gas column head pressure, 70 kPa; and injector port and transfer line temperatures, 250°C. The electron impact (EI) MS conditions were as follows: ionizing energy, 70 eV; emission current, 300 μA; and source temperature, 200°C. The following ions were monitored by selected ion monitoring: TBDMS-$CO(NH_2)_2$ [mass-to-charge ratio (m/z) 231.1], TBDMS-$^{13}CO(NH_2)_2$ (m/z 232.1), TBDMS-$^{13}CO(^{15}NH_2)_2$ (m/z 234.1), TBDMS-alanine (m/z 158.1), TBDMS-valine (m/z 288.1), TBDMS-leucine (m/z 200.2), TBDMS-isoleucine (m/z 200.2), TBDMS-norleucine (m/z 200.2), and TBDMS-L-[3,3,3-$^2H_3$]alanine (m/z 161.1). TBDMS-leucine eluted at 15 min, TBDMS-isoleucine at 15.6 min, and TBDMS-norleucine at 15.9 min.

The [^{13}C]urea, although 99% ^{13}C, was also 8% [^{18}O,^{13}C]urea, a phenomenon noted by previous investigators (Matthews and Downey, 1984). The same was true of the [$^{13}C,^{15}N_2$]urea used as an internal standard, which was 9% [$^{18}O,^{13}C,^{15}N_2$]urea. The resulting M+5 mass could be used to advantage as an internal standard in quantitating plasma urea. Standard curves were constructed to measure the concentration of [^{12}C]urea and the mole ratio of [^{13}C]urea to [^{12}C]urea (M+1/M). Varying amounts of [^{12}C]urea (0-40 μg) were added to tubes containing 20 μg of [$^{13}C,^{15}N_2$]urea which were then derivatized and analysed by EI GC-MS, and an areas ratio standard curve relating the

intensity ratio 231.1/234.1 (M/M+3) and urea was constructed. Plasma and urine [^{12}C]urea concentrations were determined from their corresponding M/(M+3) intensity ratios using the areas ratio standard curve. Similar results were obtained when the signal intensity at m/z 236.1 (M+5) was used as an internal standard to quantitate [^{12}C]urea. Total urea was calculated as the sum of [^{12}C]urea and [^{13}C]urea.

To measure plasma urea enrichment due to the administered tracer, a calibration curve was constructed by preparing varying mole ratios of [^{13}C]urea to [^{12}C]urea, as described by Tserng and Kalhan (1983). The tracer-to-tracee ratio (TTR) was calculated by subtracting the baseline mole ratio from the sample mole ratio:

$$TTR = [(M+1)/M]sample - [(M+1)/M]baseline$$

where M is the signal intensity of m/z 231.1 and M+1 the signal intensity at m/z 232.1.

Plasma glucose (mmol/L) was analysed using the Beckman Glucose Analyzer II.

Calculations

True urea input into the circulation was defined as the sum of exogenous urea infused and endogenous urea production. The latter was measured as the basal urea Ra after 4 h of primed continuous tracer urea infusion. Urea Ra was measured using two methods. The *first method*, termed "nonisotopic", calculated urea Ra as its urinary excretion rate corrected for changes in the amount of urea in body water (Geiseler et al, 1985; Kay et al, 1986). Urinary urea excretion was normalized for urinary creatinine excretion rates to correct for any time delay in its excretion, as described by Cheema-

Dhadli and Halperin (1993). Briefly, assuming creatinine clearance to be constant, "expected" creatinine excretion over any hour was one-twelfth the total creatinine excretion over the 12 hours. The urea excretion for any hour was multiplied by the corresponding expected/measured creatinine excretion. The nonisotopic method does not account for urea that is hydrolysed in the gut or leaves the body by a nonurinary route. To correct for this, we measured the recovery of [^{13}C]urea in body water and urine, on the assumption that fractional nonurinary losses of tracer and tracee urea are the same. Thus, "corrected" nonisotopic urea appearance was equal to nonisotopic urea appearance divided by the fractional recovery of administered tracer in body water and urine over a specified time period. Tracer urea concentration in plasma and urine was calculated as the product of [^{12}C]urea concentration and TTR.

The *second method* for measuring urea Ra used steady- and non-steady-state tracer kinetics. Steady-state urea Ra was calculated using the equation Ra = i/TTR, where i is the tracer infusion rate in μmol/kg.h. Non-steady state Ra was calculated using Steele's equation (Steele, 1959):

$$Ra = \frac{i - [\{((U_{t1} + U_{t2})/2)/\text{body weight}\} \times \{(TTR_{t2} - TTR_{t1})/(t_2 - t_1)\}]}{[(TTR_{t1} + TTR_{t2})/2]}$$

where U_{t1} and U_{t2} are the urea pool sizes at time points 1 and 2 in μmol, body weight in kg, TTR_{t1} and TTR_{t2} the tracer-to-tracee ratios at time points 1 and 2, and t_1 and t_2 the time in h at points 1 and 2, respectively. The urea pool size was calculated using the formula:

$$U = p \times TBW \times \text{plasma urea concentration}$$

where p is the pool fraction.

Plasma urea concentrations were fitted to smoothing functions to minimize the effects of small analytical errors on total body urea. Plasma urea values between hours 0 and 12 for the control infusion and hours 0 and 4 for the three other infusions were fitted to the function $y(t) = a + bt$ (GraphPad Inplot 4.0; GraphPad, San Diego, CA), where t refers to time. For the step infusion, values between hours 4 and 12 were fitted to the function $y(t) = a(1-e^{-k1t}) + b(1-e^{-k2t}) + c$. For the pulse infusion, values between hours 4 and 8 were fitted to the linear function, whereas those between hours 8 and 12 were fitted to the exponential decay equation $y(t) = a.e^{-kt} + b$. For the alanine infusion, data between hours 4 and 9 were fitted to the polynomial equation $y(t) = a + bt + ct^2 + dt^3 + et^4$.

Statistical analyses

Unpaired Student's t-test and one-way analysis of variance (ANOVA) were used to determine significant differences between different infusion protocols. Paired Student's t-test and repeated-measures ANOVA were used to determine significance between different methods within the same protocol. When ANOVA results showed significance, Newman-Keuls test was used post hoc to determine the source of difference. Differences between data were considered significant at $P \leq 0.05$. All results are presented as mean ± standard error of the mean (SEM).

RESULTS

Control infusion

Under control conditions, the plasma urea concentration demonstrated a slight,

albeit statistically significant ($P < 0.05$), downward trend over 12 h (Figure 1). When calculated as i/TTR, urea Ra was similar at hour 4 (181 ± 16 μmol/kg.h) to that at hour 12 (175 ± 33 μmol/kg.h; Table 2). Urea Ra values as calculated using steady-state and Steele's non-steady-state equations were similar (Figure 2). The mean nonisotopic urea Ra was 72 ± 6% of tracer-determined urea Ra ($P = 0.013$). Because isotopic Ra measured after 4 h of primed-tracer infusion was similar to the value obtained after 12 h, this justified using the sum of tracer-determined endogenous Ra at hour 4 plus infused Ra to determine "true Ra" in the subsequent unlabeled urea and alanine infusion protocols.

Step infusion

After the urea infusion commenced, the plasma urea concentration increased (Figure 1) and TTR decreased (Figure 3). Nonsteady and nonisotopic urea Ra promptly increased and, after 8 h of urea infusion, were 78% and 72%, respectively, of the true Ra (both $P < 0.05$, Table 2 and Figure 4). The non-steady-state Ra profile shown in this figure was derived using a pool fraction of one, which provided the best fit of model-derived Ra to true Ra (Figure 5).

Pulse infusion

Plasma urea increased steadily after the unlabeled urea infusion commenced (Figure 1) and decreased slowly upon its cessation, whereas [^{13}C]urea enrichment showed the opposite trend (Figure 3). Nonsteady and nonisotopic Ra increased promptly upon commencement of the urea infusion and after 4 h of infusion were 67% ($P < 0.05$) and 90% [not significant (NS)], respectively, of the true Ra (Table 2). Upon the cessation of

Table 2. Urea Ra measured using different methods.

	Control	Step	Pulse
	µmol/kg.h	µmol/kg.h	µmol/kg.h
Hour 4			
i/TTR	181 ± 16	221 ± 18	193 ± 22
Nonsteady	167 ± 33 (89 ± 10)	189 ± 29 (86 ± 12)	170 ± 9 (91 ± 8)
Nonisotopic	128 ± 7 (71 ± 3)§	158 ± 30* (69 ± 9)§	121 ± 15* (63 ± 5)§
Hour 8			
True	-	553 ± 16	516 ± 18
i/TTR	176 ± 23 -	294 ± 18 ‡ (53 ± 2)§	281 ± 15 ‡ (54 ± 1)§
Nonsteady	167 ± 40 (90 ± 9)	421 ± 15*‡ (76 ± 2)§	346 ± 22*‡ (67 ± 4)§ ‖
Nonisotopic	124 ± 17 (73 ± 10)	394 ± 21*‡ (71 ± 4)§	463 ± 16*†‡ (90 ± 5)
Hour 12			
True	-	553 ± 16	193 ± 22
i/TTR	175 ± 33 -	340 ± 14‡ (61 ± 2)§	246 ± 24 (131 ± 14)¶
Nonsteady	171 ± 49 (91 ± 8)	431 ± 26*‡ (78 ± 5)§	210 ± 48 (114 ± 30)
Nonisotopic	99 ± 16 (60 ± 10)§	400 ± 15*‡ (72 ± 2)§	174 ± 31 (89 ± 13)

Data are presented as mean ± SEM, n = 6 subjects. Units for urea production rate (Ra) are µmol/kg.h. Urea was infused at 333 µmol/kg.h for 8 h (Step) or 4 h (Pulse). "True" urea Ra is the sum of urea infused plus steady-state Ra determined at hour 4 of the basal condition [tracer infusion (i) divided by tracer-to-tracee ratio (TTR)]. Data in parentheses are % of true urea Ra.

* Significantly different from i/TTR, $P < 0.05$.
† Significantly different from Nonsteady, $P < 0.05$.
‡ Significantly different from True, $P < 0.05$.
§ Significantly different from 100%, $P < 0.05$.
‖ Significantly different from Control, $P < 0.05$.
¶ Significantly different from Step, $P < 0.05$.

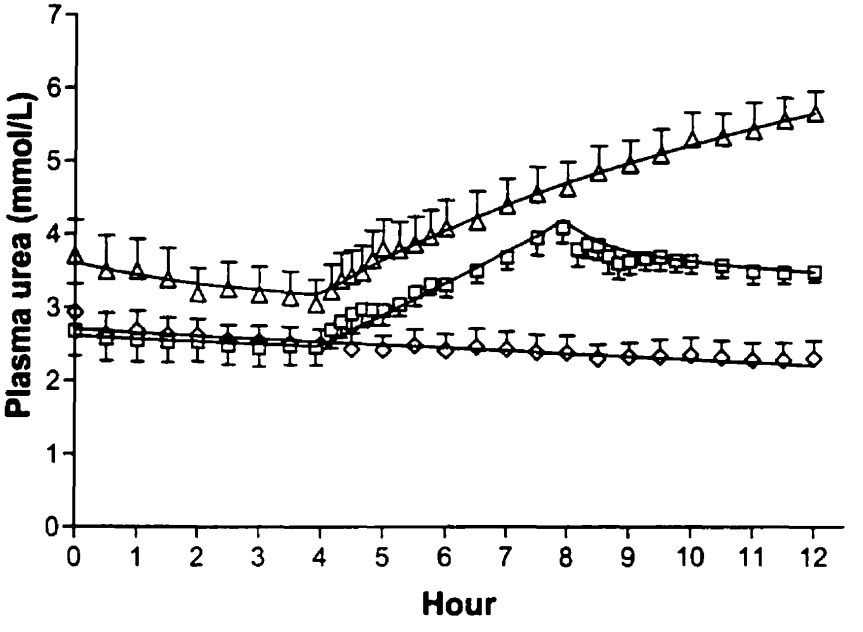

Figure 1. Plasma urea concentration (mmol/L) between hours 0 and 12 for the step (△), pulse (□), and control (◇) infusions.

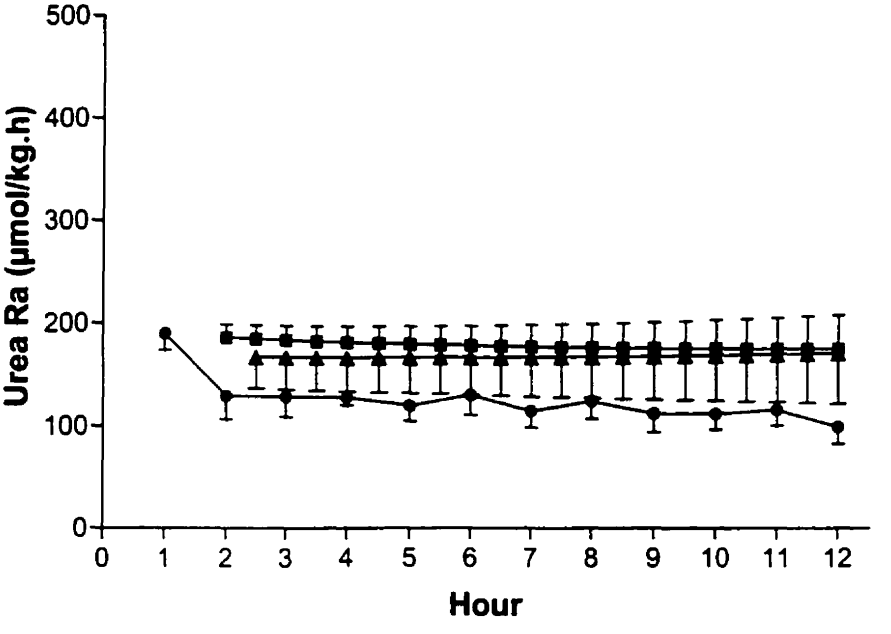

Figure 2. Urea production rate (Ra, µmol/kg.h) for the control infusion calculated using the tracer [i/TTR (■) and nonsteady (▲)] and nontracer (●) methods.

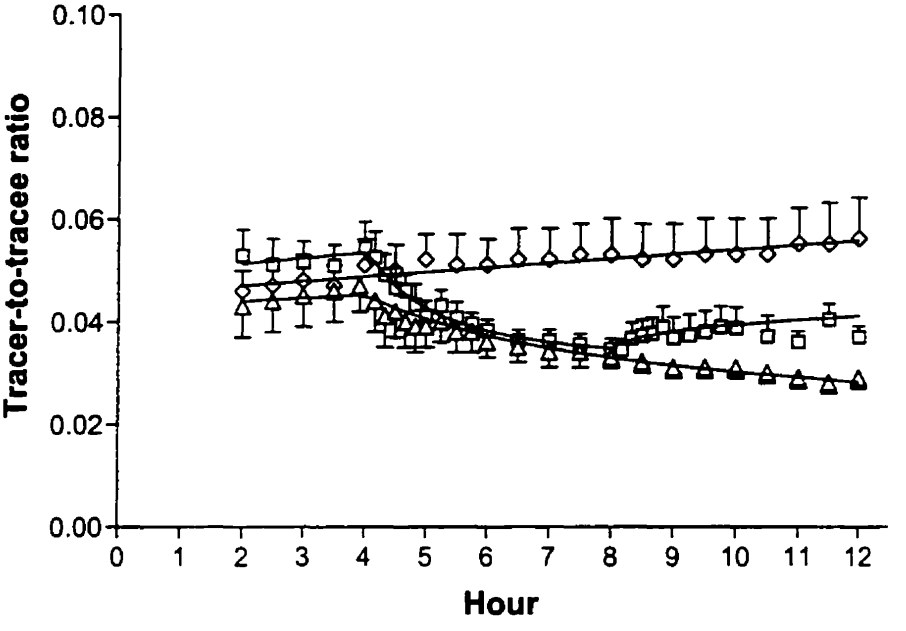

Figure 3. Plasma urea TTR between hours 2 and 12 for the step (△), pulse (□), and control (◇) infusions.

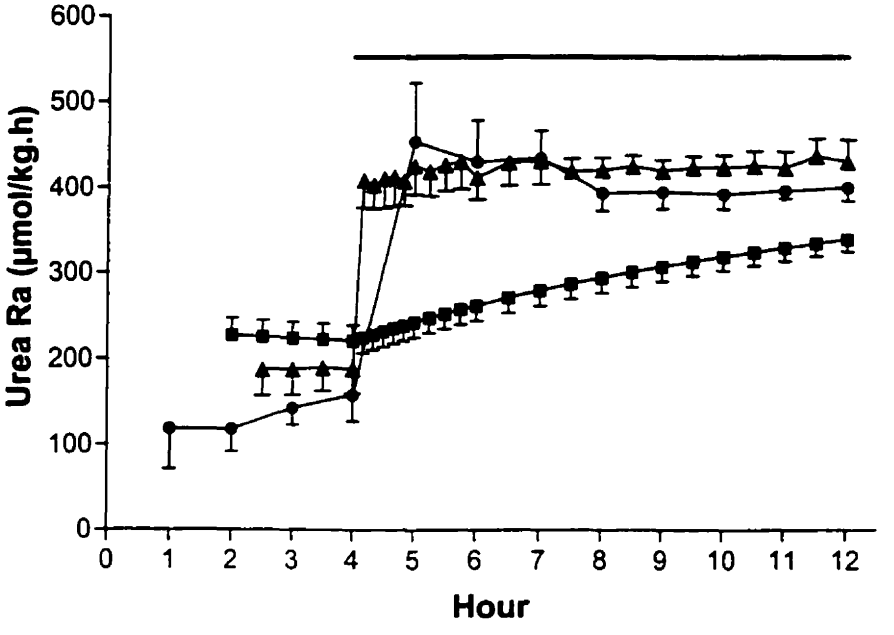

Figure 4. Urea Ra (µmol/kg.h) for the step infusion calculated using the tracer [i/TTR (■) and nonsteady (▲)] and nontracer (●) methods compared with true urea Ra (bar).

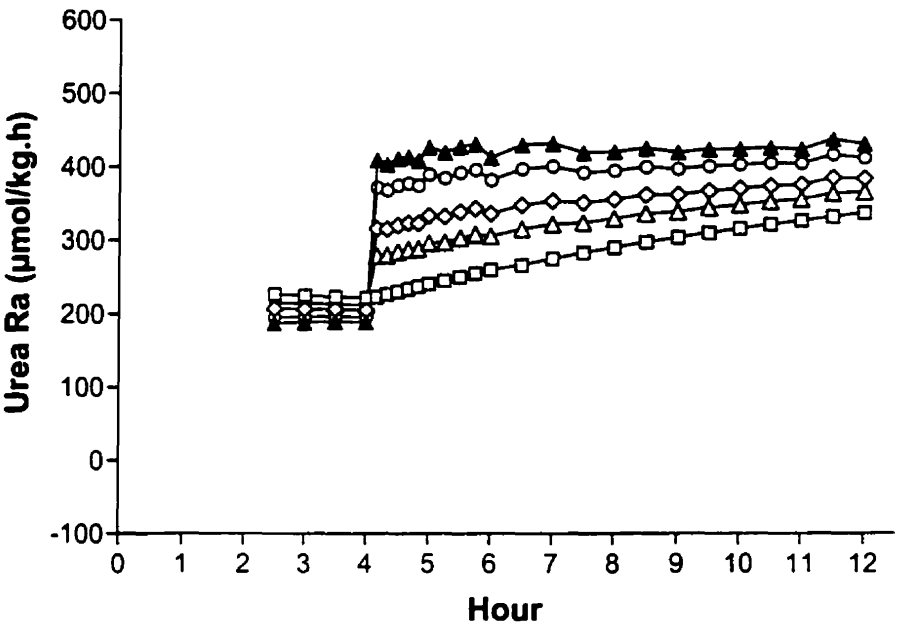

Figure 5. Non-steady-state Ra profiles for the step infusion using different pool fraction values (p): p = 0 (□), p = 0.3 (△), p = 0.5 (◇), p = 0.8 (○), and p = 1.0 (▲).

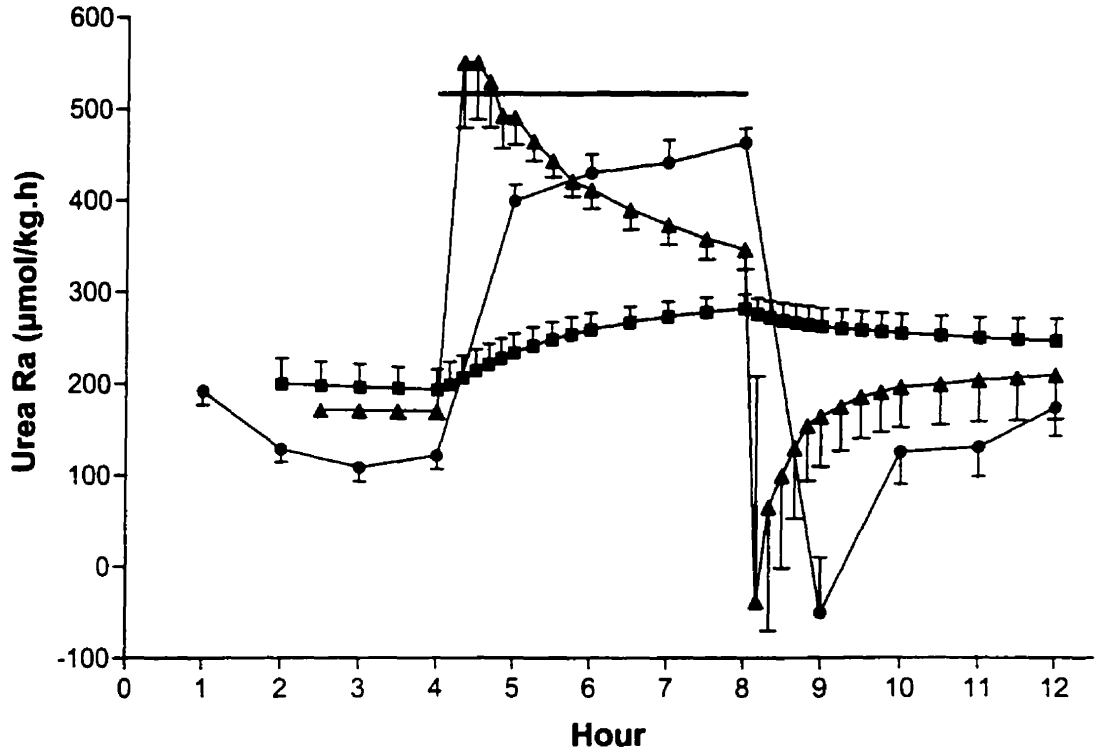

Figure 6. Urea Ra (µmol/kg.h) for the pulse infusion calculated using the tracer [i/TTR (■) and nonsteady (▲)] and nontracer (●) methods compared with true urea Ra (bar).

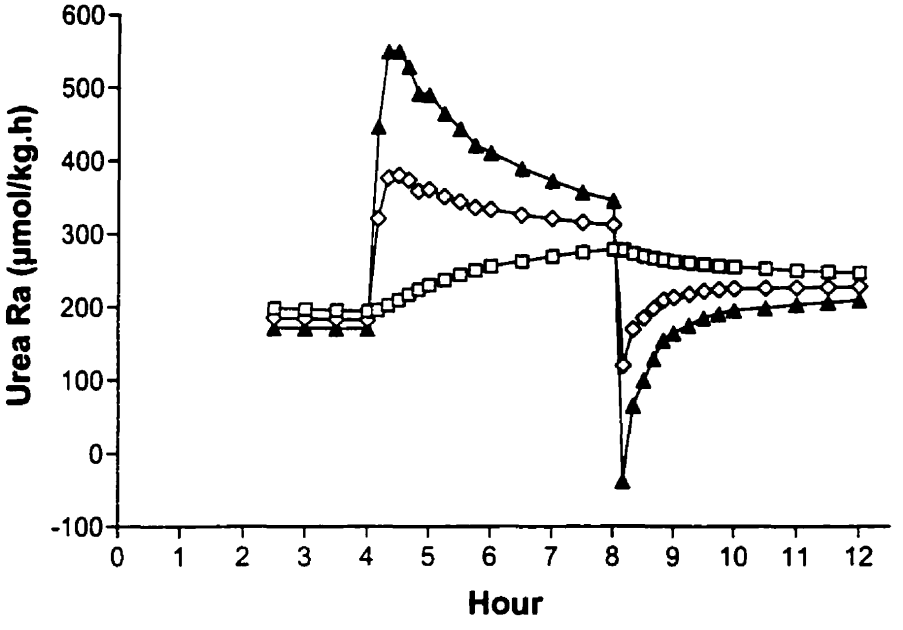

Figure 7. Non-steady-state Ra profiles for the pulse infusion using different pool fraction values (p): p = 0 (□), p = 0.5 (◇), and p = 1.0 (▲).

the unlabeled urea infusion, i/TTR decreased at a slower rate than its increase in the previous 4 h, whereas nonsteady and nonisotopic Ra rapidly decreased (Figure 6). Nonsteady and nonisotopic Ra values were 114% and 89% of the true Ra at hour 12, respectively (NS). As with the step urea infusion, the best non-steady-state profile was obtained using a pool fraction value of one (Figure 7).

Cumulative nonisotopically measured urea production

Table 3 depicts the cumulative urea production (urinary excretion corrected for changes in body pool) over the entire period of urea infusion. For the step infusion, urea production over the 4 h of unlabeled urea infusion was 73% of the true urea input into the body during that period ($P < 0.001$). For the pulse infusion, cumulative urea production over the 8 h of unlabeled urea infusion was 83% of known total urea input during that period ($P = 0.012$). These results ignore non-urinary losses from body water. When these were corrected for by dividing the values by fractional tracer urea recovery in urine and plasma (see Methods), cumulative urea production during the urea infusions was 85% ($P = 0.03$) and 93% (NS) of the true input rate for the step and pulse infusions, respectively (Table 3).

Alanine infusion

Alanine concentrations increased from 0.24 ± 0.02 mmol/L to 2.89 ± 0.23 mmol/L within 90 min of the start of the primed continuous alanine infusion and remained constant thereafter (Figure 8). Plasma glucose remained constant at 5.0 ± 0.02

Table 3. Cumulative nonisotopic urea production for the step and pulse urea infusions.

	Step	Pulse
	mmol./kg.8h	mmol/kg.4h
Urea input	4.50 ± 0.13	2.10 ± 0.07
Urea produced	3.29 ± 0.18*	1.73 ± 0.07*
	(73.2 ± 3.8)†	(82.8 ± 4.5)†
Corrected urea produced	3.81 ± 0.23*	1.95 ± 0.17
	(84.9 ± 5.3)†	(92.8 ± 8.0)

Data are presented as mean ± SEM, n = 6 subjects. Urea input is the sum of endogenous and exogenous sources. Urea production is the sum of urinary excretion and increase in urea in body water. Corrected urea production is urea produced divided by the fraction of [^{13}C]urea infused over the measurement period that was recovered in urine and body water. Data in parentheses are % of input.

* Significantly different from urea input, $P < 0.05$.

† Significantly different from 100%, $P < 0.05$.

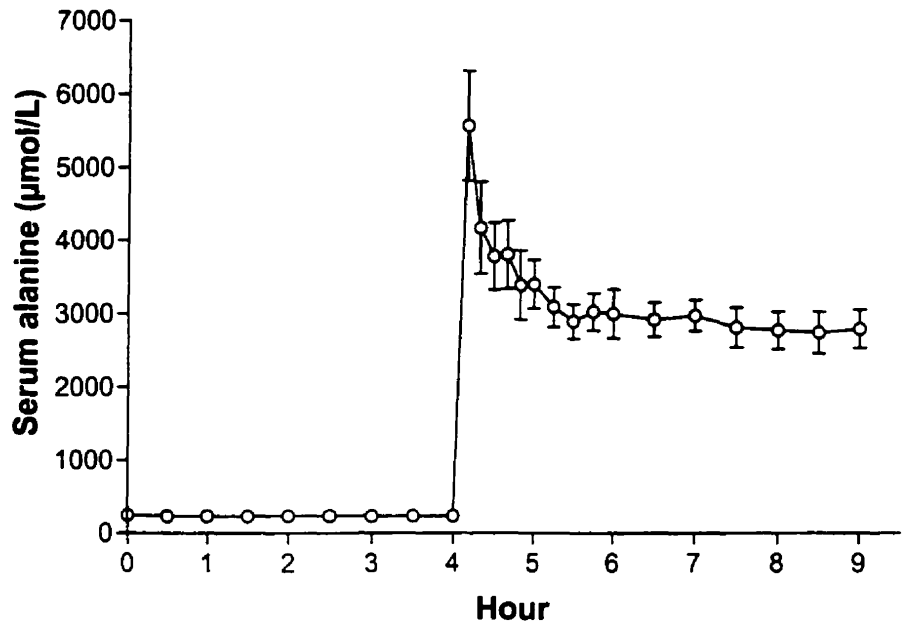

Figure 8. Plasma alanine concentration (µmol/L) for the alanine infusion protocol.

mmol/L throughout the alanine infusion. Plasma leucine, isoleucine, and valine concentrations did not change (data not shown). The alanine infusion induced a steady rise in plasma urea concentration (5.88 ± 0.40 mmol/L at 5 h of alanine infusion) similar to the slower increase observed with the step urea infusion (4.97 ± 0.40 mmol/l at 5 h of step urea infusion; Figure 9). This steeper increase corresponded to a larger amount of urea precursor than the urea infused in the step urea infusion. Nonisotopic urea production increased in a manner similar to the step urea infusion, with i/TTR slowly increasing over the 5-h unlabeled alanine infusion (Figure 10). Both the nonisotopic and Steele's non-steady-state calculations depicted an abrupt increase in urea appearance rate and an apparently constant urea Ra within 2 h of alanine infusion, as previously described (Wolfe et al, 1987).

DISCUSSION

We tested the accuracy of measuring steady-state urea Ra after a 4-h primed continuous tracer infusion as well as the accuracy of this method to determine changes in whole body urea appearance produced by intravenous urea infusions that simulated postprandial changes in urea production. We also measured the short-term effect on urea Ra of infusing alanine, a precursor for urea synthesis.

When only tracer urea was infused, there was no difference between the steady and nonsteady tracer calculations of urea Ra as observed over 12 h. The steady-state Ra at hour 4 was therefore accepted as a valid measure of endogenous urea Ra when no perturbations in urea metabolism occur, in agreement with an earlier conclusion by

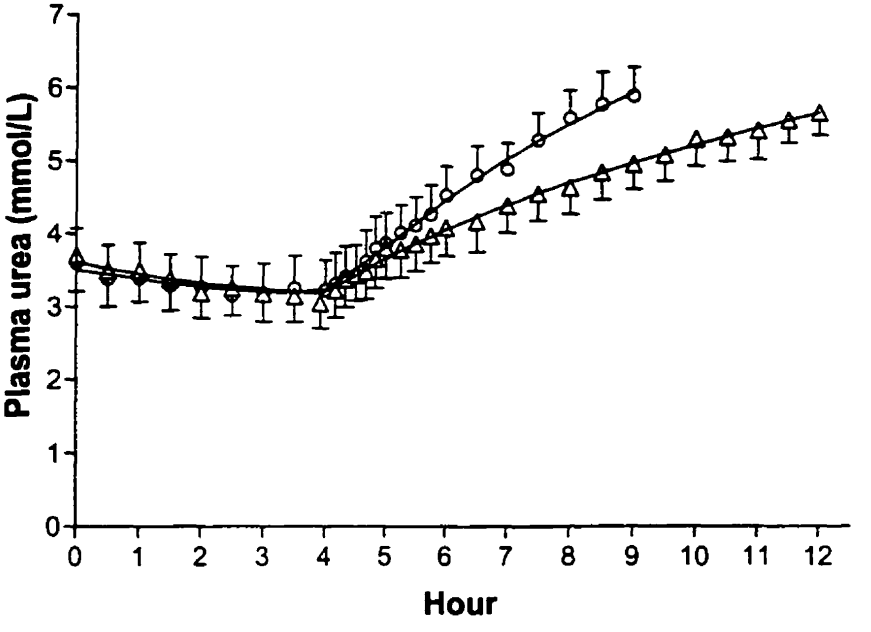

Figure 9. Plasma urea concentration (mmol/L) between hours 0 and 12 for the step (△) and alanine (○) infusions.

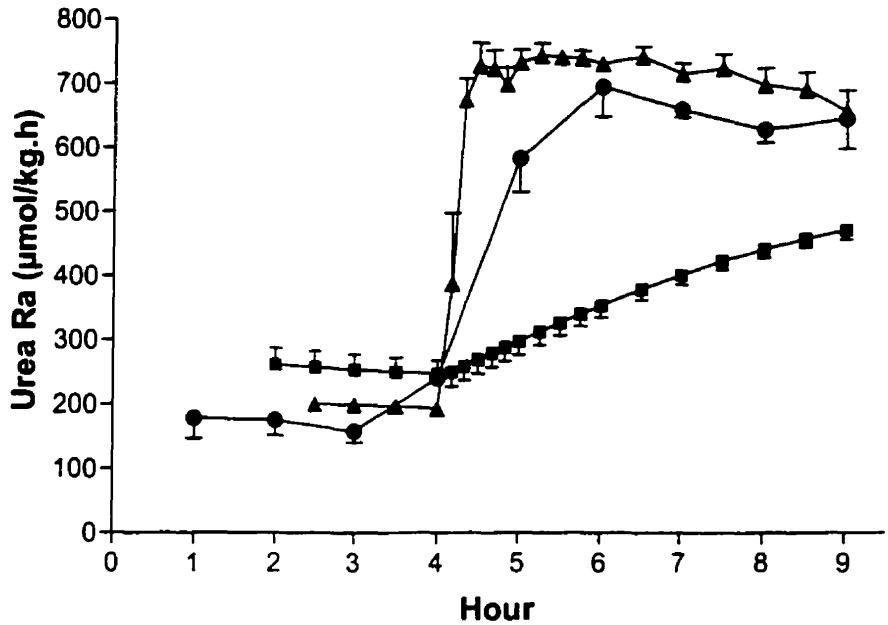

Figure 10. Urea Ra (µmol/kg.h) for the alanine infusion calculated using the tracer [i/TTR (■) and nonsteady (▲)] and nontracer (●) methods.

Jahoor and Wolfe (1987). The nonisotopic Ra was 28% less than the true Ra, also as reported by other researchers (Jones et al, 1969; Long et al, 1978).

When urea was infused to simulate a single meal (pulse infusion) or nibbling for 8 hours (step infusion), i/TTR increased only slowly so that the Ra indicated by the steady-state calculation was only 61% and 54% of the true Ra after 8 h (step) and 4 h (pulse) of true urea input, respectively (both $P < 0.05$). This failure to observe an isotopic steady state even 8 h after urea input increased can be explained by urea's large pool size and slow fractional turnover rate, approximately 9%/h (Coleman et al, 1972; Walser and Bodenlos, 1959). However, Steele's non-steady-state equation and the nontracer method also underestimated the true urea rate of appearance, although to a lesser degree (Table 2).

It should be noted that our calculations assume that the unlabeled urea infusion did not change endogenous urea Ra. It is possible that the urea infusion increased endogenous urea synthesis by increasing urea recycling. If this occurred, it would mean that the tracer and nonisotopic methods underestimate changes in urea production to an even greater extent than the present results indicate.

Cumulative urea production, measured nonisotopically as urinary excretion plus increase in body urea, was 73% ($P < 0.05$) of the true urea input over the 8-h step urea infusion and 83% ($P < 0.05$) of true urea input over the 4-h pulse urea infusion. However, this calculation ignores non-urinary urea losses. Correcting cumulative urea productions for their corresponding tracer urea recoveries improved these estimates to 85% ($P < 0.05$) and 93% (NS) of true urea input for the step and pulse infusions,

respectively (Table 3).

During the alanine infusion, plasma alanine levels were constant over the ninth hour of alanine infusion, indicating that alanine was being disposed of at the rate it was being administered. When given alone, alanine would not be expected to stimulate protein synthesis (as confirmed by the unchanging branched-chain amino acid concentrations), so it may be assumed that alanine N was also being eliminated at the rate it was being administered. If all alanine N was converted to urea, the nonisotopic and non-steady-state calculations at hour 9 underestimated true urea Ra by 40% and 39% (both $P < 0.001$), respectively. This is close to the extent by which these methods underestimated true urea Ra during the urea infusions, strongly suggesting that urea production after alanine administration is also underestimated when both isotopic and nonisotopic approaches are used (Wolfe et al, 1987). In an earlier study in which alanine was infused at the same rate as in this study, urea Ra accounted for an even smaller fraction of the total alanine N infused than we found (Wolfe et al, 1987). We therefore conclude that true urea Ra is significantly underestimated even by the non-steady-state tracer method during acute administration of a urea precursor.

The present conclusions are important for interpreting the results of studies in which urea Ra was measured to determine the effect of an acute intervention, such as exercise, feeding, or hormone administration (Beaumier et al, 1995; Carraro et al, 1993; El-Khoury et al, 1996; El-Khoury et al, 1994; Ensinger et al, 1994; Wolfe et al, 1982; Wolfe et al, 1987). The failure to observe an increase in urea Ra during acute exercise, despite increased amino acid oxidation, is consistent with insensitivity of the model to

detect acute increases in urea Ra (Carraro et al, 1993; Wolfe et al, 1982). In feeding studies (El-Khoury et al, 1996; El-Khoury et al, 1994), the increase in urea Ra reported to occur in the fed state using continuous [$^{15}N_2$]urea infusion might have underestimated a true fed-state increase in urea Ra, resulting in an underestimate of urea recycling.

We conclude that the primed continuous tracer urea infusion method can provide a valid measure of urea Ra in a 4-h study, but only under basal conditions. The method is insensitive for detecting changes in urea Ra, even 8 h after urea Ra increases, when either non-steady-state kinetics or nonisotopically measured urea excretion is used to measure it. The accuracy of the nonisotopic measurement is improved if corrections are made to account for non-urinary urea losses by measuring recovery in urine and plasma of the dose of tracer urea introduced into the body over the period of analysis.

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CONNECTING TEXT I

Our results from chapter 3 determined that under basal conditions the primed continuous tracer urea infusion method provides a valid measure of urea Ra in 4 h. Under non-steady conditions, the method is insensitive for detecting changes in urea Ra. even 8 h after urea Ra increases, when either non-steady-state kinetics or nonisotopically measured urea excretion is used to measure it. However, the accuracy of the nonisotopic measurement is improved if corrections are made to account for non-urinary urea losses by measuring recovery in urine and plasma of the dose of tracer urea introduced into the body over the period of analysis.

Having determined that changes in whole-body amino acid catabolism could not be reliably determined by measuring changes in urea Ra, our next objective was to investigate whether sulfate production was a valid measure of whole-body amino acid catabolism. Sulfate is the major end-product of the sulfur amino acids (SAA), methionine, cysteine and cystine, and its urinary excretion over 24-hour periods closely matches both dietary SAA intake and N excretion. Unlike urea, which distributes throughout total body water, sulfate distributes in the considerably smaller extracellular fluid volume (ECF) and is not subject to important losses in the gut. Short-term sulfate kinetics might be more accurately determined than those of urea. However, it has not been determined whether sulfate production can be measured accurately over periods of only a few hours, such as those following a typical meal.

The nontracer method calculates sulfate production as its urinary excretion

corrected for changes in ECF. To do this, we needed first to establish a reliable method for measuring the sulfate distribution volume, a measure of the ECF. Although the ECF is considered best approximated by the sulfate space, the methodology involves the administration of radioactive sulfur which limits its usefulness. We therefore investigated the use of negative electropsray ionization tandem mass spectrometry to measure the stable isotope $^{34}SO_4$ in biological fluids.

CHAPTER IV

Measurement of Sulfate Concentrations and Tracer/Tracee Ratios in Biological Fluids by Electrospray Tandem Mass Spectrometry

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Measurement of Sulfate Concentrations and Tracer/Tracee Ratios in Biological Fluids by Electrospray Tandem Mass Spectrometry

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ABSTRACT

A reproducible and very sensitive method is described for the quantitation of inorganic sulfate in biological fluids by negative electrospray ionization tandem mass spectrometry. After addition to the sample of ^{34}S-labeled sodium sulfate internal standard and deproteinization with methanol, interfering bicarbonate anions are removed by acidification and chloride and phosphate by means of a single filtration step. The tandem mass spectrometer is used in neutral loss mode to detect HSO_4^- ions free of interference from residual isobaric $H_2PO_4^-$ ions. Organic sulfates do not interfere with the measurement. Serum urinary inorganic sulfate concentrations measured with this technique agree closely with determinations by ion-exchange chromatography with conductivity detection. Unlike the latter method, this technique does not require dedicated equipment. The method is also suitable for measuring the ratio of ^{34}S-labeled sulfate to unlabeled sulfate in serum and hence represents and attractive alternative for the use of the radioactive ^{35}S isotope in human studies of body composition and oxidation of sulfur-containing substrates to sulfate.

INTRODUCTION

Inorganic sulfate metabolism has long interested biologists, including the renowned chemist Folin (1905). In many studies, including the classic one on human body composition by Reifenstein et al (1947), a close relationship was found between nitrogen and sulfate balance that closely conformed to the N/S molar ratio in body proteins. Further, there is a close relationship between sulfur amino acid intake and urinary sulfate excretion in humans for intakes above the requirement level (Sabry et al, 1965). Thus, urinary sulfate excretion can be used to assess the short- and long-term balance between sulfur amino acid ingestion and its elimination (Cheema-Dhadli and Halperin, 1993). Because it is excreted almost entirely by the kidneys, sulfuric acid accumulates in renal failure, contributing importantly to the accompanying acidosis (Freeman and Richards, 1979). Finally, sulfate conjugation is an important step in the metabolism of phenolic drugs, whose elimination is prolonged in states of sulfate depletion (Morris and Levy, 1983). Previous methods for measuring sulfate use either turbidimetry (Lundquist et al, 1980; Sorbo, 1987) or ion-exchange chromatography with conductivity detection (IEC-CD) (Cole and Scriver, 1981; Hoffman et al, 1991; Sullivan, 1987).

In this paper we describe the use of negative electrospray ionization tandem mass spectrometry (ESI-MS/MS) in two analytical methods involving sulfate. In the first, we describe its use in a simple and highly sensitive stable isotope dilution assay for serum and urinary sulfate. We compare values obtained with those from samples analyzed by IEC-CD. In the second we describe its use in the measurement of tracer/tracee ratios in

studies of sulfate distribution and metabolism with ^{34}S-labeled sulfate.

MATERIALS AND METHODS

Instrumentation

The ion chromatograph used was a Dionex 2110i (Dionex, Sunnyvale, CA) equipped with a conductivity detector, a 25-μL sample loop, a 4-mm AMMS-II anion micromembrane suppressor, an ion-exchange Ionpac AG4A-SC precolumn, and an AS4A-SC analytical column. Peak integration was performed with a Waters 740 data module (Milford, MA).

A Quattro II triple quadrupole (Micromass, Manchester, UK) was configured for negative-ion analysis and used with cone voltage -25 V, source temperature 120°C, sample infusion rate 40 μL/min, nitrogen bath gas flow rate 300 L/h, nebulizer gas flow rate 18 L/h, collision cell energy 23 eV, with argon pressure in the collision cell at 1.3 x 10^{-3} mbar.

Materials

All the water used in this report was Type 1 or ultrapure water (resistivity higher than 18 Mohms/cm), purified by treatment with Milli-RO Plus and Milli-Q UF Plus systems (Millipore, Bedford, MA) and was found to have insignificant amounts of sulfate. Anhydrous unlabeled sodium sulfate was obtained from Fisher scientific (Montreal, QC, Canada). Icon Services Inc (Summit, NJ) supplied ^{34}S-labeled sodium sulfate that was specified to be 93% labeled, but analysis by fast atom bombardment and

by the method described here showed this to be 94% ^{34}S-labeled. Sodium sulfate standards were heated at 120°C for a minimum of 24 h to ensure that they were weighed in their anhydrous form. Sodium D-glucosamine 2-sulfate and potassium *p*-nitrocatechol sulfate were from Sigma Chemical Co. (St. Louis, MO). Solvents and miscellaneous chemicals and buffers were obtained from local sources. Standard Vacutainer blood collection tubes were obtained from Becton-Dickinson (Franklin Lakes, NJ) and because they were found to contain traces of sulfate, they were rinsed with water and dried before use. OnGuard-Ag cartridges (No. 39637) were obtained from Dionex, syringe filters (0.22 μm) were from Chromatographic Specialties (Brockville, ON, Canada), and MPS micropartition cartridges with a YMT 10,000-Da molecular weight cutoff membrane filter came from Amicon (Beverley, MA). Prior to use, each Amicon filter was soaked in water for 1 h with three water changes to remove traces of sulfate.

Ion-Exchange Chromatography

Operating conditions

A carbonate-bicarbonate buffer (1.7 mM $NaHCO_3$; 1.8 mM Na_2CO_3, pH 8.75) was used as the mobile phase at a flow rate of 2.0 mL/min as suggested by the instrument manufacturer (Dionex Document No. 034528-03, January 1992). The suppressor regenerant (25 mM H_2SO_4) was percolated by gravity at 2 mL/min. The output range of the conductivity detector was set at 10 μS. Background conductivity was usually approximately 17 μS. The retention times for chloride, bromide, phosphate, and sulfate were 1.8, 2.9, 5.7, and 7.5 min, respectively, under these conditions. Injection volumes

were 25 µL.

Sample analysis

Although previous reports describe the direct injection of diluted serum or plasma into the ion chromatograph (Cole and Scriver, 1981; Sullivan, 1987), this severely shortened column life. Deproteinization with trichloroacetic acid, sulfosalicylic acid, or perchloric acid-potassium hydroxide produced interfering chromatographic peaks, whereas acetonitrile reduced the recovery of added known amounts of sulfate by 40-50%. Methanol deproteinization gave a better recovery (80-100%) but still lower measured concentrations in biological samples than obtained using the filtration method ultimately adopted.

Among several filtration membranes tested, the Amicon MPS cartridges yielded the best recoveries and were least contaminated with sulfate which was easily eliminated by water washings. Although either plasma or serum could be used, serum was easier to filter.

Clotted venous blood was centrifuged (1400 *g*, 15 min) and the serum separated and frozen at -30°C. At the time of analysis, 125 µL of serum was diluted 10- to 40-fold with water and passed through the Amicon filters by centrifugation (1400 *g*). The filtrate obtained in the first 15 min (from 300 µL to 500 µL) was discarded to avoid sample dilution by traces of water left in the filter membrane by the washing technique. Centrifugation was resumed for a further 60 min and the filtrate kept for analysis. Urine samples of 50 µL were diluted 100-fold, filtered through 0.22-µm syringe filters, and

treated like the serum final filtrates.

Electrospray Mass Spectrometry

Elimination of sample interferences

Preliminary experiments with a Rheodyne injector and Hamilton glass syringe to load samples into the injection loop generated severe sulfate contamination in subsequent water blanks. We elected to introduce each sample with a new disposable syringe directly linked to the electrospray probe. Becton-Dickinson 1-mL disposable plastic syringes were modified by removing 4 mm from the luer tips with a bench grinder. The remaining tips were threaded with a 1/8-inch-diameter die (32 threads per inch), and a 1/8-inch swagelock nut was then used with a 2-mm-i.d. Viton "O" ring to seal a 10-cm length of 1/16-inch PEEK tubing (0.17 mm i.d.) onto the threaded tip. This modified syringe was filled with the sample and mounted onto a single-syringe infusion pump (Cole-Parmer Model E-74900, Vernon Hills IL), and the solution was infused through the electrospray probe via the PEEK tubing at 40 μL/min. The syringes are sufficiently inexpensive and easy to modify that a new syringe could be used for each sample.

Another source of cross-contamination was desorption of sulfate from previous samples from the probe and counterelectrode surfaces. This was eliminated by manual injection through the ESI probe of three 1-mL aliquots of water at a high flow rate (approximately 2 mL/min) between samples.

Sulfate anion intensities in biological fluids cannot be measured by simple scanning because the phosphate anion $H_2PO_4^-$ is isobaric with $H^{32}SO_4^-$ at 97 Da.

Phosphate, bicarbonate, and chloride ionic strengths in serum and urine are high enough to completely inhibit sulfate detection in ESI. Furthermore, these salts are not volatile and will crystallize in the source. Bicarbonate was eliminated by sample acidification with 100 μL of 1N HCl. Phosphate and chloride concentrations were then reduced to acceptable levels by filtering the samples through Dionex No.39637 OnGuard-Ag cartridges. However, enough phosphate remained to produce apparently incorrect ratios of 99 to 97 Da for $H^{34}SO_4^-$ and $H^{32}SO_4^-$, respectively. This last problem was resolved by using an MS/MS method instead of simply scanning with MS1. Metzger et al (1995) showed that under collision-induced dissociation (CID) conditions, $H_2PO_4^-$ loses H_2O (loss of 18 Da) to give PO_3^- which appears at 79, while $H^{32}SO_4^-$ loses neutral 17 Da (loss of $OH^\cdot$) to give $SO_3^{-\cdot}$ which appears at 80 Da. Contrary to Metzger et al who used product ions of 97 ($H^{32}SO_4^-$) and 99 Da ($H^{34}SO_4^-$), we selected scanning for neutral loss of 17 Da over the range 92 to 102 Da as it ignores phosphate which loses an 18-Da neutral. Acquisitions of 2 min each were made in triplicate in multichannel acquisition mode (MCA). Neutral loss scanning with the Quattro II in MCA yields the intensities of ions 80 and 82 formed from precursors 97 and 99 recorded in the same data file which simplifies subsequent data manipulation. Peak intensities in arbitrary units were corrected for natural abundance isotope inclusion and for ^{32}S isotopic impurity in the $Na_2{}^{34}SO_4$.

We examined the possibility that sulfate may be freed from sulfate-containing organic compounds under ESI conditions and interfere with the analysis. Solutions of sodium D-glucosamine 2-sulfate and potassium *p*-nitrocatechol sulfate as model com-

pounds were subjected to analysis under the conditions described above.

Calibration

Calibration standards for sulfate quantitation by stable isotope dilution techniques were made with $Na_2{}^{32}SO_4$ concentrations varying from 0 to 600 μM. To 1.0 mL of each standard, 0.5 mL of 600 μM $Na_2{}^{34}SO_4$ was added as internal standard followed by 5 mL of methanol.

Because the natural abundance of the ^{34}S isotope is slightly dependent upon diet and area of residence (Szabo et al, 1950), baseline samples obtained before administration of ^{34}S-enriched sodium sulfate were used to calculate the tracer/tracee ratio, which is defined as the ratio of the intensities of masses 99 and 97 for a given sample minus the same ratio for a natural abundance sample obtained before tracer administration (Tserng and Kalhan, 1983).

Standard solutions for measurement of tracer/tracee ratios following the administration of $Na_2{}^{34}SO_4$ were made having $(Na_2{}^{34}SO_4)/(Na_2{}^{32}SO_4)$ molar ratios of 0 (natural abundance), 0.00495, 0.00996, 0.0340, 0.0519 and 0.1095 with a constant total sulfate concentration of 7 mM. These stock calibration standards were then diluted by addition of 50 μL of each standard to 1450 μL of water and 5 mL of methanol to produce final sulfate concentrations of 54 μM. A calibration curve relating the known versus measured tracer/tracee ratios was constructed as described by Tserng and Kalhan (1983).

Administration of $Na_2{}^{34}SO_4$

A volunteer consumed 50 mg of $Na_2{}^{34}SO_4$ dissolved in 150 mL of water. Serum samples were taken immediately prior to administration, every 30 min for the first 2 h, and then every 60 min for the last 3 h.

Preparation of serum and urine samples

To measure serum sulfate concentrations, 1.0-mL aliquots of serum were mixed with 0.5 mL of the 600 μM $Na_2{}^{34}SO_4$ internal standard solution, followed by 5.0 mL of ice cold methanol. These were mixed well and kept on ice for 10 min. Precipitated proteins were removed by centrifugation at 4°C (1400 *g*, 10 min). The supernatants were acidified with 0.1 mL 1 M HCl and then passed through an OnGuard-Ag cartridge previously rinsed with 25 mL water to remove traces of sulfate. The first 3 mL of filtrate were discarded and the rest collected for analysis. Complete recovery is not necessary as the ratio of labeled to unlabeled sulfate will remain invariant. Methanol proved to be an effective deproteinization agent that also enhanced the sulfate signal strength by a factor of approximately 100 relative to solutions of equal concentration in water, presumably by increasing the efficiency of droplet formation and evaporation in the ion source.

Urine samples were first diluted 20- to 40-fold with water, and then 1.0 mL was mixed with 0.5 mL of the 600 μM $Na_2{}^{34}SO_4$ internal standard and 5 mL methanol and treated in the manner of the serum supernatant samples following the first centrifugation step, except that the sample is not acidified.

For isotopic enrichment measurements in serum, 0.5 mL of water replaced the 0.5

mL of the 600 μM $Na_2{}^{34}SO_4$ internal standard.

Data acquisition

Acquisitions were made in triplicate in MCA (2 min each), as described above. Peak intensities were recorded in arbitrary units for calculation of the $^{34}S/^{32}S$ ratio for the enrichment measurements or the $^{32}S/^{34}S$ ratio for the concentration measurements. Four samples could be analyzed per hour.

Concentrations were read from a calibration curve in which measured $^{32}S/^{34}S$ intensity ratios were plotted for samples containing constant amounts of $Na_2{}^{34}SO_4$ and varying amounts of natural abundance sodium sulfate as described above. Similarly, isotope enrichments were read from a plot of known enrichments vs measured intensity ratios.

Statistical Analysis

F-tests were conducted (PRISM, Graph Pad, Inc.) to test whether the slopes for the correlations between ESI-MS/MS and IEC-CD values in serum and urine were significantly different from zero. The p value obtained for each slope was calculated from an F-test. P values of ≤ 0.05 are considered significant.

RESULTS AND DISCUSSION

IEC-CD Analyses

Standard curves were linear over the range 0 to 105.6 μM ($r^2 = 0.999$).

Recoveries of sulfate added to serum treated with the Amicon filters or to urine were 99.5% and 101%, respectively (CV = 1.5%, n = 4).

Differences between peak areas from repeat injections of the same treated sample were usually less than 5%. The relative standard deviation of peak areas for the same samples treated and analyzed on different days was 1-4%. The lowest concentration measured was 7 μM.

ESI-MS/MS Analyses

Figure 1A represents superimposed product ion spectra for the 97-Da $H_2PO_4^-$ and HSO_4^- ions in collision with argon and shows the lack of interference by phosphate at 79 Da.

A typical mass spectrum obtained for naturally abundant sulfate with this method is shown in Figure 1B. The ratio of the intensity of m/z 99 to 97 is 5.3% and conforms to the calculated ratio obtained for isotopes of hydrogen, sulfur, and oxygen in their natural abundances. Serial dilutions of ^{34}S-labeled sulfate in water were made, and this produced no change in the 99/97 ratio demonstrating that the introduction of contamination by ^{32}S-labeled sulfate in the water used in these experiments was insignificant. The sensitivity of the method is such that 2.0 μM solutions of natural abundance sodium sulfate provided 97- and 99-Da intensities with signal to noise ratios 100 and 20, respectively, which allowed accurate determinations of the natural $^{34}S/^{32}S$ ratio.

To determine whether sulfate-containing organic compounds would liberate sulfate in the ESI source and thereby interfere, we selected as a model sulfate ester,

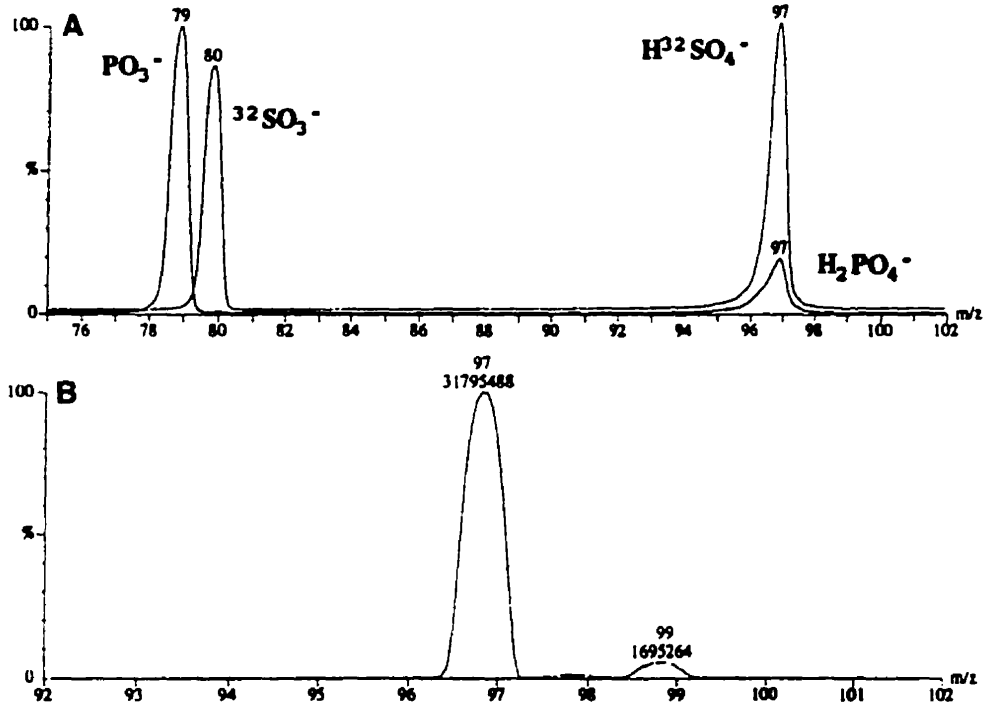

Figure 1. (A) Superimposed negative product ion spectra for the 97- Da ions corresponding to $H^{32}SO_4^-$ and $H_2PO_4^-$. The former loses OH˙ radical, while the latter loses H_2O to yield product ions with masses 80 and 79, respectively. (B) Negative-ion electrospray mass spectrum for natural abundance sodium sulfate in neutral loss of 17 (OH˙) mode. The ion intensities conform with the expected common $^{34}S/^{32}S$ ratio of 5.3%.

nitrocatechol sulfate, which showed only the expected $(M-H)^-$ ion at 234 Da (data not shown). A collision energy of 50 eV with argon was required to induce fragmentation of the 234-Da ion and produce a product ion at 154 Da $(M-SO_3)^-$ and a very weak 80 Da-$SO_3^{-\cdot}$ ion (Figure 2A). This collision energy is twice that used for inorganic sulfate determination, and the 80-Da ion does not appear at 23 eV. This compound was also submitted to neutral loss of 17-Da scanning and the resulting 97-Da intensity is approximately three times the noise and is similar to that obtained with the water used in these studies (Figure 2B).

Another model compound, sodium D-glucosamine 2-sulfate (a sulfamate), in full-scan mode (data not shown) showed an $(M-H)^-$ ion at 258 Da and moderately intense ions with masses 138 ($OHCCH_2NHSO_3^-$), 97 (HSO_4^-), 96 ($NH_2SO_3^-$), and 80 ($SO_3^{-\cdot}$). A moderate amount of free sulfate was easily detected as the 97-Da ion noted above. Analysis of this sample by IEC-CD, however, revealed that it contained approximately 7% free sulfate as received from the manufacturer. It is unlikely that HSO_4^- can originate from the sulfamate by fragmentation in the source, as it is not a product ion of the 258-Da quasimolecular anion at 23 eV collision energy, and it is only at 40 eV collision energy that a 97-Da ion could first be detected (Figure 2C). Furthermore, since the sulfur is bound up in a $-NH-SO_3^-$ moiety, it would seem unlikely that it would easily fragment under ESI conditions to release an HSO_4^- ion. D-Glucosamine 2-sulfate was also submitted to neutral loss of 17-Da scanning and the result is presented in Figure 2D. It is interesting to note that the $NH_2SO_3^-$ anion (96 Da) also loses 17 Da. While ammonia is isobaric with $OH^\cdot$, $NH_2SO_3^-$ has too few hydrogen atoms to undergo ammonia loss, and

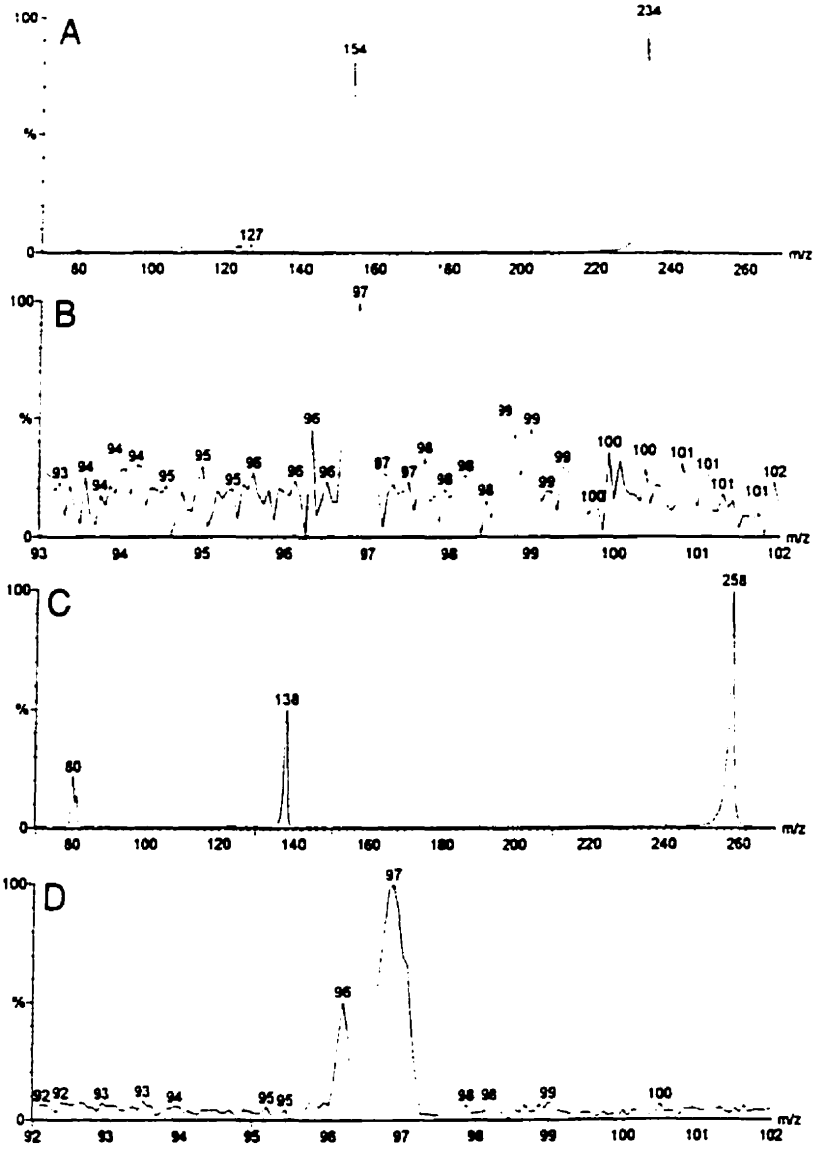

Figure 2. (A) Collision-induced product ion spectrum of the 234-Da $(M-H)^-$ quasimolecular anion of *p*-nitrocatechol sulfate with argon at 50 eV energy. A HSO_4^- anion is not detected, and at the 23-eV collision energy used in the quantitative work, the 80-Da ion is not a product of the 234-Da ion. (B) Neutral loss of 17-Da spectrum for *p*-nitrocatechol sulfate. The level of sulfate detected is consistent with that found in the water used in the analysis. (C) Collision-induced product ion spectrum of the D-glucosamine 2-sulfate quasimolecular anion (258 Da) with argon at 40 eV energy. An 80-Da fragment is apparent, along with a very weak 97-Da ion. The latter ion is not detectable at 23 eV collision energy. Under neutral 17 loss conditions therefore, the 80-Da ion would not be detected at 23 eV. An ion with apparent composition $NH_2SO_3^-$ (96 Da) is able to lose 17 Da (see text). (D) Neutral loss of 17-Da spectrum for D-glucosamine 2-sulfate. The only contributing ion appears to be $H^{32}SO_4^-$ (97 Da) and arises from free sulfate determined by IEC-CD to be present in the glucosamine sulfate in the amount of 7% by weight as received from the supplier.

we suggest that the loss is that of $OH^{\cdot}$.

We are therefore confident that the 97-Da ion corresponding to $H^{32}SO_4^-$ passed by quadrupole 1 in neutral 17 loss mode originates not by fragmentation of organically bound sulfate in the ion source, but entirely from free inorganic sulfate. It appears then that sulfate-containing organic compounds do not interfere with the determination of the isotopic ratio for inorganic sulfate.

The results obtained for the quantitation of sulfate by the IEC-CD and ESI-MS/MS methods are compared in Figures 3 and 4 for 52 sera and eight urines, respectively, from normal volunteers under a variety of fasting or fed conditions.

The ESI-MS/MS method also permits the quantitation of tracer/tracee ratios of less than 0.01 with excellent precision following ^{34}S-labeled sulfate administration. Figure 5 illustrates the appearance of ^{34}S-labeled sulfate in serum after the oral ingestion of 50 mg of $Na_2{}^{34}SO_4$ by a normal volunteer. Enrichment was maximum after 2 h and declined thereafter, in accordance with previous results with ^{35}S-labeled sulfate in humans (Bauer, 1976). The sulfate distribution volume, which conforms to the volume of the extracellular fluid compartment (Pierson et al, 1982), was calculated as described by Bauer (1976) by log-linear extrapolation of ^{34}S-labeled sulfate enrichment to zero time using values for the samples drawn at hours 3, 4 and 5.

This ESI-MS/MS method may be used to determine both serum sulfate concentrations and isotopic enrichments in the same sample even in the presence of administered ^{34}S-labeled sulfate. For the tracer enrichment measurement, a sample aliquot without internal standard is analyzed as described. $Na_2{}^{34}SO_4$ internal standard is

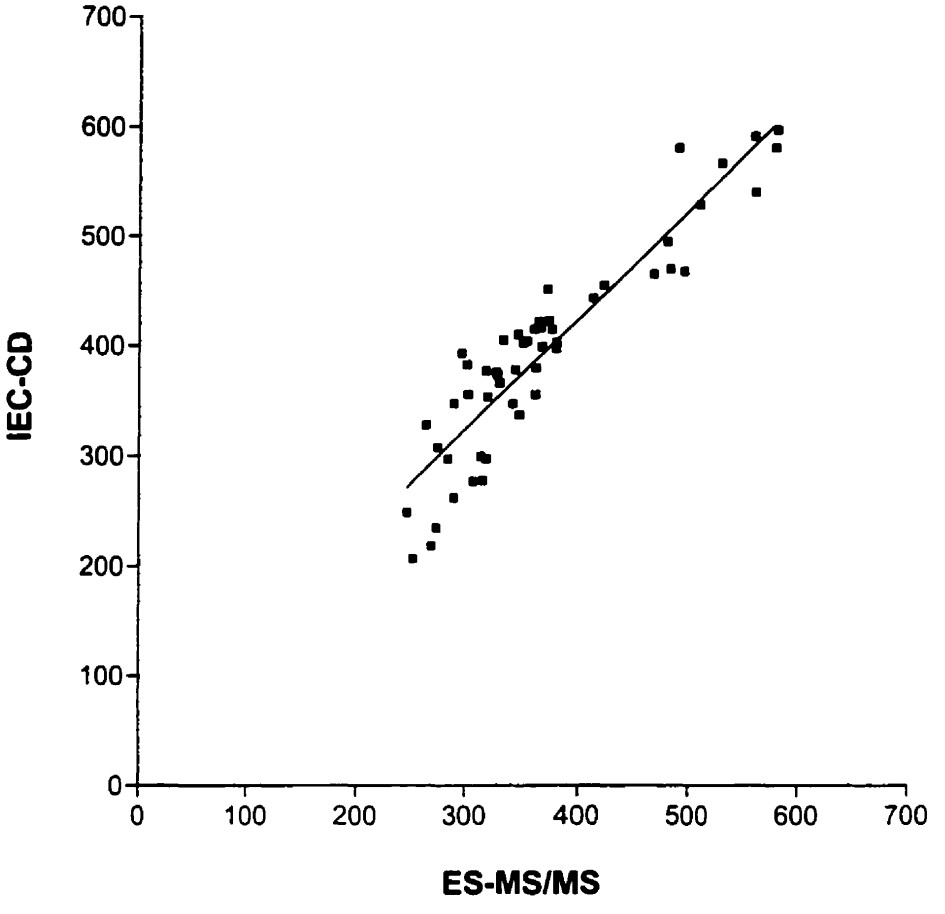

Figure 3. Comparison of sulfate concentration determinations (μM) by ion-exchange chromatography and negative-ion electrospray tandem mass spectrometry in 52 sera. The line of best fit is represented by $y = (0.99 \pm 0.06)x + (27 \pm 22)$; $r = 0.92$, $P < 0.0001$.

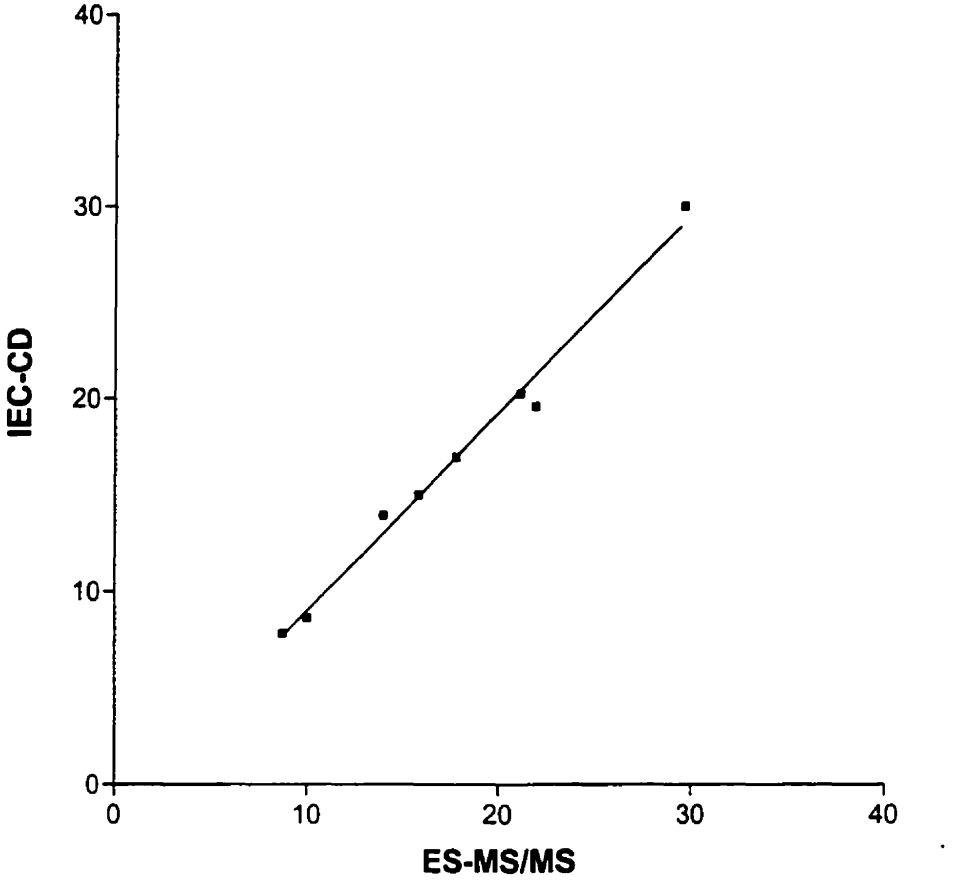

Figure 4. Comparison of sulfate concentration determinations (mM) in eight urine samples by ion exchange chromatography and negative-ion electrospray tandem mass spectrometry. The line of best fit is represented by $y = (1.03 \pm 0.05)x - (1.3 \pm 0.9)$; $r = 0.99$, $P < 0.0001$.

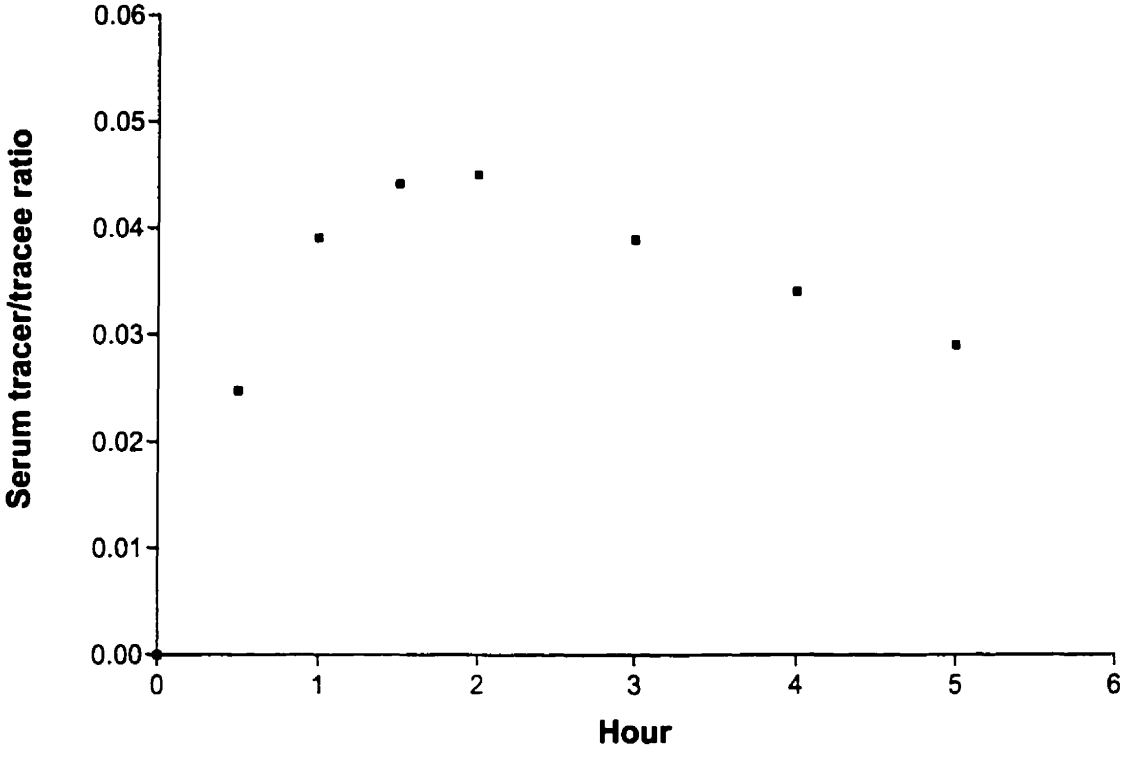

Figure 5. Serum $^{34}S/^{32}S$ sulfate tracer/tracee ratios in a 73-kg normal man after ingestion of 50 mg of $Na_2{}^{34}SO_4$. His calculated extracellular fluid volume was 17.1 L (23.4% of body weight).

added to a second aliquot of the same sample which is then analyzed in the manner of the stable isotope dilution ESI-MS/MS assay described above but with correction for the presence of the small amount of ^{34}S contributed by the tracer.

The sulfate measurement method described here has a number of advantages. It can be used in two modes: sulfate quantitation in biological fluids in the manner of a stable isotope dilution assay, as well as in a tracer/tracee mode using ^{34}S-labeled sulfate as the tracer. It is far more sensitive than IEC-CD measurements which cannot be used for tracer studies. A principal advantage is that it may do away with the need to administer sulfate labeled with the radioactive isotope ^{35}S, currently a standard technique for measuring extracellular fluid volumes in humans and animals (Pierson et al, 1982). Since the ^{34}S isotope of sulfur is stable, it eliminates the requirement for difficult and expensive radiosyntheses of the short half-life ^{35}S isotope-containing substrates shortly before use in metabolic studies.

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CONNECTING TEXT II

Our results from chapter 4 determined that $^{34}SO_4$ could be accurately measured in biological fluids using ESI-MS/MS, doing without the use of radiolabeled sulfate. Using this method, both sulfate concentration and $^{34}SO_4/^{32}SO_4$ TTR could be measured in serum and urine.

However, prior to meeting our objective to investigate the use of sulfate production as a valid measure of whole-body amino acid catabolism, we need first to establish a reliable method for measuring the sulfate distribution volume, a measure of the ECF, since the nontracer method calculates sulfate production as its urinary excretion corrected for changes in ECF.

Therefore, our next objective was to investigate the use of the stable isotope sodium [^{34}S]sulfate to measure SS and compare the results, determined by selecting the optimum sampling interval, to the simultaneously measured CBS.

CHAPTER V

Human Extracellular Fluid Volume Can Be Measured Using the Stable Isotope $Na_2{}^{34}SO_4$

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Human Extracellular Water Volume Can Be Measured Using the Stable Isotope $Na_2{}^{34}SO_4$

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Running Title: ECW measured with $Na_2{}^{34}SO_4$

Key words: extracellular water volume, stable isotope, corrected bromide space, sulfate space, body composition

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ABSTRACT

The volume of human extracellular water (ECF) may be estimated from the sulfate space (SS). Although it may better approximate ECF volume than the bromide space, a common alternative, SS measurement is limited by the need to administer a radioactive substance, sodium [^{35}S]sulfate. In this paper, we demonstrate the measurement of the SS using the stable isotope, sodium [^{34}S]sulfate. Eight healthy, non-obese men ingested 0.50-0.78 mg (3.47-5.42 μmol) $Na_2{}^{34}SO_4$/kg body weight and 30 mg NaBr/kg body weight. Sulfate concentrations and $^{34}SO_4$ enrichments were measured by electrospray tandem mass spectrometry prior to and during the 5 h following tracer administration. SS was calculated by linear extrapolation of the natural logarithm of serum $^{34}SO_4$ concentrations obtained at h 2, 3, and 4 compared with h 3, 4, and 5. The SS obtained using values between h 3 and 5 (187 ± 17 mL/kg) was similar to published determinations using intravenous or oral radiosulfate, and was 80 % of the simultaneously measured corrected bromide space (234 ± 10 mL/kg, P = 0.01). Oral sodium [^{34}S]sulfate administration is a suitable technique for measuring ECF and avoids radiation exposure.

INTRODUCTION

According to most authors, the extracellular fluid volume (ECF) of humans is best approximated by the sulfate space (SS) as measured by $^{35}SO_4$ isotope dilution (Bauer 1976; Bauer et al. 1975; Kragelund and Dyrbye 1967; Lacroix et al. 1965; Malpartida and Moncloa 1967; Omvik et al. 1979; Pierson et al. 1982; Ryan et al. 1956; Waki et al. 1991; Walser et al. 1953). The tracer is well absorbed and may be administered orally (Bauer 1976; Omvik et al. 1979). However, the radioactivity and short half-life of ^{35}S limit its usefulness. We have developed a method to measure serum sulfate concentrations and $^{34}SO_4$ enrichments using electrospray tandem mass spectrometry (ESI-MS/MS). In this paper, we describe the use of this methodology to measure the SS of normal humans.

This study was designed to select the appropriate dose of orally administered sodium [^{34}S]sulfate to determine the SS and to select the optimum sampling interval. The ECF was calculated from extrapolation to time zero of serum $^{34}SO_4$ concentrations using the natural logarithm (ln) of serum $^{34}SO_4$ between h 2 and 4 and h 3 and 5 of their $^{34}SO_4$ decay slopes to identify the optimum sampling interval. We compared the best estimate of the SS with simultaneously measured corrected bromide space (CBS), a common alternative for estimating ECF.

MATERIALS AND METHODS

Subjects

Eight healthy, non-obese men with normal blood biochemistries were studied at

the Clinical Research Unit of the Jewish General Hospital of Montreal (**Table 1**). All gave written consent for the study which was approved by the Research Ethics Committee.

Materials

$Na_2{}^{34}SO_4$ was purchased from Icon Services, Mt. Marion, NY (93% ^{34}S) and from Isoflex USA, San Francisco, CA (99% ^{34}S). Using the methods described in this paper, the isotope from Icon Services was determined to be 94% ^{34}S. NaBr was purchased from A&C American Chemicals, Montreal, QC, Canada. All the water used was Type 1 or ultrapure water (resistivity of 18.2 megohms/cm), purified by treatment with Milli-RO Plus and Milli-Q UF systems (Millipore, Bedford, MA). OnGuard-Ag cartridges (#39637) were obtained from Dionex, Oakville, ON, Canada, MPS micropartition cartridges (YMT 10,000 Da molecular weight cut-off membrane filter) from Amicon, MA, and 0.22 μm syringe filters from Chromatographic Specialties, Brockville, ON, Canada. To remove traces of sulfate before use, the OnGuard-Ag cartridges were rinsed with 30 mL water and the Amicon filter membranes were soaked in water for 1 h with three water changes.

Study design

Subjects were studied at 0700 h in the postabsorptive state. Their body weight was recorded and total body water (TBW) measured by bioimpedance analysis (BIA-101A; RJL Systems, Mt. Clemens, MI) (Kushner and Schoeller 1986).

Table 1. Subject characteristics.

Subject	Age	Weight	BMI	TBW	TBW/Wt
	(y)	(kg)	(kg/m^2)	(L)	(%)
1	40	79.9	24.8	46.7	58.4
2	26	72.9	21.5	46.3	63.5
3	27	86.1	26.3	51.2	59.5
4	28	63.8	21.6	36.5	57.2
5	30	78.0	24.8	48.9	62.7
6	46	68.8	20.8	41.5	60.3
7	25	61.9	25.2	34.8	56.2
8	30	84.4	24.9	49.8	59.0
Mean	31.5	74.5	23.7	44.5	59.6
± SD	7.5	9.1	2.1	6.2	2.5

BMI, body mass index; TBW, total body water; TBW/Wt, (total body water/body weight) x 100%.

Experimental protocol

Each of the first four subjects consumed 50 mg $Na_2^{34}SO_4$ (0.58-0.78 mg $Na_2^{34}SO_4$/kg body weight) and 30 mg NaBr/kg body weight dissolved in 160 mL of deionized water. Because this resulted in more than adequate serum enrichments, the tracer sulfate dose was reduced to 0.5 mg $Na_2^{34}SO_4$/kg body weight for the final four subjects. Blood samples were drawn prior to and 30, 60, 90, 120, 180, 240 and 300 min following tracer administration. Volunteers refrained from drinking water before and throughout this period. The blood sampling catheter was kept patent by infusing 0.45% (77mmol/L) NaCl at a rate of 20-24 mL/h. Urine was collected over 5 h.

Analytical methods

Clotted blood was centrifuged at 1400 *g* for 30 min; the serum transferred into screw cap vials and stored at -30°C. Serum sulfate was analyzed by ESI-MS/MS for sulfate concentration and $^{34}SO_4/^{32}SO_4$ enrichment, as described elsewhere (Boismenu et al. 1998). The sensitivity of this method is such that at 2 μmol/L, the 97 and 99 ions are easily detected at signal-to-noise ratios of 100 and 20, respectively. As a consequence, serum sulfate concentrations <200 μmol/L and tracer/tracee ratios <0.01 can be quantified with excellent precision. Briefly, to measure $^{34}SO_4/^{32}SO_4$ enrichment, 1.0 mL of serum was mixed with 0.5 mL of water and 5.0 mL of ice-cold methanol, kept on ice for 10 min, then centrifuged at 1400 *g* for 10 min at 4°C. The supernatant was acidified with 0.1 mL of 1 mol/L HCl to remove bicarbonate anions, then passed through an OnGuard-Ag cartridge to remove inorganic anions other than sulfate. The first 3 mL of

filtrate were discarded and the rest saved for analysis. To measure $^{32}SO_4$ concentration, 0.5 mL of 600 μmol/L $Na_2{}^{34}SO_4$ (0.3 μmol) internal standard replaced the 0.5 mL of water above.

ESI-MS/MS analyses were performed using a Quattro II triple quadrupole (Micromass, Manchester, UK) configured for negative ion analysis. Samples were introduced directly into the electrospray probe at 40 μL/min with a 1-mL disposable syringe under the following conditions: neutral loss mode, 17 Da; range, 92 to 102 Da; cone voltage, 25 V; source temperature, 120°C; sample infusion pump, 40 μL/min; nitrogen bath gas flow rate, 300 L/h; nebulizer gas flow rate, 18 L/h; collision cell energy, 23 eV; and argon pressure in the collision cell, 1.3×10^{-1} Pa. Acquisitions of 2 min each were made in triplicate in multichannel acquisition mode. The following ions were monitored: $H^{32}SO_4^-$ (m/z 97), and $H^{34}SO_4^-$ (m/z 99).

To measure serum sulfate enrichment due to the administered tracer, a calibration curve was constructed by preparing varying mole ratios of $^{34}SO_4/^{32}SO_4$, as described by Tserng and Kalhan (1983). The tracer to tracee ratio (TTR) was calculated by subtracting the baseline mole ratio from the sample mole ratio:

$$TTR = [(M+2)/M]_{sample} - [(M+2)/M]_{baseline}$$

where M is the signal intensity of m/z 97 and M+2 the signal intensity of m/z 99.

To measure serum $^{32}SO_4$ concentration, 0.3 μmol of $^{34}SO_4$ internal standard was added to tubes containing concentrations of $Na_2{}^{32}SO_4$ ranging from 0 to 600 μmol/L and an areas ratio standard curve constructed. Serum $^{32}SO_4$ concentrations were then determined using this standard curve after subtracting the contribution at mass 99 due to

the tracer as determined in a sample to which no internal standard was added. Serum $^{34}SO_4$ concentration was calculated as the product of sample TTR and $^{32}SO_4$ concentration.

Bromide was measured by ion exchange high performance chromatography with conductivity detection (IEC-CD) using a Dionex 2110i chromatography system (Dionex, Sunnyvale, CA) under the following conditions: carbonate-bicarbonate buffer mobile phase flow rate, 2.0 mL/min; suppressor regenerant, 25 mmol/L H_2SO_4; regenerant flow rate, 2 mL/min; background conductivity, 17 μSi; conductivity detector output range, 10 μSi; injection volume, 25 μL; 4 mm AMMS-II anion micromembrane suppressor; ion exchange Ionpac AG4A-SC pre-column; and AS4A-SC analytical column. Peak integration was performed with a Waters 740 Data Module (Milford, MA). Before injection onto the column, serum was diluted 40-fold in water and passed through a MPS micropartition cartridge. Urine was diluted 100-fold and filtered through 0.22-μm syringe filters. Samples were injected into the IEC-CD with a 1-mL disposable syringe (Becton Dickinson, Franklin Lakes, NJ). All samples were analyzed in duplicate.

Calculations

The SS was calculated as SS = [(dose of $^{34}SO_4$)/(zero-time serum $^{34}SO_4$ concentration)] x 0.95 x 0.94, where SS is the sulfate space in liters, the dose of $^{34}SO_4$ is expressed in μmol, zero-time serum $^{34}SO_4$ concentration is in μmol/L, 0.95 the correction factor for the Donnan equilibrium, and 0.94 the correction factor for the water content of serum (Bell et al. 1984; Forbes 1987). Zero-time $^{34}SO_4$ concentration was calculated by

submitting the natural logarithm (ln) of $^{34}SO_4$ concentration to a linear function and extrapolating to time zero, as described by Bauer (1976) and Ryan et al. (1956), using values either between h 2 and 4 ($\ln[^{34}SO_4]h2\text{-}4$) or between h 3 and 5 ($\ln[^{34}SO_4]h3\text{-}5$). Zero-time $^{34}SO_4$ concentration is the theoretical $^{34}SO_4$ concentration value that would be obtained if the $^{34}SO_4$ dose distributed instantaneously in the ECF.

The corrected bromide space (CBS) was calculated as described by Bell et al. (1984) as follows:

$$CBS = \frac{(\text{Br dose} - \text{Br excreted}) \times 0.90 \times 0.95 \times 0.94}{[\text{serum Br}]_{h\,5} - [\text{serum Br}]_{\text{baseline}}}$$

where CBS is in liters, Br dose in mmol, Br excreted is the mmol of Br excreted over 5 h, $[\text{serum Br}]_{h\,5}$ is the serum Br concentration at h 5 in mmol/L, $[\text{serum Br}]_{\text{baseline}}$ is the baseline serum Br concentration in mmol/L, 0.90 is the correction factor for the nonextracellular distribution of Br, 0.95 is the correction factor for the Donnan equilibrium (Forbes 1987), and 0.94 is the correction factor for the water content of serum (Bell et al. 1984; Forbes 1987; Leth and Binder 1970; Vaisman et al. 1987).

Statistical analysis

The paired Student's t-test was used to compare SS calculated using $\ln[^{34}SO_4]h2\text{-}4$ and $\ln[^{34}SO_4]h3\text{-}5$. The F-test was used to compare the variability in data fits for individual determinations of SS as well as between-subject variability for SS determined as $\ln[^{34}SO_4]h2\text{-}4$ and $\ln[^{34}SO_4]h3\text{-}5$. The paired t-test was used to compare SS by $\ln[^{34}SO_4]h3\text{-}5$ with CBS. Differences between data were considered significant at $P \leq$

0.05. Results are presented as mean ± standard deviation (SD) unless otherwise indicated.

RESULTS

On average, tracer enrichment reached a maximum 2 h after oral administration (Figure 1) but there was variability among the subjects. The time course of individual serum $^{34}SO_4$ concentrations are shown in Figures 2 (higher tracer dose) and 3 (lower tracer dose). Results with the lower tracer dose were comparable to those with the higher one. Figure 1 also shows that average serum $^{32}SO_4$ concentrations decreased by 18 % ($P < 0.0001$) over the 5-h study period.

As suggested by simple visual inspection of Figures 2 and 3, the log-linear reduction in $^{34}SO_4$ concentration was better described using concentrations at h 3, 4, and 5 than concentrations at h 2, 3, and 4. Thus, the variance for the average coefficient of determination (a measure of goodness of data fit for a linear function) based on h 2-4 was 0.1901, but only 0.0002 for values between h 3 and 5 ($P < 0.0001$, F test). In addition, the use of the later sampling times resulted in a significantly smaller between-subject standard deviation of the calculated SS ($P = 0.04$) and a smaller estimate of its size (Table 2).

The $\ln[^{34}SO_4]$h3-5 SS was strongly correlated with CBS ($r = 0.82$, $P = 0.012$). As illustrated in Figure 4, it was consistently less than CBS with all but one value lying to the right of the line of identity. SS determined in this way was 79.7 ± 5.7 % of CBS ($P = 0.008$).

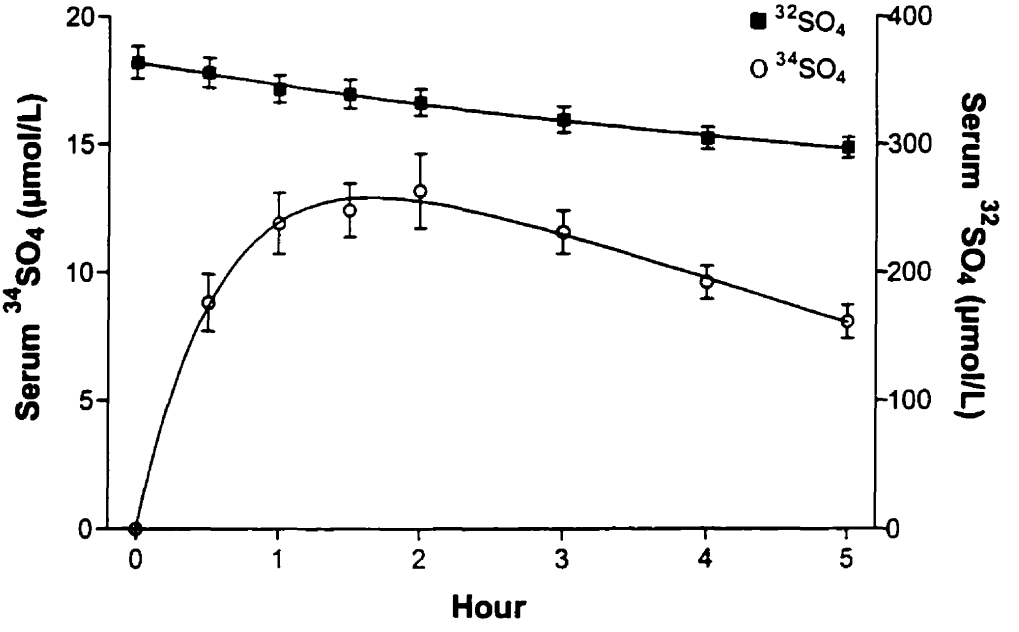

Figure 1. Serum $^{34}SO_4$ (○; µmol/L) and $^{32}SO_4$ (■; µmol/L) following oral administration of 0.50-0.78 mg (3.47-5.42 µmol) $Na_2{}^{34}SO_4$/kg body weight in eight healthy, non-obese men. Values are means ± SEM.

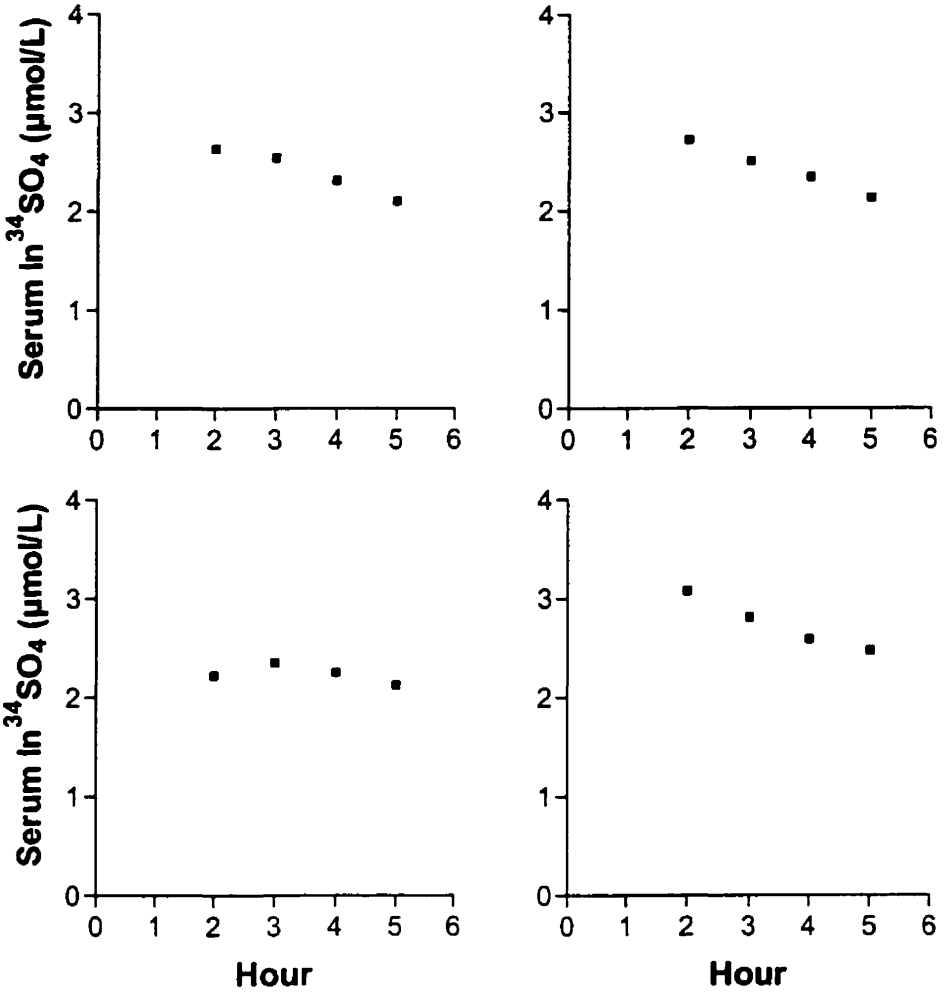

Figure 2. Individual serum $^{34}SO_4$ concentrations for four healthy men following oral administration of 50 mg (347 µmol) of $Na_2{}^{34}SO_4$.

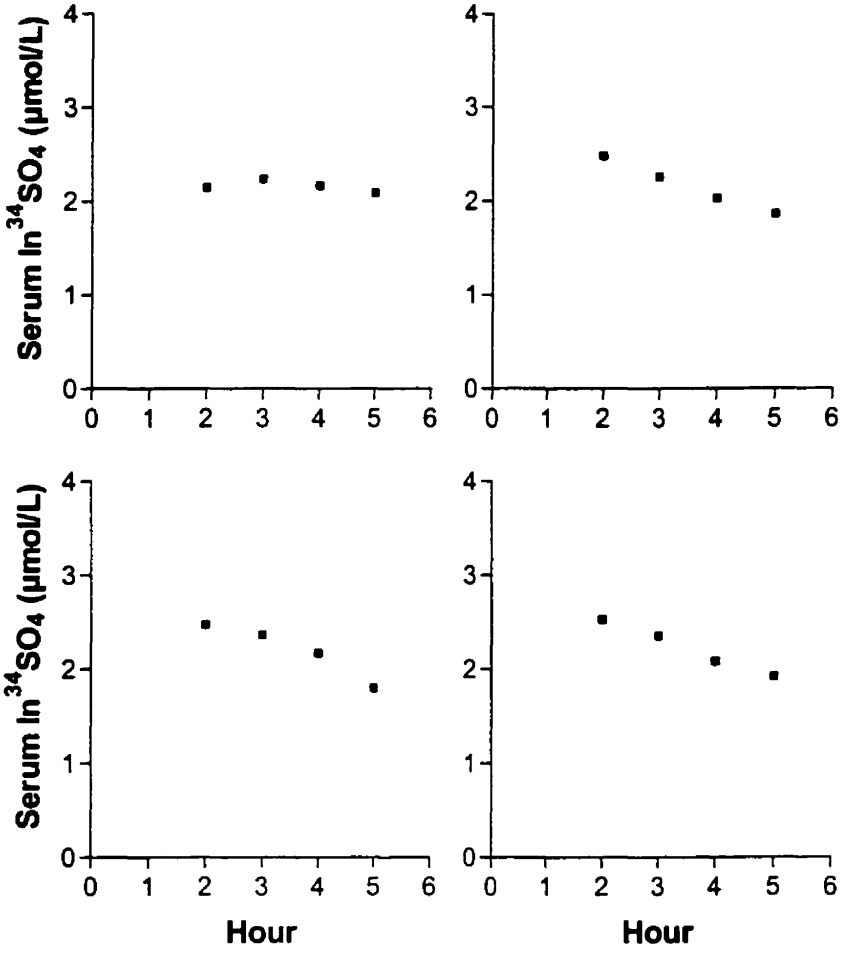

Figure 3. Individual serum $^{34}SO_4$ concentrations for four healthy men following oral administration of 0.50 mg (3.47 µmol) of $Na_2{}^{34}SO_4$/kg body weight.

Table 2. Extracellular fluid volume (ECF) in eight healthy, non-obese men as measured using $^{34}SO_4$ and Br following oral administration of $Na_2{}^{34}SO_4$ and NaBr.

Subject	ECF, L			ECF, mL/kg		
	ln[$^{34}SO_4$]h2-4	ln[$^{34}SO_4$]h3-5	CBS	ln[$^{34}SO_4$]h2-4	ln[$^{34}SO_4$]h3-5	CBS
1	15.7	12.6	17.9	196.1	157.0	224.3
2	14.1	14.3	17.8	193.7	195.8	244.5
3	33.0	20.8	18.7	383.7	241.7	217.6
4	8.8	11.4	14.4	137.8	179.2	225.8
5	28.0	20.6	21.7	359.3	264.3	277.8
6	11.3	12.5	18.6	164.0	181.8	270.5
7	11.8	7.5	12.0	190.7	120.6	193.7
8	13.1	13.2	18.4	155.1	156.7	218.0
Mean	17.0	14.1	17.4	222.5	187.2	234.0
± SD	8.7	4.5	3.0	94.4	46.8	28.5

Sulfate space was measured using values between h 2 and 4 (ln[$^{34}SO_4$]h2-4) and h 3 and 5 (ln[$^{34}SO_4$]h3-5).

ECF, extracellular fluid volume; CBS, corrected bromide space.

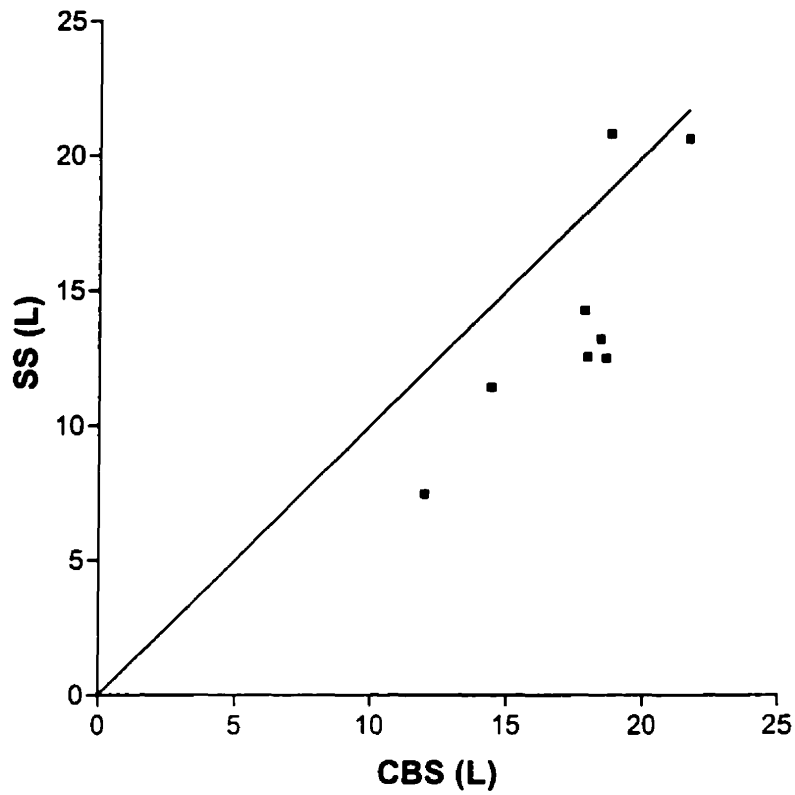

Figure 4. Relationship between the corrected bromide space (CBS) and sulfate space (SS) measured in eight healthy, non-obese men using the ln[$^{34}SO_4$]h3-5 method [y = (1.26 ± 0.36)x - (7.90 ± 6.32), r = 0.82, P = 0.012). SS and CBS were measured following oral administration of 0.50-0.78 mg (3.47-5.42 μmol) $Na_2{}^{34}SO_4$/kg body weight and 30 mg NaBr/kg body weight. The line of identity is shown.

DISCUSSION

We have demonstrated the use of an orally administered stable isotope of sulfate to determine ECF in eight healthy men. We determined the optimal serum sampling time for measuring SS and compared the resulting best estimate of SS with simultaneously measured CBS. Our data indicate that SS is optimally measured using serum $^{34}SO_4$ values between h 3 and 5, and this SS is approximately 80 % of CBS.

Mean enrichment 5 h folloiwng oral administration of $Na_2{}^{34}SO_4$ was TTR = 0.027 ± 0.006, well within the sensitivity of the method. Indeed it is likely that a dose one-half to one-fourth the lowest dose used in this study would provide excellent precision.

Although, on average, $^{34}SO_4$ enrichment reached a maximum 2 h following oral tracer administration, there was variability among the subjects, with some reaching a maximum at h 3 (Figures 1-3). In this respect, our data differ from those of Bauer (1976) who obtained the maximum $^{35}SO_4$ concentration 60-105 min after an oral dose of radiosulfate. This could be explained by slower gastrointestinal absorption of a 30- to 50-mg dose of $^{34}SO_4$ than the much smaller mass (2-3 MBq, or 60-80 μCi) of a dose of radioactive $^{35}SO_4$. In our study, serum concentrations that included h 2 were variably influenced by the tracer absorption phase, whereas those between h 3 and 5 consistently represented the elimination phase of serum $^{34}SO_4$ (Figures 2 and 3). SS calculated using h 3-5 was associated with a better data fit for individual studies and a smaller between-subject variance than SS calculated using h 2-4. We conclude that using data points before h 3 following oral tracer administration may result in greater variability and the overestimation of SS.

It is noteworthy that the serum $^{32}SO_4$ concentration decreased gradually over the course of the SS measurement. This has not been noted in previous studies, in which serum radioactivity rather than sulfate was measured, but it is to be expected because sulfate is cleared into the urine and sulfur intake is zero during this measurement. This does not invalidate an isotope dilution technique based on zero-time extrapolation. In fact, if SS is calculated by extrapolation of ln serum TTR rather than $^{34}SO_4$ concentration, the resulting SS is insignificantly different: 182 ± 46 mL/kg for extrapolation of tracer enrichment versus 187 ± 47 mL/kg for tracer concentration (P = 0.26).

As shown in Table 3, the SS determined in this study is similar to values obtained by other researchers using oral or intravenous radiosulfate. The CBS values obtained are also similar to ones reported by other researchers for normal persons. The SS was 79.7 ± 5.7 % of CBS, confirming results obtained by Lacroix et al. (1965) in men and women (85 ± 10 %), Yu et al. (1996) in men (83.9 %), and Barratt and Walser (1969) in rats (80 ± 0.4 %) when SS and CBS were compared directly. The reason for this constant difference between the SS and the CBS is unknown.

The use of radiosulfate in this study might have further confirmed the validity of our new method. However, we were persuaded that a direct comparison between the radioactive and stable isotope methods was technically unnecessary because they are both based on the same principle of isotope dilution and zero-time extrapolation, and valid comparison data for SS and CBS are readily available from the literature. For these reasons, and the availability of the stable isotope alternative in our laboratory, we could not ethically justify exposing normal research volunteers to radioactive sulfate.

We conclude that the oral administration of $^{34}SO_4$ provides a practical alternative for measuring SS, with the advantage of avoiding oral administration of a radioactive tracer. Serum samples are best obtained at h 3, 4, and 5 following tracer ingestion. Our results confirm that SS is 20% smaller than CBS in adults of normal body composition ven after standard correction for Br penetration into erythrocytes.

Table 3. Extracellular fluid volume (ECF) in humans using radiosulfate or sodium [^{34}S]sulfate and bromide as reported by different research groups.

Research group	Sample size	Age	Weight (BMI)	Route of administration	SS	CBS
		(y)	kg (kg/m^2)		(mL/kg)	(mL/kg)
Present study	8 ♂	32 ± 3	75 ± 3 (24 ± 1)	oral bromide oral sulfate	187 ± 17	234 ± 10
Lacroix et al, 1965	11 ♂, ♀	42 ± 5	58 ± 3	i.v. bromide i.v. sulfate	217 ± 7	255 ± 8
Yu et al, 1996	7 ♂			oral bromide oral sulfate	16.7 ± 3.1 L	19.9 ± 1.0 L
Walser et al, 1953	10 ♂ 8 ♀	41 ± 3 36 ± 4	71 ± 4 (24 ± 1) 60 ± 3 (22 ± 1)	i.v.	151 ± 8 140 ± 11	
Ryan et al, 1956	11 soldiers ♂, ♀ 9 young ♂ 5 elderly ♂ 8 elderly ♀	19 ± 0 26 ± 1 63 ± 1 65 ± 3	70 ± 1 72 ± 3 74 ± 6 70 ± 5	i.v.	191 ± 1 167 ± 1 149 ± 1 155 ± 1	

continued

continued, Table 3

Research group	Sample size	Age	Weight (BMI)	Route of administration	SS	CBS
		(y)	kg (kg/m^2)		(mL/kg)	(mL/kg)
Lacroix, et al, 1965	32 ♂ 10 ♀	42 ± 3 38 ± 5	63 ± 2 56 ± 3	i.v.	224 ± 6 189 ± 11	
Kragelund and Dyrbye 1967	11 ♂ 14 ♀	42 (25-60) 43 (20-61)	73 60	i.v.	208 180	
Malpartida and Moncloa 1967	14 ♂	19-27	56-74	i.v.	153 ±5	
Bauer et al. 1975	4		76 ± 8	i.v.	188-195	
Bauer 1976	8 ♂		77 ±16	oral i.v.	201 ± 9 220 ± 7	
Omvik et al. 1979	12 ♂, ♀			oral	189 ± 7	
Pierson, Jr et al. 1982	58 ♂, ♀	19-80	51-96 (18-30)	i.v.	230	

continued

continued, Table 3

Research group	Sample size	Age	Weight (BMI)	Route of administration	SS	CBS
		(y)	kg (kg/m^2)		(mL/kg)	(mL/kg)
Waki et al. 1991	26 ♀	38 ± 3	57 ± 1 (21 ± 1)	i.v.	214	
Lacroix, et al. 1965	8 ♂ 4 ♀	42 ± 6 38 ± 7	63 ± 3 50 ± 1	oral		269 ± 7 232 ± 7
Leth and Binder. 1970	7 ♂, ♀ 16 ♂, ♀	53 ± 3 49 ± 4		oral i.v.		252 ± 14 236 ± 10
Vaisman et al. 1987	7 ♂	26-35		oral i.v.		253 ± 8 242 ± 2
Miller et al. 1989	28 ♂ 54 ♀	30 ± 2 29 ± 2	85 ± 3 73 ± 3	oral		227 ± 6 213 ± 5
McCullough et al. 1991	8 ♂ 6 ♀	35 ± 4 33 ± 3	80 ± 3 (24 ± 1) 63 ± 2 (22 ± 1)	oral		209 224

continued

continued, Table 3

Research group	Sample size		Age	Weight (BMI)	Route of administration	SS	CBS
			(y)	kg (kg/m^2)		(mL/kg)	(mL/kg)
Mørkeberg et al. 1992	10 ♂		28 ± 1	77 ± 3	oral		268 ± 6
van Kreel 1994	6 ♂, ♀			72 ± 8 (23 ± 2)	oral		242 ± 10
Deurenberg et al. 1995	139 ♂, ♀	♂	26 ± 1	75 ± 1 (22 ± 0)	oral		243
		♀	26 ± 1	66 ± 1 (23 ± 0)			218
Ma et al. 1996	5 ♂		42 ± 4	70 ± 3 (24 ± 1)	oral		186 ± 9

Values are presented as means ± SEM; ECF, extracellular fluid volume; BMI, body mass index; SS, sulfate space; CBS, corrected bromide space; i.v., intravenous.

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CONNECTING TEXT III

Our results from chapter 5 determined that the oral administration of $^{34}SO_4$ provides a practical alternative for measuring SS, with the advantage of avoiding oral administration of a radioactive tracer. Serum samples are best obtained at h 3, 4, and 5 following tracer ingestion. The SS is 20% smaller than CBS in adults of normal body composition even after standard correction for Br penetration into erythrocytes.

Having determined in chapters 4 and 5 that the stable isotope $^{34}SO_4$ could be accurately measured in biological fluids using ESI-MS/MS and that the tracer could be used to measure SS in humans (80% of CBS), we proceeded with our objective to evaluate the use of sulfate production as an indicator of whole-body SAA oxidation. To do this, we determined whether tracer and nontracer methods accurately depict sulfate production under basal steady state conditions and following changes in sulfate production achieved by sulfate infusion. We measured sulfate recovery following intravenous administration of a known amount of magnesium sulfate and sulfate recovery from methionine following oral methionine. We also compared balances of urea-N and S following oral methionine and intravenous administration of a mixture of essential amino acids containing methionine.

CHAPTER VI

Use of Sulfate Production as a Measure of Short-Term Sulfur Amino Acid Catabolism in Humans

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Use of Sulfate Production as a Measure of Short-Term Sulfur Amino Acid Catabolism in Humans

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ABSTRACT

There is no fully satisfactory method for measuring amino acid catabolism in the non-steady state that follows normal protein consumption. Since sulfate is the major product of sulfur amino acid catabolism, we tested whether its production can be accurately depicted using simple tracer or non-tracer approaches under basal conditions and following the intravenous administration of a known amount of sulfate. In the basal, postabsorptive state serum sulfate concentration and urinary sulfate excretion remained constant for many hours, but the apparent steady-state serum sulfate Ra achieved with primed continuous oral administration of sodium [^{34}S]sulfate was 20% higher than urinary sulfate excretion. By contrast, after magnesium sulfate infusion the increase in sulfate production above basal accounted for 95% over 6 h and 98% over 9 h of the administered dose when measured simply as urinary inorganic sulfate excretion, corrected for changes in its extracellular fluid content. Using the latter method, we measured sulfate production following oral methionine and the intravenous infusion of methionine in a mixture of other essential amino acids. Sulfate production above basal accounted for 59% over 6 h and 75% over 9 h of the oral methionine dose. Similar results were obtained with the mixed amino acid infusion, but interpretation of the latter experiment was limited by the mild protein sparing (and hence, reduced endogenous sulfate production) induced by the amino acid infusion. We conclude that a simple non-tracer method can provide an accurate measure of sulfate production and hence, sulfur amino acid catabolism, over collection periods as short as 6 h following a test meal. A significant portion of the S derived from methionine appears to be retained in non-protein

compounds immediately following its ingestion.

INTRODUCTION

The body's allocation of dietary amino acids for catabolism or protein synthesis largely takes place in the fed state, and for this reason accurate measurement of fed-state amino acid catabolism is important for understanding the factors regulating whole-body protein economy (El-Khoury et al, 1994; McNurlan et al, 1989; Owen et al, 1992; Quevedo et al, 1994; Taveroff et al, 1994; Tessari et al, 1988). Both urea production and measures of essential amino acid (usually leucine) oxidation have been used for this purpose. However, the amino acid oxidation technique requires an accurate measurement of expired tracer carbon dioxide, and we have recently shown that short-term changes in urea production, as typically occur in the normal fed state, may not be reliably depicted by either tracer or non-tracer methods (Hamadeh and Hoffer, 1998). Sulfate production is a potentially useful adjunct to urea production and essential amino acid oxidation when measuring whole-body amino acid catabolism.

Catabolism of the sulfur amino acids (SAA), methionine, cysteine and cystine, leads predominantly to the production of sulfate, since mercaptolactate, mercaptoacetate, thiocyanate, thiosulfate and taurine contribute only very small amounts to the total (Martensson, 1982; Martensson and Hermansson, 1984; Stipanuk, 1999). When measured over 24-hour periods, urinary sulfate excretion closely matches both dietary SAA intake (Sabry et al, 1965; Zlotkin and Anderson, 1982) and N excretion (Jourdan et al, 1980; Lakshmanan et al, 1976; Zlotkin and Anderson, 1982). However, it has not been determined whether sulfate production can be measured accurately over periods of only a few hours, such as those following a typical meal. Short-term sulfate kinetics

might be more accurately determined than those of urea, since unlike urea, which distributes throughout total body water, sulfate distributes in the considerably smaller extracellular fluid volume (ECF) (Ryan et al, 1956); moreover, unlike urea, it is not subject to important losses in the gut (Bauer, 1976; Cakmakjian and Bethune, 1966; Omvik et al, 1979; Ryan et al, 1956).

The present research was carried out to determine whether tracer and non-tracer methods accurately depict sulfate production under basal steady state conditions and after the change in sulfate production created by a sulfate infusion designed to mimic the amount of sulfate that would be produced after a typical protein meal. Specifically, we measured basal sulfate rate of appearance (Ra) using primed continuous oral administration of sodium [^{34}S]sulfate and compared this result with a non-tracer technique based on urinary excretion. We determined the accuracy of the non-tracer method to detect sulfate production following intravenous administration of a known amount of magnesium sulfate. We tested whether basal sulfate production as measured over a 3-h baseline period is an accurate predictor of sulfate production over the subsequent 9 h in subjects whose recent previous protein intake was low or high. We measured sulfate production following oral administration of methionine and the intravenous infusion of a mixture of essential amino acids containing methionine under conditions in which baseline endogenous SAA catabolism would be expected to be constant or nearly constant. This was done to determine whether exogenous surfeit methionine is completely catabolized to sulfate within a short time after ingestion or its S retained in a non-protein form in the body. The measurement periods were both 6 and 9

h, with the aim of finding the most convenient valid collection periods for future studies.

METHODS

Subjects and protocols

Eleven healthy men with normal serum biochemistries and taking no medications participated in 6 study protocols. Their physical characteristics are presented in Table 1. When the same subject was studied more than once, at least 6 weeks separated testing days. All volunteers provided written consent for the study, which was approved by the Research and Ethics Committee of the Jewish General Hospital in Montreal.

The subjects were admitted to the clinical research unit at 08:00 h one day prior to the study day. Breakfast (08:30), lunch (12:30), and dinner (17:30) at maintenance energy (38kcal/kg body weight) were consumed following anthropometric measurements. When used, a low-protein diet (0.3 g/kg body weight.d) consisted of low protein foods (juice, butter, jam, mashed potatoes, green beans, carrots, tea, coffee, ginger ale, and sugar), and low-protein bread and wafers. Except for a multiple vitamin-mineral tablet (Centrum Forte, Whitehall-Robins, Mississauga, ON, Canada), consumption of other foods and beverages was not permitted. The studies were carried out the next morning with subjects overnight-fasted for 12-14 h. Body weight was recorded and total body water (TBW) measured by bioimpedance analysis (RJL Systems BIA-101A, Mt Clemens, MI) (Kushner and Schoeller, 1986). In every study, 30 mg NaBr/kg body weight (A & C American Chemicals, Montreal, QC, Canada) was administered by mouth to determine the corrected bromide space (CBS), a measure of the

Table 1. Subject characteristics

	Model validation studies			Methionine administration studies		
	Fasting-LP	Sulfate infusion	Fasting-HP	High-dose methionine	Oral methionine	Amino acid infusion
Sample size	5	5	5	5	5	5
Age (y)	33 ± 5	33 ± 5	36 ± 4	39 ± 3	35 ± 4	37 ± 3
Weight (kg)	73 ± 4	71 ± 3	77 ± 3	73 ± 4	73 ± 3	75 ±3
Height (cm)	174 ± 5	173 ± 5	179 ± 2	176 ± 2	177 ± 2	175 ± 3
BMI (kg/m^2)	24 ± 1	24 ± 1	24 ± 1	24 ± 1	23 ± 1	25 ± 1
TBW (% body weight)	57 ± 1	56 ± 2	56 ± 2	57 ± 2	56 ± 2	56 ± 2
FFM (% body weight)	78 ± 1	77 ± 2	77 ± 2	78 ± 3	77 ± 3	77 ± 3
CBS (ml/kg)	210 ± 6	204 ± 7	205 ± 9	209 ± 10	210 ± 10	204 ± 11

Data are presented as mean ± SEM. The data were not significantly different between groups. BMI, body mass index; TBW, total body water; FFM, fat free mass; CBS, corrected bromide space.

ECF (Vaisman et al, 1987). During the measurement periods, 77 mmol/L NaCl was infused at a rate of 50 mL/h and the subjects consumed 250-350 mL of water hourly to maintain adequate urine flow (355 ± 13 mL/h).

The 6 studies were carried out in 2 groups. Three *model validation studies* were carried out in subjects who remained fasting over the entire measurement period. These studies were designed with three aims. First, to measure basal sulfate rate of appearance (Ra) using simultaneous tracer and non-tracer methods after one day of a restricted protein intake ("fasting-LP"). The one day of protein restriction prior to the study day was not used to induce nutritional adaptation, but rather to reduce endogenous sulfate production and its variability, thereby permitting a sharp comparison between the tracer and non-tracer methods. Second, to compare the sulfate production value generated by the non-tracer technique with true sulfate appearance resulting from intravenous administration of a known amount of magnesium sulfate. Third, to determine whether sulfate production remains constant for many hours in fasting subjects even when their dietary protein intake had not been restricted the previous day ("fasting-HP"). Such constancy would justify using the value of sulfate production measured over a 3-h baseline period as an accurate predictor of endogenous sulfate production over the subsequent 9 h in subjects with a wide range of prior habitual protein intakes. For these studies the protein intake the day before the study was 1.5 g/kg body weight, a customary surfeit level for healthy young men (Gray-Donald et al, 2000).

Three *methionine administration* studies were carried out. In a dose-finding study ("high-dose methionine"), 0.45 mmol/kg of methionine was taken orally. In the two

subsequent studies, 0.15 mmol/kg of methionine was administered following one day of a normal protein diet (1.5 g/kg body weight.d), either as oral methionine taken alone or intravenous methionine within a mixture of non-sulfur essential amino acids.

Model validation studies

Fasting-LP study. Following a priming dose of 0.5 mg/kg body weight (3.47 μmol/kg), 0.05 mg/kg.h of anhydrous sodium [^{34}S]sulfate (99% ^{34}S, Isoflex USA, San Francisco, CA) in water was administered by mouth every 30 min for 9 h to measure endogenous sulfate Ra and determine whether steady-state sulfate Ra agrees with non-tracer determined sulfate production, as measured using hourly urinary sulfate excretion corrected for changes in ECF sulfate.

Sulfate infusion study. Endogenous sulfate production was measured using the non-tracer method for 3 h, after which 0.15 mmol/kg of $MgSO_4 \cdot 7H_20$ (Sabex Inc., Boucherville, QC, Canada) was infused. A Harvard Syringe Infusion Pump 681 E (Harvard Apparatus Co, Milford, MA) was adjusted to administer 60 μmol/kg.h magnesium sulfate for 2.5 h from a stock solution with a concentration of 400 μmol/mL. The amount of magnesium sulfate infused was verified by weighing the infusate container before and after each infusion. Urine was collected hourly for the first 3 h and in two separate collections thereafter (hours 4-9, and hours 10-12).

Fasting-HP study. Serum sulfate concentration and urinary sulfate excretion were measured over 12 h with hourly urine collections.

Methionine administration studies

Oral methionine. Basal sulfate production was measured for 3 h, after which subjects consumed a "high dose" 0.45 mmol (67.05 mg)/kg or a "low dose" 0.15 mmol (22.35 mg)/kg of methionine in water over 30 min (Tanabe USA Inc., San Diego, CA).

Amino acid infusion study. Basal sulfate production was measured for 3 h, after which 0.15 mmol/kg of methionine was administered intravenously at a constant rate as AMINOSYN-RF 5.2% (Ross Laboratories, St Laurent, QC) over 2 h. AMINOSYN-RF 5.2% contains (in mg/100 mL): isoleucine, 462; leucine 726; lysine, 535; methionine, 726; phenylalanine, 726; threonine, 330; tryptophan, 165; valine, 528; arginine, 600; histidine, 429. The product was infused using a Baxter Flo-Gard volumetric infusion pump adjusted to administer 11.2 mg of methionine/kg.h (75 μmol/kg.h) for 2 h from a stock solution with a concentration of 726 mg/100 mL (4.87 mmol/100 mL). The amount infused was verified by weighing the infusate container before and after each infusion. For the methionine administration studies, urine was collected hourly for the first 3 h and in two separate collections thereafter (hours 4-9, and hours 10-12).

Analytical methods

Inorganic sulfate

Unless referred to otherwise, all sulfate measurements refer to the inorganic sulfate anion. Blood collection tubes were centrifuged at 1400 *g* for 30 min and the serum transferred into screw-cap vials and stored at -30°C. Serum and urine were analyzed by ion exchange high performance chromatography with conductivity detection

(IEC-CD) using a Dionex 2110i chromatography system (Dionex, Sunnyvale, CA, USA) for inorganic sulfate and bromide concentrations, as previously described (Boismenu et al, 1998; Hamadeh et al, 1999). Serum was diluted 10-fold with water, passed through a 10,000 Da molecular weight cut-off Amicon filter (Beverley, MA) by centrifugation (1400 *g*), and the filtrate injected into the IEC-CD. Urine was diluted 50-fold, filtered through 0.45-μm syringe filters, and injected into the IEC-CD. Samples were analyzed in triplicate.

$^{34}SO_4/^{32}SO_4$ enrichment

$^{34}SO_4/^{32}SO_4$ enrichment was measured by electrospray tandem mass spectrometry (ESI-MS/MS), as previously described (Boismenu et al, 1998). In brief, 1.0 mL of serum was mixed with 0.5 mL of water and 5.0 mL of ice-cold methanol, kept on ice for 10 min, then centrifuged at 1400 *g* for 10 min at 4°C. The supernatant was acidified with 0.1 mL 1 M HCl to remove bicarbonate anions, then passed through an OnGuard-Ag cartridge (Dionex, Oakville, ON, Canada) to remove inorganic anions other than sulfate. The first 3 mL of filtrate were discarded and the rest saved for analysis.

ESI-MS/MS analyses were performed using a Quattro II triple quadrupole (Micromass, Manchester, UK) configured for negative ion analysis. Samples were introduced directly into the electrospray probe at 40 μL/min with a 1 mL disposable syringe under the following conditions: neutral loss mode, 17 Da; range, 92 to 102 Da; cone voltage, 25 V; source temperature, 120°C; sample infusion pump, 40 μL/min; nitrogen bath gas flow rate, 300 L/h; nebulizer gas flow rate, 18 L/h; collision cell

energy, 23 eV; and argon pressure in the collision cell, 130 Pa. Acquisitions of 2 min each were made in triplicate in multi-channel acquisition mode. The following ions were monitored: $H^{32}SO_4^-$ (m/z 97), and $H^{34}SO_4^-$ (m/z 99).

To measure sulfate enrichment due to the administered tracer, a calibration curve was constructed by preparing varying mole ratios of $^{34}SO_4/^{32}SO_4$, as described by Tserng and Kalhan (1983).

Organic (ester) sulfate

Inorganic sulfate was eliminated from urine samples by barium chloride precipitation, as described by Lundquist et al (1980). Three mL of a solution of 0.977 g $BaCl_2.2H_2O$ and 4.1 mL concentrated HCl in 100 mL were mixed with 1.5 mL urine, centrifuged at 1400 *g* for 10 min at 20°C, and 1.5 mL of the supernatant applied to 1 mL columns of cation exchange resin (Dowex 50W-X8, 100-200 mesh hydrogen form, Bio-Rad Laboratories, Richmond, CA). Water (2.5 mL) was added and the eluate was collected in screw-cap glass tubes. The sealed tubes were heated at 100°C for 30 min in a water bath to release organic sulfate esters (Lundquist et al, 1980), and 0.5 mL of the resulting solution was diluted 10 fold. To 1 mL of this diluted solution, 5 mL of ice-cold methanol was added and sulfate measured.

Other measurements

Plasma amino acid concentrations were measured by high performance liquid chromatography (HPLC) after derivatization with phenylisothiocyanate (Robitaille and

Hoffer, 1988). Serum bromide was measured by IEC-CD as previously described (Hamadeh et al, 1999) in order to calculate the ECF. The ECF was measured as the corrected bromide space (CBS) (Vaisman et al, 1987). Serum insulin was measured using a human insulin specific radioimmunoassay kit (Cat. # HI-14K, Linco Research Inc., St. Charles, MO). Samples were counted using a Cobra II Auto-Gamma (model D5002, Canberra-Pakard Canada, St. Laurent, QC, Canada/Pakard Instrument Company Inc., Downers Grove, IL). Serum and urine urea and creatinine were analyzed using Hitachi 917 automated analyzer (Laval, QC, Canada).

Calculations

Sulfate Ra, enrichment and recovery

Sulfate Ra was calculated as i/TTR, where i is the rate of tracer sulfate administration in μmol/kg.h. The tracer to tracee ratio (TTR) was calculated by subtracting the baseline mole ratio from the sample mole ratio:

$$TTR = [(M+2)/M]_{sample} - [(M+2)/M]_{baseline}$$

where M is the signal intensity of m/z 97 and M+2 the signal intensity of m/z 99.

Serum inorganic $^{34}SO_4$ concentration was calculated as follows:

$$[^{34}SO_4] = \frac{[SO_4] \cdot TTR}{1 + TTR}$$

where $[^{34}SO_4]$ is inorganic $^{34}SO_4$ concentration in μmol/L, $[SO_4]$ is the concentration of inorganic sulfate as measured by IEC-CD in μmol/L, and TTR is the tracer to tracee ratio.

The non-tracer method calculates cumulative inorganic sulfate production between time t_0 and t as its urinary excretion rate corrected for a change in ECF sulfate over this period:

Cumulative SO_4 production between times t_0 and t =

urinary SO_4 excretion over that period + $([SO_4]$ at time t - $[SO_4]$ at time $t_0) \cdot ECF$

When measured hourly, urinary sulfate excretion was normalized for urinary creatinine excretion to correct for incomplete bladder emptying, as described by Cheema-Dhadli and Halperin (1993). The studies to measure the recovery of infused intravenous magnesium sulfate as sulfate production, and the increase in sulfate production after methionine administration, had to take into account ongoing sulfate production from the catabolism of endogenous SAA. Thus, sulfate production due to exogenous methionine = total sulfate production - sulfate production due to endogenous SAA catabolism occurring over the same period. To do this we measured endogenous sulfate production over a 3 h baseline period before administering the test dose, and extrapolated that value to the subsequent 6 or 9 h. This assumes that (1) basal sulfate production is constant over a 6 or 9 h collection period, and (2) the test substrate does not alter endogenous sulfate production by increasing or decreasing proteolysis. The first assumption was tested in the fasting studies in which basal sulfate production was monitored for 9 or 12 h the morning after consuming low or conventional protein diets. The second was indirectly tested by measuring urea N balance.

Urea production

Urea production was calculated as its urinary excretion rate corrected for changes in total body urea as in the equation above for sulfate production (Cheema-Dhadli and Halperin, 1993; Geiseler et al, 1985; Hamadeh and Hoffer, 1998; Kay et al, 1986).

Total body water and bromide spaces

Body electrical resistance was measured after the patient voided and before placement of the intravenous catheters. TBW was then calculated using the equation of Kushner and Schoeller (1986). For the fasting-LP study, sodium bromide was given orally at time 0. For the other studies, it was given at h 6 and serum and urinary bromide measured at h 9. The corrected bromide space (CBS) was calculated as described by Vaisman et al (1987):

$$CBS = \frac{[\text{Br dose} - \text{Br excreted}] \cdot 0.90 \cdot 0.95 \cdot 0.94}{[Br]_{h9} - [Br]_{h6}}$$

where CBS is in liters, the Br dose is in mmol, Br excreted is mmol of Br excreted over 3 h, $[Br]_{h9}$ is the serum Br concentration at hour 9 in mmol/L, $[Br]_{h6}$ is the baseline serum Br concentration in mmol/L, 0.90 the correction factor for the non-extracellular distribution of Br, 0.95 the correction factor for the Donnan equilibrium, and 0.94 the correction factor for the water content of serum.

Statistical analyses

One-way analysis of variance (ANOVA) was used to determine significant

difference in subject characteristics between different protocols (Table 1). One-way repeated measures ANOVA was used to determine significant difference over time within the same protocol (Figures 1, 2, 3 and 6). Two-way repeated measures ANOVA was used to determine significant difference in sulfate production rate (time vs. method of measurement) for the fasting-LP study (Figure 1), and in serum sulfate and insulin (time vs. method of methionine administration) between the oral methionine and amino acid infusion studies (Figures 5 and 7). When ANOVA results showed significance, Newman Keuls test was used post hoc to determine the source of difference. The unpaired t test was used to compare data between the oral methionine and amino acid infusion studies (Tables 3 and 4). Differences between data were considered significant at $P \leq 0.05$. All results are presented as mean ± standard error of the mean (SEM).

RESULTS

Unless specified otherwise, sulfate measurements refer to the inorganic sulfate anion. Average postabsorptive serum sulfate concentrations ranged between 250-330 μmol/L for the different protocols, similar to previously reported values for healthy normal humans (Cheema-Dhadli and Halperin, 1993; Cole and Scriver, 1980; Hoffman et al, 1990).

Model validation studies

Fasting-LP study

When studied in the postabsorptive state and having consumed a low protein diet

the previous day, normal subjects maintained a constant serum sulfate concentration (Figure 1). Urinary sulfate excretion over the final 6 h was 8.3 ± 5.8% less than during the initial 3 h, but this difference was not statistically significant (P = 0.23).

Serum $^{34}SO_4$ enrichment reached apparent steady state soon after commencing the primed continuous tracer administration (Figure 1). However, the sulfate Ra calculated from these enrichments was 20 ± 2% higher than indicated by urinary sulfate excretion (P = 0.006).

To investigate the reason for this, we measured the $^{34}SO_4$ enrichment in organic sulfate esters in the urine, and found that it appeared rapidly in this fraction and increased over subsequent hours, but did not reach equilibrium with the inorganic sulfate pool over the time course of the study (Figure 2). Sulfate ester excretion was constant (0.99 ± 0.05 μmol/kg.h) as measured during hours 3, 6 and 9, representing 15% of total urinary sulfate excretion.

Sulfate infusion study

The serum sulfate concentration increased 2.4 fold during the magnesium sulfate infusion, then returned to near baseline by hour 12 (Figure 3). Assuming basal sulfate production over the 3-h baseline period remained constant over the subsequent 9 h, recovery of the infused sulfate in urine and ECF accounted for 95.4% over 6 h and 97.9% over 9 h after the start of the infusion (both NS from 100%; see Table 2). Going by the results of the fasting-LP study, we could not rule out a slight decrease in endogenous sulfate production over this time period. If correction is made for this, however, recovery

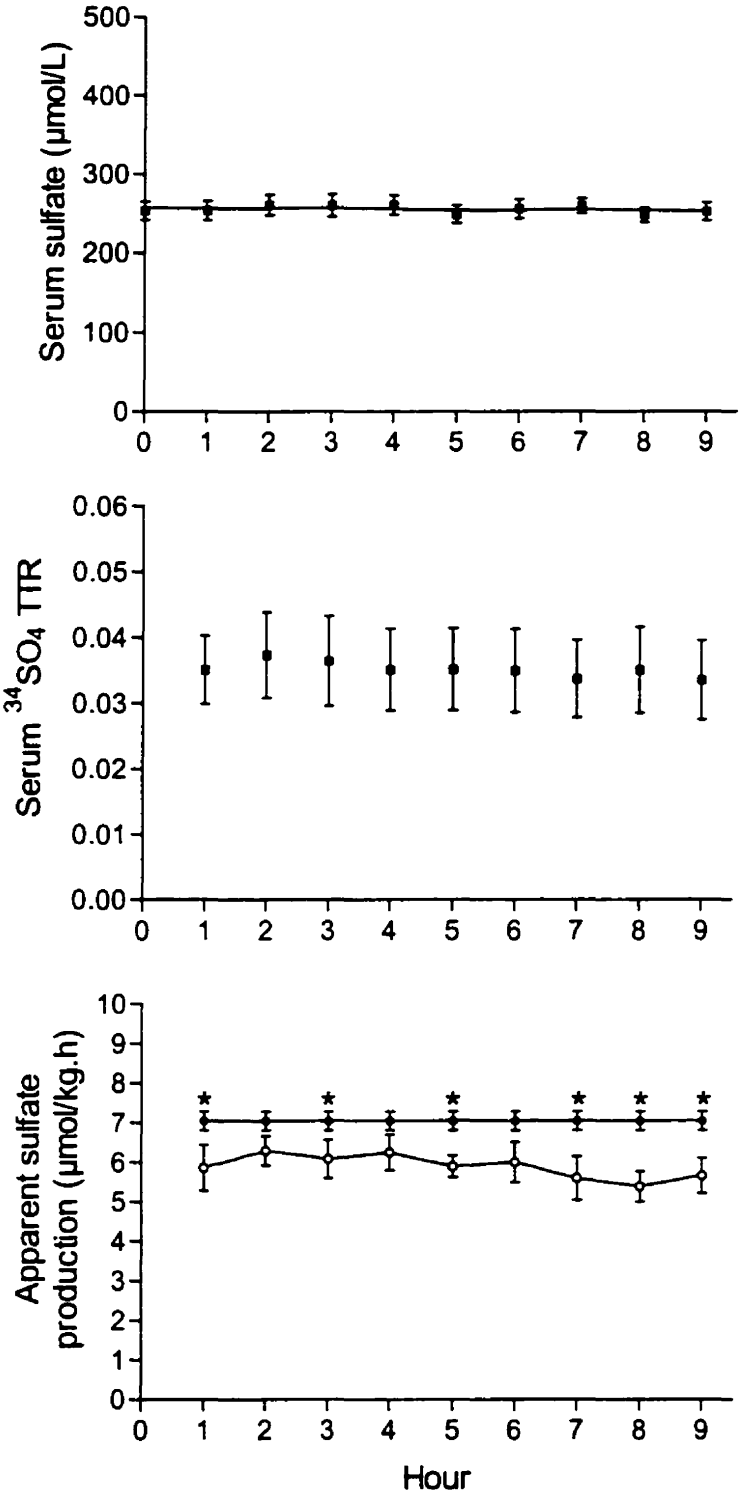

Figure 1. Serum sulfate (µmol/L), serum $^{34}SO_4$ enrichment (TTR), and sulfate production (µmol/kg.h) over 9 h as measured using the tracer (●, i/TTR) and non-tracer methods (○) in fasting subjects.

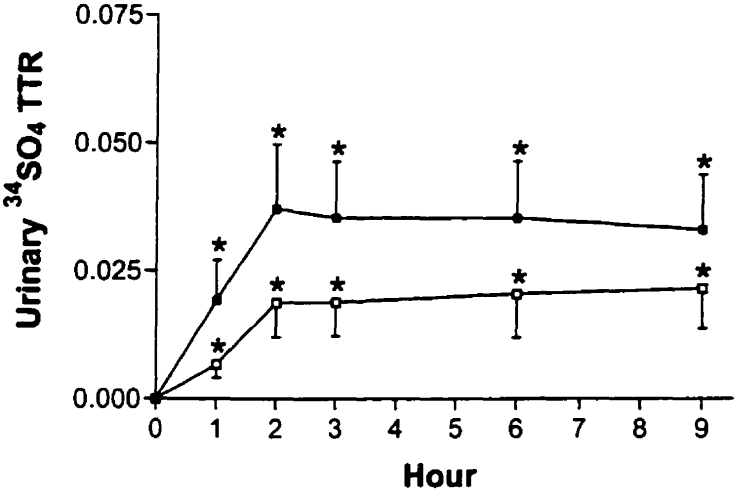

Figure 2. Urinary inorganic (■) and organic (□) $^{34}SO_4$ enrichments for the same experiment as in Figure 1.

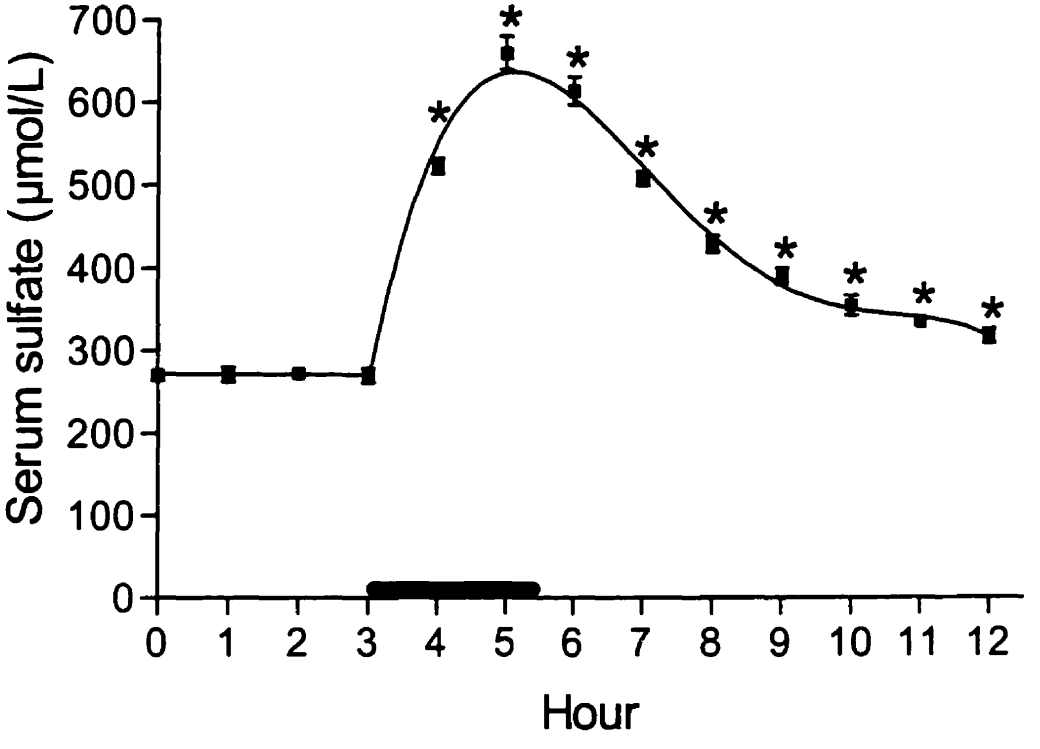

Figure 3. Serum sulfate (µmol/L) 3 h prior to and 9 h following intravenous administration of magnesium sulfate (sulfate infusion study). The bolded bar on the x-axis indicates time period of substrate administration.

Table 2. Cumulative sulfate production (μmol/kg) over 6 and 9 h following administration of sulfate or methionine.

		Sulfate infusion	Oral methionine
S input (A)		141.5 ± 6.8	150.3 ± 0.8
Basal sulfate production normalized (B)			
	to 6 h	33.9 ± 1.9	50.8 ± 4.0
	to 9 h	50.8 ± 2.8	76.2 ± 6.0
Total sulfate produced (C)			
	per 6 h	168.9 ± 7.6	140.2 ± 9.4
	per 9 h	189.2 ± 7.5	189.3 ± 10.9
Sulfate production above basal (C - B = D)			
	per 6 h	135.0 ± 7.9	89.4 ± 6.4
	per 9 h	138.4 ± 7.1	113.1 ± 6.4
Exogenous S retained (A - D)			
	per 6 h	6.5 ± 3.4	60.9 ± 5.6
	per 9 h	3.1 ± 2.6	37.2 ± 5.7
Sulfate recovery (%) [D/A].100%			
	per 6 h	95.4 ± 2.4	59.4 ± 3.9[1]
	per 9 h	97.9 ± 1.8	75.2 ± 3.9[1]

Data are presented as mean ± SEM.

[1] Statistically different from 100%.

of the infused sulfate improves to 97.3% over the 6 h and 100.6% over the 9 h collection periods.

Fasting-HP study

Serum sulfate concentrations and urinary sulfate excretion remained constant over the 12 h observation period when the previous diet was high in protein (Figure 4). Urinary sulfate excretion over the final 6 h (3.6 ± 0.4 mmol) or 9 h (5.3 ± 0.6 mmol) was closely similar to the value predicted from sulfate excretion over the initial 3 h (3.6 ± 0.5 mmol/6 h; 5.4 ± 0.7 mmol/9 h).

Methionine administration studies

High-dose (0.45 mmol/kg) oral methionine

The serum methionine concentration increased 29 fold ($P < 0.0001$) within 1 h following methionine administration, and was still ~6 fold higher than baseline at h 12, indicating incomplete methionine absorption by the gut and/or incomplete tissue metabolism during the 9 h following its consumption. The serum sulfate concentration increased ~2 fold over the 7 h following methionine administration and was still at approximately this level at h 12 ($P < 0.0001$). The increase in sulfate production above its endogenous level accounted for 36.8 ± 6.4% (at 6 h) and 54.9 ± 6.4% (at 9 h) of the S in the administered methionine. In subsequent studies the methionine dose was reduced to 0.15 mmol/kg.

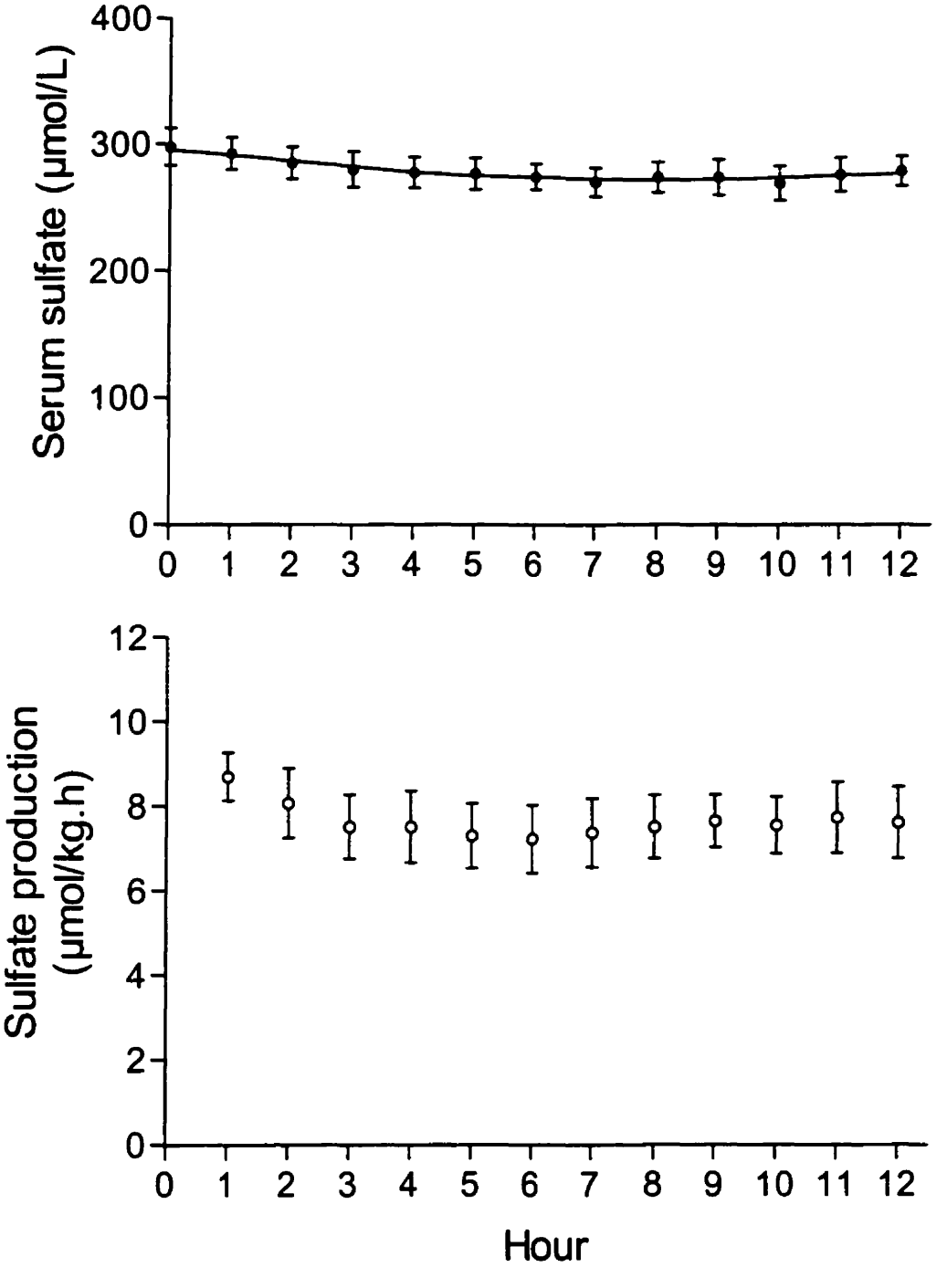

Figure 4. Serum sulfate (●; µmol/L) over 12 h and sulfate production (○; µmol/kg.h) between hours 1 and 12 for the fasting-HP study.

Oral methionine and methionine plus mixed amino acid infusion

Oral and intravenous administration of 0.15 mmol/kg methionine resulted in a similar serum sulfate response, with an initial 1.5 fold increase, followed by a gradual decrease towards baseline (Figure 5). Oral methionine increased serum methionine 7.6 fold 1 h following its administration (Figure 6); alanine, aspartate, glutamate, serine, threonine, leucine, isoleucine and valine concentrations changed minimally. The mixed amino acid infusion transiently increased serum methionine, threonine, leucine, isoleucine and valine concentrations (Figure 6), whereas alanine, aspartate, glutamate, and serine concentrations did not increase.

The increase in sulfate production above baseline accounted for 59% (at 6 h) and 75% (at 9 h) of the S in the administered methionine (Table 2). Whole body S balances were insignificantly different for both methionine administration methods (Table 3), but urea N balances were slightly less negative for the mixed amino acid infusion (Table 4). Unlike methionine alone, the intravenous amino acids stimulated insulin secretion (Figure 7). Figure 8 illustrates directional similarity between 9 h urea N and S balances in individual subjects.

DISCUSSION

This study evaluated the use of sulfate production as an indicator of whole-body SAA catabolism so as to be able to examine nutritional factors regulating it using short, clinically practical protocols. We measured sulfate Ra under basal conditions using a primed continuous oral administration of sodium [^{34}S]sulfate; we also measured non-

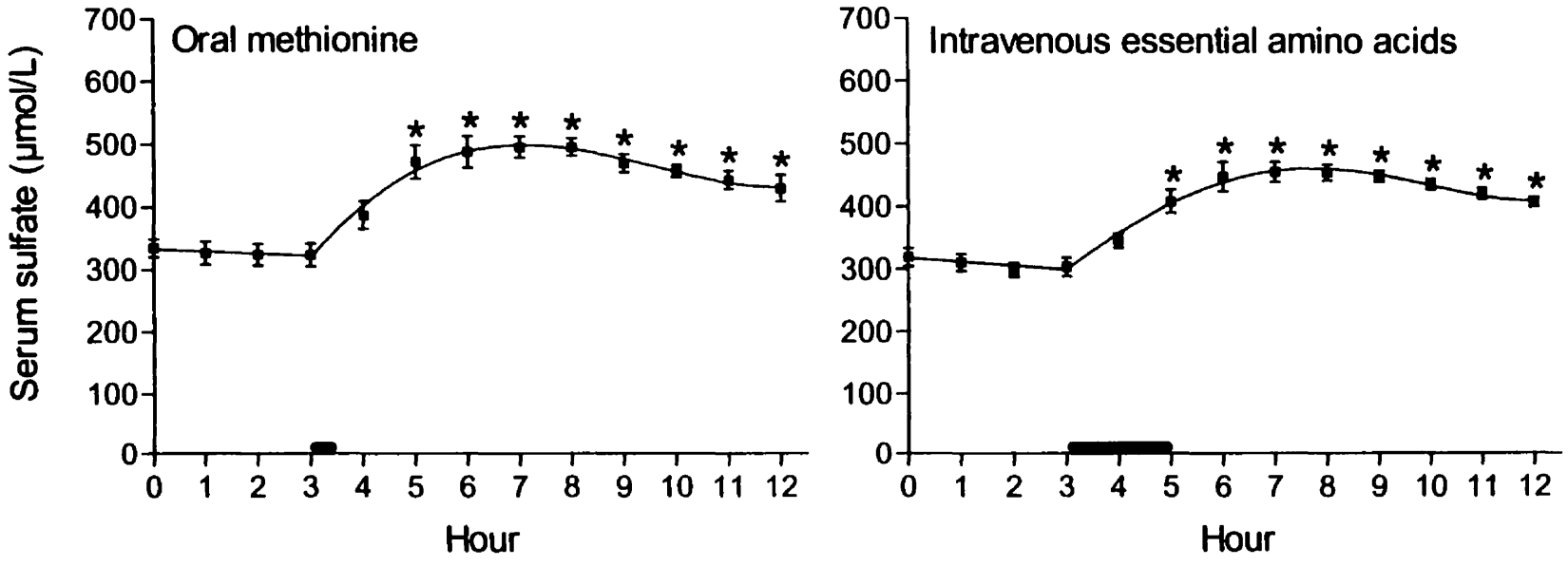

Figure 5. Serum sulfate concentrations (µmol/L) 3 h prior to and 9 h following oral (oral methionine study) and intravenous (amino acid infusion study) administration of methionine. Bolded bars on the x-axis indicate time period of substrate administration.

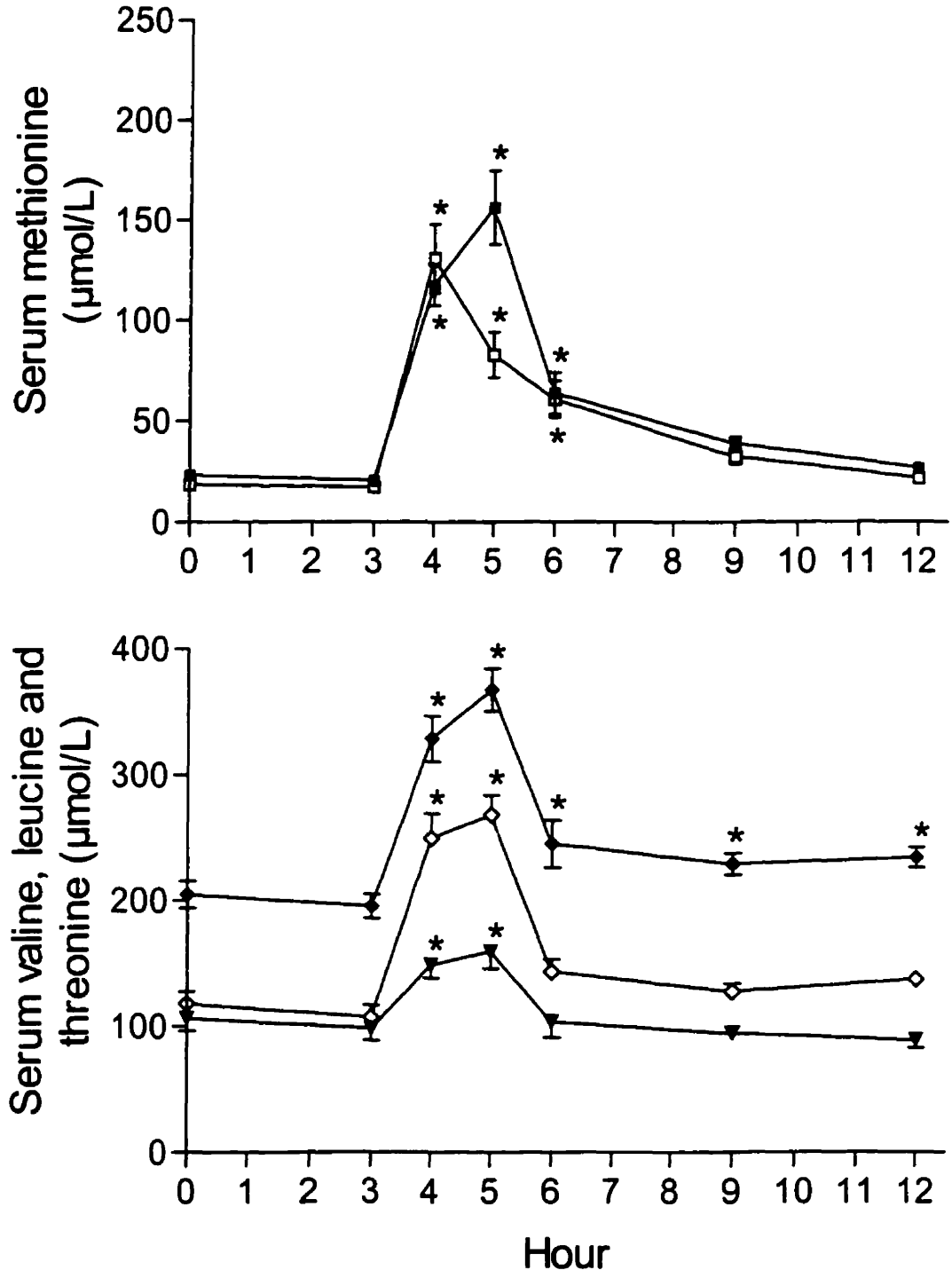

Figure 6. Serum amino acid concentrations (µmol/L) for the oral methionine (□) and amino acid infusion (■, methionine; ♦, valine; ◇, leucine; ▼, threonine) studies.

Table 3. Cumulative sulfur balance (μmol/kg) over 6 and 9 h following administration of methionine.

Measurement		Oral methionine	Amino acid infusion
S in (A)		150.3 ± 0.8	158.2 ± 3.9
S out (B)			
	per 6 h	140.2 ± 9.4	134.7 ± 22.6
	per 9 h	189.3 ± 10.9	181.8 ± 22.4
S balance (A-B)			
	per 6 h	10.1 ± 8.8	23.5 ± 7.2
	per 9 h	-39.1 ± 10.4	-23.7 ± 6.9

Data are presented as mean ± SEM. S out was measured as sulfate production. There was no significant differences between corresponding mean values in the oral methionine and amino acid infusion studies.

Table 4. Cumulative urea N balance (mmol/kg) over 6 and 9 h following administration of methionine.

Measurement		Oral methionine	Amino acid infusion
N in (A)		0.15 ± 0.00	1.77 ± 0.04*
N out (B)			
	per 6 h	1.92 ± 0.14	2.75 ± 0.22*
	per 9 h	2.86 ± 0.20	3.95 ± 0.18*
N balance (A-B)			
	per 6 h	-1.77 ± 0.14	-0.98 ± 0.19*
	per 9 h	-2.71 ± 0.20	-2.19 ± 0.15

Data are presented as mean ± SEM.

* Significantly different from the oral methionine study, t test $P < 0.05$.

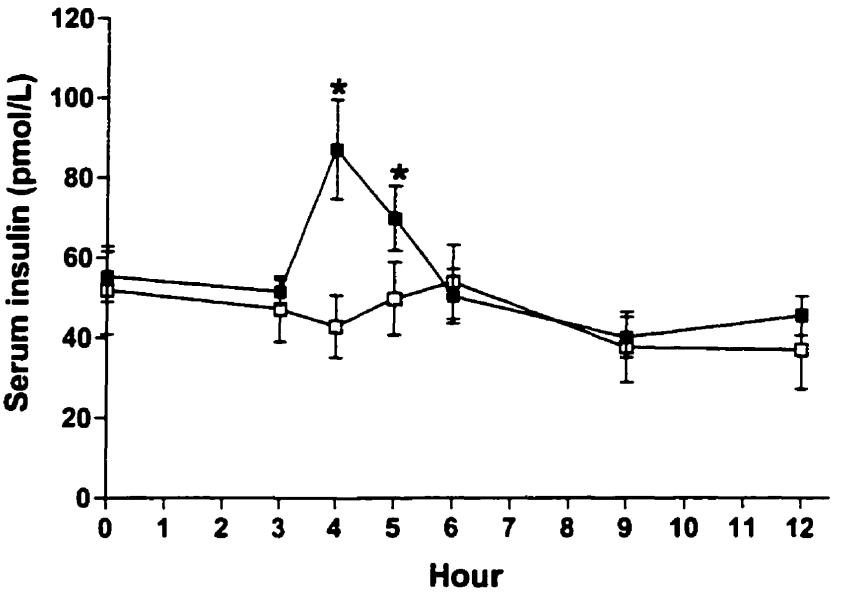

Figure 7. Serum insulin (pmol/L) over 12 h for the oral methionine (□) and amino acid infusion (■) studies.

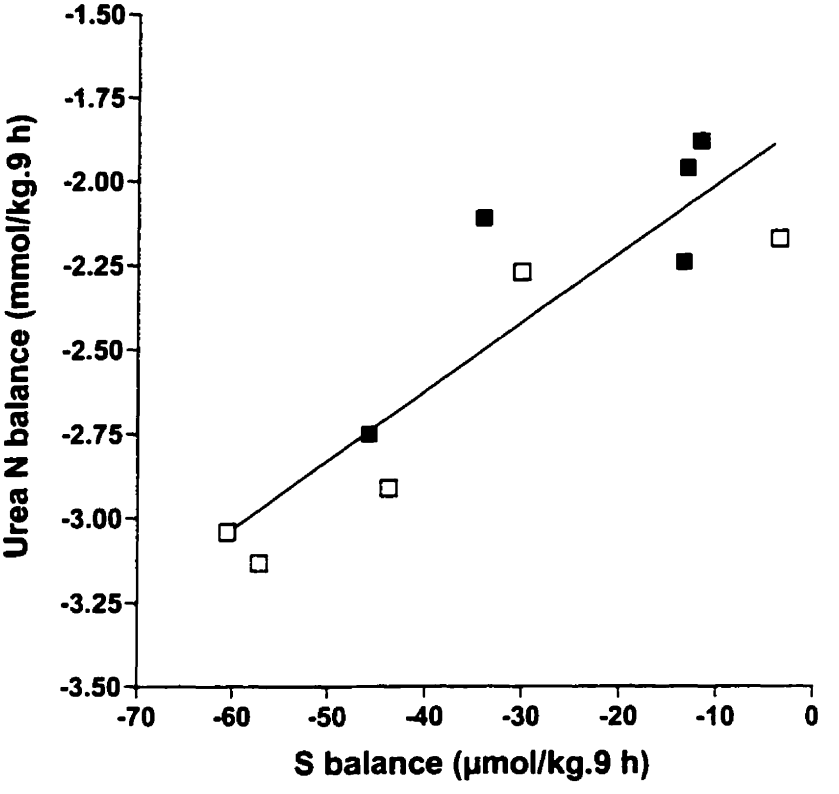

Figure 8. Correlation between 9 h cumulative urea N and S balances for individuals consuming oral methionine (□) or intravenous methionine plus other essential amino acids (■). r = 0.89, P = 0.0005.

tracer sulfate production after administering known amounts of intravenous sulfate, oral methionine, and an intravenous mixture of essential amino acids including methionine.

To test whether the tracer method accurately depicts sulfate production, we compared the serum sulfate Ra indicated from a primed continuous oral administration of sodium [^{34}S]sulfate with sulfate production as measured by its urinary excretion. The value obtained was 20% higher with the tracer approach; possible reasons for this are considered below. The non-tracer method, which calculates sulfate production as its urinary sulfate excretion corrected for changes in ECF sulfate, accurately depicted short-term changes in sulfate production following intravenous administration of a known, physiologically relevant amount of magnesium sulfate. Baseline sulfate production, as measured over a 3-h baseline period, accurately predicted basal production over the subsequent 6 or 9 h when the preceding diet was either normal or low in protein. Using this assumption, we calculated sulfate production above basal following methionine administration. If it can be assumed that this treatment has little effect to increase or decrease net body protein balance, then a considerable portion of the S in the administered methionine appeared to be retained in a non-protein form.

Sulfate production could provide a useful measure of short-term SAA catabolism. Urea production and tracer-determined amino acid oxidation are commonly used to measure whole-body amino acid catabolism over short periods of time (Beaumier et al, 1995; Carraro et al, 1993; El-Khoury et al, 1994; Ensinger et al, 1994; Quevedo et al, 1994; Wolfe et al, 1987), but these methods have disadvantages. First, 15-30% of synthesized urea is not excreted directly into the urine, but is hydrolyzed in the gut with

partial recycling of the resulting ammonia for new urea synthesis (Jones et al, 1968; Long et al, 1978; Walser and Bodenlos, 1959). Second, the plasma urea concentration tends to change under basal and fed conditions, and since body water is a large pool, small analytical errors in the plasma urea measurement can lead to large errors in apparent urea retention or excretion. Under steady state conditions, plasma urea turnover can be accurately measured using the technique of primed continuous tracer infusion, but both this method and the non-tracer approach are unreliable during non-steady state conditions which are of particular physiological interest (Hamadeh and Hoffer, 1998; Taveroff and Hoffer, 1993). There are theoretical advantages to using sulfate to measure SAA catabolism in non-steady state situations. Like urea, sulfate is excreted in the urine, but unlike urea, it distributes in a smaller body compartment (the ECF) and does not appear to be subject to important losses in the gut (Bauer, 1976; Chakmakjian and Bethune, 1966; Omvik et al, 1979; Ryan et al, 1956).

The tracer approach we adopted for this study was modeled after the primed-continuous infusion urea tracer method, and appeared to indicate rapid attainment of steady state (Figure 1). However, the resulting sulfate Ra was significantly higher than the sulfate production rate indicated by the non-tracer method. More than one factor could account for this. First, in the interest of developing the most practical and convenient model, we administered the tracer orally, and it could have been incompletely absorbed from the gut. We consider this unlikely since small oral doses of sodium sulfate are well absorbed (Cocchetto and Levy, 1981; Florin et al, 1991; Krijgsheld et al, 1979). In light of the present findings, however, future studies are indicated using

intravenous tracer.

We detected $^{34}SO_4$ enrichment in urinary sulfate esters within a short time after commencing tracer administration (Figure 2). Sulfate esters make up a relatively constant and small (9-15%) fraction of total sulfate excretion (Beach et al, 1942; Martensson, 1982; Martensson et al, 1984); they have been thought to arise mainly in the large intestine from conjugation of sulfate with phenolic compounds formed by bacterial action on aromatic amino acids (Lundquist et al, 1980). This last assumption warrants re-examination. The ^{34}S-enriched ester sulfate detected in our study is unlikely to have arisen as a result of colonic bacterial action because of its rapid appearance, and in light of recent data indicating that sulfate-metabolizing intestinal bacteria target sulfomucins rather than exogenous sulfate (Deplancke et al, 2000).

Partial first-pass splanchnic extraction of the tracer (with release into the circulation as sulfate esters) could explain the present findings. Another possibility is uptake of sulfate in the general circulation into the liver and other tissues with concurrent release of some of it into the ECF in the form of sulfate esters. The last possibility implies that the apparent steady-state enrichment shown in Fig 2 is a pseudo-plateau and that it will overestimate sulfate Ra until the organic and inorganic sulfate pools came into isotopic equilibrium, which they clearly were not over the time course of the present study. Ryan et al (1956) reported that following intravenous [^{35}S]radiosulfate injection, radioactivity disappears from the plasma faster than it appears in the urine, suggesting the existence of an intracellular sulfate compartment that dynamically exchanges its sulfate with the ECF. The present observation of the prompt appearance of label in sulfate esters

supports this possibility.

To determine whether the non-tracer method predicts short-term acute changes in sulfate production, an amount of magnesium sulfate corresponding to the amount of amino acid S in a normal meal was administered intravenously following a 3-h baseline period (Figure 3). Sulfate production following the sulfate infusion was corrected for endogenous sulfate production as extrapolated from sulfate production over this baseline period. Sulfate production above basal accounted for 95% (at 6 h) and 98% (at 9 h) of the sulfate infused (Table 2, both NS from 100%). This agrees with the 90-100% urinary recovery of sulfate reported by Chakmakjian and Bethune (1966) 24 h following intravenous administration of an extremely large dose (362 mmol) of sodium sulfate.

Sulfate production over a 3-h baseline period accurately predicted sulfate production over the subsequent 9 h in men adapted to a normal protein intake (fasting-HP study). Hourly sulfate production was strongly correlated with hourly urinary sulfate excretion ($r = 0.96$, slope = 0.95, $P < 0.0001$), as expected when the serum sulfate concentration is unchanging. Thus in the subsequent methionine administration studies, baseline sulfate production was used to extrapolate endogenous cumulative sulfate production over the subsequent 6 or 9 h.

Whether or not subjects consumed a low or high protein diet the day before they were studied, their serum sulfate concentrations were unchanging over the 9-12 h observation period, so there was no need to account for changes in ECF sulfate (Figures 1 and 4). This suggests that simple urinary sulfate excretion could be used in many settings as a convenient measure of baseline sulfate production. Furthermore, results

obtained at h 6 corresponded well with those obtained at h 9. Thus, acceptably accurate results can be obtained following acute short-term changes in sulfate production in study protocols that last only 6 h, an important practical advantage when carrying out metabolic studies in the clinical setting.

Theoretically, the sulfate space (SS) would most accurately depict sulfate distribution. This space is ~20% smaller than the CBS (Hamadeh et al, 1999). However, sulfate recovery following magnesium sulfate infusion was most accurate when the CBS was used to estimate the ECF volume. We conclude that the CBS (or even body weight x 0.2) (Hamadeh et al, 1999) provides a suitable and convenient measure of ECF for determining changes in body sulfate content.

Having determined that the non-tracer approach can provide accurate short-term information about sulfate production, we investigated what fraction of the S in a dose of methionine can be accounted for as increased sulfate production over the hours following its administration, under conditions in which baseline endogenous SAA catabolism would be expected to be constant or nearly constant. Since sulfate production is a measure of the sum of the catabolism of all the SAA, any inference about the catabolism of an exogenous dose of methionine requires that endogenous SAA catabolism is not affected by this treatment. A methionine dose of 0.15 mmol/kg was administered after it was found that a 0.45 mmol/kg dose was associated with incomplete absorption and/or tissue metabolism 9 h following consumption. Using the lower methionine dose, serum methionine concentration returned to the baseline level 9 h following its administration (Figure 6). The resulting increase in sulfate production accounted for only 59% (at 6 h)

and 75% (at 9 h) of the S in the administered methionine (Table 2).

The amount of methionine that remained as methionine following its administration can be presumed to be small. Intracellular and plasma methionine concentrations are not greatly different (Bergstrom et al, 1990), and if serum methionine is extrapolated to TBW, changes in serum methionine amount to 4.8% of oral methionine over 6 h and 1.7% over 9 h. Since oral methionine was administered alone and did not stimulate insulin secretion, we presume it effected no change in the negative net body protein balance typical of the postabsorptive state. It is therefore likely that the S retained in the body after oral methionine was stored in non-protein sulfur-containing compounds, such as glutathione, an important cysteine reservoir (Cho et al, 1981; Fukagawa et al, 1996; Tateishi et al, 1981).

We also measured the effect of an infusion of methionine plus other essential amino acids on sulfate production. The resulting serum sulfate and methionine concentrations were similar to those observed after oral methionine (Figures 5 and 6). If it is assumed that basal sulfate production was unchanged by this treatment, then 58% of the administered methionine S was accounted for by the increase in sulfate production above baseline after 6 h and 74% after 9 h. These values are similar to those observed with oral methionine alone. In this case, however, endogenous sulfate production must have been affected by the mixed amino acid infusion. This treatment increased serum methionine, threonine, leucine, isoleucine, valine and insulin concentrations, and was associated with a more positive S balance and less negative urea N balance than occurred with oral methionine alone (Figures 6, 7, 8, and Tables 3 and 4). Hypocaloric infusions

of leucine or mixtures of branched-chain or essential amino acids are mildly protein sparing (Louard et al, 1990; Nair et al, 1992; Pacy et al, 1988), and hence will reduce net proteolysis and endogenous SAA catabolism and sulfate production. The extent of protein sparing is minor, however. This can be illustrated by comparing 9-h urea N balances following oral methionine (-2.71 mmol/kg) and amino acid infusion (-2.19 mmol/kg). This suggests a relative retention of body proteins of 0.52 mmol N/kg (46 mg whole-body protein/kg body weight) due to the amino acid infusion. If it is assumed that the body protein spared by the amino acid infusion had an N/S molar ratio of 40 (Food Policy and Food Science Service, NDF, 1970; Reifenstein et al, 1947), then baseline sulfate production was reduced by 13 μmol/kg over 9 h, equivalent to 8.2% of the S administered in the mixed amino acid infusion. Since 25% of the sulfur in the oral methionine dose was retained in the body over the same period of time, we conclude, as with the administration of methionine alone, that most of the S retained in the body following the mixed amino acid infusion must have been in a non-protein form.

The conclusion that a significant fraction of the S in an oral methionine dose or an intravenous mixture of methionine and essential amino acids is retained in non-protein bound form is further supported by comparing urea N and S balances, calculated on the assumption that short-term changes in N and S production are approximately captured by urea N and inorganic sulfate production, respectively. Positive cumulative S balances over 6 h for both the oral methionine and amino acid infusion studies indicated S retention over this period, whereas urea N balances indicated net N loss (Tables 3 and 4). Dividing the 9-h urea N balance by the S balance results in a N/S molar ratio of 69.3

following oral methionine and 92.4 following amino acid infusion, higher than in body proteins. These results suggest a greater retention of S than N in the postprandial period. This temporal offset between sulfate and urea production has been described after whole meal ingestion by Cheema-Dhadli and Halperin (1993).

There are some disadvantages to measuring sulfate. First, the measurement is not routinely available in clinical laboratories. Second, even if it accurately depicts SAA catabolism, sulfate production may not always provide information on whole body proteolysis because of its potential storage in a non-protein form, as suggested by Cheema-Dhadli and Halperin (1993), and by the present results after methionine administration. Finally, certain medications metabolized by sulfation could interfere with accurate sulfate measurements (Levy, 1986).

In conclusion, a simple ^{34}S-sulfate tracer approach to measuring whole-body sulfate production was inaccurate. By contrast, a simple non-tracer approach of measuring urinary sulfate excretion, corrected for changes in ECF sulfate, appears to accurately and conveniently depict sulfate production over observation periods as short as 6 h. Endogenous sulfate production measured over a 3-h baseline period can be used to measure endogenous production over subsequent hours when subjects are studied in the normal postabsorptive state. Following ingestion of methionine, either alone or together with other essential amino acids, a significant fraction of the methionine S appears to be stored in the body in a non-protein bound form, presumably as glutathione.

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CONNECTING TEXT IV

Our results from chapter 6 determined that the nontracer approach of measuring urinary sulfate excretion corrected for changes in ECF accurately depict sulfate production over observation periods as short as 6 h. The ^{34}S-sulfate tracer approach to measuring whole-body sulfate production is inaccurate, likely owing to incomplete exchange of the tracer with intracellular organic compounds. Endogenous sulfate production measured over a 3-h basal period can be used to measure endogenous production over subsequent several hours when subjects are studied in the normal postabsorptive state. Following ingestion of methionine, either alone or together with other essential amino acids, a significant fraction is stored within a nonprotein bound form, which could represent glutathione.

Our next objective was to investigate the use of sulfate production as an alternative to urea to measure the adaptive decrease in whole-body amino acid oxidation that occurs with reductions in protein intake. To do this, we tested the reproducibility of postprandial urea and sulfate production upon repeat testing following the nonsteady state consumption of a protein-restricted test meal in persons previously adapted to high and low protein diets.

We also tested whether the abnormal protein turnover present in conventionally treated IDDM affects body protein economy.

CHAPTER VII

Sulfate Production Depicts Fed-State Adaptation to Protein Restriction in Humans

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Sulfate Production Effectively Depicts Fed-State Adaptation to Protein Restriction in Humans

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ABSTRACT

Normal adaptation to dietary protein restriction requires an appropriate reduction in dietary and endogenous amino acid oxidation and urea production. We previously showed that the postprandial reduction in urea production that occurs as a part of the normal adaptation to protein restriction is impaired in conventionally treated insulin-dependent diabetes mellitus (IDDM) when a high protein (0.5 g/kg) test meal is used (Hoffer et al, Am J Physiol 1997;272:E59-E67). The present study was undertaken to test the reproducibility of a more discriminating test meal lower in protein, and to include as a main outcome variable the production of sulfate, a specific indicator of sulfur amino acid oxidation. Six healthy, non-obese men consumed a mixed test meal containing 0.25 g protein/kg and 10 kcal/kg while adapted to high (HP, 1.5 g/kg.d) and low (LP, 0.3 g/kg.d) protein intakes. They followed this procedure twice: once when both test meals included tracer [^{15}N]alanine ([^{15}N]ALA) and once when both test meals included tracer [^{15}N]*Spirulina platensis*. Six subjects with IDDM receiving conventional insulin therapy consumed this test meal with added [^{15}N]ALA while adapted to a high protein intake. Urinary urea and sulfate were measured over 6 and 9 hours following the test meal with a correction for changes in body pool. Protein restriction decreased cumulative urea-N and S production over 9 h following the test meal by 22-29% (mean, HP 3.5-3.7 mmol/kg; LP, 2.6-2.7 mmol/kg) and 49-52% (HP, 88-90 μmol/kg; LP, 42-46 μmol/kg), respectively (both $P < 0.05$). Similar results were obtained for a collection period of 6 h following the test meal (urea-N: HP, 2.5-2.6 mmol/kg and LP, 1.9 mmol/kg, 26% reduction; S: HP, 63-66 μmol/kg and LP, 30-34 μmol/kg, 48-53% reduction). The data

obtained with IDDM subjects were similar to those obtained with the normal subjects adapted to high protein intake. We conclude that postprandial changes in urea and sulfate production following normal adaptation to protein restriction are reproducible on repeat testing irrespective of the ^{15}N tracer source, and are evident using a collection period as short as 6 h following the test meal. Sulfate production effectively depicts fed-state adaptation to protein restriction and is less prone to error.

INTRODUCTION

Urea production is commonly used to measure short-term changes in whole body amino acid oxidation (Beaumier et al, 1995; Carraro et al, 1993; El-Khoury et al, 1996; El-Khoury et al, 1994; Ensinger et al, 1994; Wolfe et al, 1982; Wolfe et al, 1987). We have previously used a 0.5 g/kg protein test meal to show that the decrease in postprandial cumulative urea production that occurs in healthy subjects following adaptation to protein restriction that was less effective in persons with insulin-dependent diabetes mellitus (IDDM) (Hoffer et al, 1997). Though this high protein test meal was able to detect changes in the efficiency to retain dietary protein in the fed state following adaptation to dietary protein restriction, the sensitivity of the system in the fine control of dietary protein still needs to be investigated. This could be accomplished using a test meal limiting in dietary protein, which will impose a requirement on the body to efficiently conserve the amino acids in the test meal.

Although widely used in clinical studies, urea production has not been tested for reproducibility. Urinary excretion of urea corrected for changes in total body water (TBW) underestimates true production by 15-30% mainly due to its hydrolysis (with partial recycling) in the gut (El-Khoury et al, 1994; Jones et al, 1968; Long et al, 1978; Walser and Bodenlos, 1959). As well, small analytical errors in the measurement of serum urea are magnified when multiplied by TBW. While tracer-determined urea rate of appearance (Ra) in serum accurately depicts urea production under basal steady state conditions, it underestimates short-term changes in urea production as occur in the fed state (Hamadeh and Hoffer, 1998). Sulfate is the predominant product of sulfur amino

acid (SAA) oxidation and might be a better alternative, since, unlike urea, it distributes in the extracellular water, is not subject to losses in the gut, and is excreted almost entirely through the renal route (Bauer, 1976; Chakmakjian and Bethune, 1966; Hamadeh and Hoffer, 2001a; Omvik et al, 1979; Ryan et al, 1956).

We have previously included [^{15}N]alanine in test meals and used the extent of transfer of ^{15}N into urea as a measure of postprandial first pass amino acid oxidation (Hoffer et al, 1997). Although rapidly transaminated (Battezzati et al, 1999), [^{15}N]alanine may not represent the extent to which absorbed dietary amino acids in general, as constituents of whole dietary proteins, are immediately retained for protein synthesis or transaminated and oxidized, and their N converted to urea. Therefore, in order to validate the use of [^{15}N]alanine as a measure of first pass dietary amino acid metabolism, we compared the results of feeding our subjects a mixed test meal containing either [^{15}N]alanine or [^{15}N]*Spirulina platensis*, a ^{15}N-labeled intact protein. These results are discussed in the accompanying paper (Hamadeh and Hoffer, 2001b).

The measurements described in this paper tested, first, whether postprandial urea production is reproducible upon repeat testing (the only difference being the ^{15}N tracer source) and, second, to investigate the validity of using sulfate production as an alternative or adjunct to urea as a measure of whole-body amino acid oxidation following consumption of a low protein test meal. To do this, we measured urea and sulfate production 6 and 9 h following a mixed test meal containing either [^{15}N]alanine or [^{15}N]*Spirulina platensis* in 6 healthy men prior to and following adaptation to protein restriction. We also measured urea-N and S production, metabolic balance and net

protein utilization (NPU). Finally, we measured whole-body protein economy following consumption of a test meal in persons with IDDM on conventional insulin therapy while adapted to a high protein intake, and compared these results with those for the normal subjects.

METHODS

Normal subjects

Subjects and protocols

Six healthy, non-smoking men with normal biochemistries and taking no medications were randomly assigned to a 5-day cross-over study (Table 1). All volunteers gave written consent for the study, which was approved by the Research and Ethics Committee of the Jewish General Hospital in Montreal.

On day 1, subjects were admitted to the clinical research unit at 0700 h and assigned a high protein diet (HP: 1.5 g/kg body weight.d) following anthropometric measurements. On day 2 (test day), a mixed test meal containing either a tracer dose of [^{15}N]alanine ([^{15}N]ALA) or [^{15}N]*Spirulina platensis* ([^{15}N]SPI) was orally administered after the overnight fast. Upon completion of the study 9 h following test meal intake, subjects consumed 2 low protein meals. On days 3 and 4, subjects consumed a low protein diet (LP: 0.3 g/kg.body weight.d). On day 5 (test day), the identical study as on day 2 was repeated. Subjects returned 10 days later to undergo the same 5-day protocol but using the alternate ^{15}N tracer.

Twenty-four hour urine collections commenced at 0700 h. On test days 2 and 5,

Table 1. Subject characteristics.

Measurement	Control	IDDM
Sample size	6 M	6 (4 M, 2 F)
Age (y)	27 ± 2	33 ± 5
Weight (kg)	70 ± 9	66 ± 5
Height (cm)	175 ± 10	172 ± 2
BMI (kg/m^2)	23 ± 2	22 ± 1
TBW (% body weight)	59 ± 3	58 ± 2
FFM (% body weight)	81 ± 4	79 ± 3
CBS (mL/kg)	202 ± 14	221 ± 8

Data are presented as mean ± SEM; BMI, body mass index;

TBW, total body water; FFM, fat free mass; CBS, corrected bromide space.

There is no significant difference between the Control and IDDM groups.

urine was collected in different pools: prior to the consumption of the test meal, over the first 6 hours following the test meal (pool 1-6), over the last 3 hours of the study (pool 7-9), and, for test day 2 only, upon completion of the study until the start of the next 24-h collection. Blood samples were drawn hourly from an arterialized arm vein kept patent with 77 mmol/L NaCl solution at 80 mL/h. Subjects drank 207 ± 23 mL (mean ± SD) of water/h to maintain adequate urine flow (mean ± SD; 279 ± 36 mL/h) during the study. Body weight was recorded daily each morning, and total body water (TBW) measured by bioimpedance analysis (RJL Systems BIA-101A, Mt Clemens, MI) (Kushner and Schoeller, 1986) on day 1 and on test days 2 and 5 prior to and upon completion of the 9-h study.

Diet

On day 1, subjects consumed high protein (1.5 g/kg body weight.d) breakfast (0830 h), lunch (1230 h) and dinner (1730 h) at maintenance energy (Table 2). On day 2 (test day), upon completion of the study 9 hours following test meal, subjects consumed 2 low protein meals at 1730 h and 2130 h, thus complementing their daily energy consumption; average protein intake on that day was 27.2 ± 1.2 g/day (0.39 ± 0.01 g/kg.d). Day 2 was considered the first day of protein restriction. On days 3 and 4, subjects consumed a LP diet (0.3 g/kg.body weight). On day 5 (test day), only the test meal was consumed. Total daily energy intake was 38 kcal/kg body weight, on the assumptions that resting energy expenditure was 24 kcal/kg (Goran et al, 1993) and sedentary activity and diet-induced thermogenesis were 14 kcal/kg.

Table 2. Diet composition of the control group.

Macronutrient	Day 1	Day 2 [1]	Days 3 and 4
Energy			
kcal/d	2740 ± 350	2720 ± 370	2710 ± 350
kJ/d	11,460 ± 1460	11,360 ± 1540	11,330 ± 1470
Carbohydrate			
(g/d)	382 ± 46	448 ± 66	453 ± 60
(% of energy)	56 ± 1	66 ± 1	67 ± 1
Fat			
(g/d)	88 ± 12	91 ± 11	90 ± 11
(% of energy)	29 ± 1	30 ± 1	30 ± 1
Protein			
(g/d)	107 ± 14	27 ± 3	22 ± 3
(% of energy)	15.6 ± 0.2	4.0 ± 1.0	3.2 ± 0.1
(g/kg.d)	1.52 ± 0.03	0.39 ± 0.01	0.31 ± 0.00

Data are presented as mean ± SD.

[1] Includes mixed test meal.

Proper hydration was maintained throughout the study period. The food consisted of low protein bread, regular low protein food (juice, butter, jam, mashed potatoes, green beans, carrots, tea, coffee, ginger ale, and sugar), and low-protein wafers. A maximum of one cup of coffee (with coffee whitener) and 2 cups of tea were allowed per day. Consumption of other foods and beverages was not permitted. While on the LP diet, subjects consumed one multiple vitamin-mineral tablet (Centrum Forte, Whitehall-Robins, Mississauga, ON, Canada) each day.

Test meal

The test meal consisted of Glucerna (Ross Laboratories, St. Laurent, QC) mixed with sufficient beet sugar (Rogers Sugar Limited, Winnipeg, MB) to provide per kg of body weight 0.25 g of protein and 10 kcal of energy (10% protein, 30% fat and 60% carbohydrate). With the test meal, 200 mg [^{13}C]urea (99%; MSD Isotopes, Montreal, QC), and either 3 mg [^{15}N]ALA/kg.body weight (99%; MSD Isotopes, Montreal, QC) or 30 mg of processed dried [^{15}N]SPI/kg.body weight (99% ^{15}N; Martek, Columbia, MD) were consumed. [^{13}C]urea was added to the test meal with the aim of using its recovery in serum and urine as a measure of the extent of nonurinary urea losses (Hoffer et al, 1997). We found in a preliminary study that, unlike cane sugar, beet sugar consumption does not increase breath $^{13}CO_2$ enrichment. The N content of Glucerna was 6.6 mg/mL and the S content determined to be 0.31 mg/mL based on data supplied by Ross Laboratories.

NaBr (30 mg/kg.body weight) was orally administered at hour 6 to determine the

corrected bromide space (CBS), a measure of the extracellular fluid volume (ECF) (Miller et al, 1989; Vaisman et al, 1987).

Subjects with diabetes mellitus

Four men and two women with insulin-dependent diabetes mellitus (IDDM) presented to the research unit at 0700 on the day of the study. The subjects had whole blood hemoglobin A_1C concentrations of 6.2 ± 0.7% (mean ± SD; normal range 3.5-5.5%) and serum insulin C-peptide concentrations of 0.24 ± 0.04 nmol/L (normal range 0.46-0.72 nmol/L). Their diet was the typical Canadian diet, which provides ~1.5 g protein/kg.day (Gray-Donald et al, 2000); they did not change their dietary habit in the days prior to the test meal. Weight and height were recorded and TBW measured by bioimpedence analysis. Fasting and postprandial capillary blood glucose concentrations were measured using the Accu-Chek III blood glucose monitor (Boehringer Mannheim, Laval, QC). Subjects self-administered their insulin 30 min prior to consuming the test meal, which consisted of Glucerna mixed with sufficient beet sugar to provide per kg of body weight 0.25 g of protein and 10 kcal of energy, and included 200 mg [^{13}C]urea and 3 mg [^{15}N]ALA/kg.body weight.

To obtain a fasting blood glucose level akin to that found in conventionally treated diabetes mellitus (10-12 mmol/L) (DCCT Research Group, 1986), subjects self-administered their normal short-acting insulin dose prior to consuming their regular dinner the evening prior to the study day but only two-thirds of their normal intermediate-acting insulin dose. The following morning, subjects administered half of

the best estimated short-acting and two-thirds of the intermediate-acting insulin dose 30 min prior to consuming the test meal.

Urine was collected in two different pools: the first 6 hours following the test meal (pool 1-6) and the last 3 hours of the study (pool 7-9). Blood was collected prior to and over 9 h following consumption of the test meal. Blood samples were drawn hourly from an arterialized arm vein kept patent with 77 mmol/L NaCl solution at 80 mL/h. Subjects also consumed 355 ± 37 mL of water/h (mean ± SD); their urine flow was 432 ± 32 mL/h (mean ±SD).

Analytical methods

Clotted arterialized venous blood was centrifuged at 1400 *g* for 30 min at room temperature and the resulting serum stored at -30°C until analysis. Urea was analyzed using a Hitachi 917 automated analyzer (Laval, Quebec): reagents were purchased from Roche-Boehringer Manheim (Laval, Quebec). Though capillary blood glucose levels were measured on-site using the Accu-Chek III blood glucose monitor, serum was subsequently analyzed for glucose using a Hitachi 917 automated analyzer. Capillary blood glucose levels as measured using Accu-Chek III blood glucose monitor strongly correlated with serum glucose ($r = 0.98$, slope = 0.90, $P < 0.0001$).

Serum and urine were analyzed for sulfate and bromide by ion-exchange chromatography with conductivity detection (IEC-CD; Dionex 2110i; Dionex, Sunnyvale, CA), as previously reported (Boismenu et al, 1998). Samples were analyzed in triplicate for sulfate and in duplicate for bromide. $^{13}C/^{12}C$ urea enrichment was

measured by gas chromatography-mass spectrometry (GCMS), as previously described (Hamadeh and Hoffer, 1998). Serum C-peptide was analyzed by radioimmunoassay in the laboratory of Dr. John Dupre, University of Western Ontario, London, ON.

Calculations

Urea production was calculated as its urinary excretion corrected for changes in TBW content. Sulfate production was calculated as its urinary excretion corrected for changes in ECF content (Hamadeh and Hoffer, 2001a). ECF was estimated from the corrected bromide space (CBS) as described by Bell et al (1984). Net protein utilization (NPU) or SAA retention was calculated as:

$$\frac{\text{(Total N (S) intake - Total N (S) produced)} \times 100\%}{\text{Total N (S) intake}}$$

Since [M+1]urea could arise from [^{13}C]urea and [^{15}N]urea, enrichment specifically due to [^{13}C]urea was calculated by subtracting the [^{15}N]urea contribution as determined by isotope ratio-mass spectrometry (IRMS), which is specific for [^{15}N]urea, from the total [M+1]urea enrichment obtained by GCMS in serum and urine. The [^{13}C]urea supplied by MSD Isotopes, although confirmed to be 99% ^{13}C, was also 8% [^{18}O,^{13}C]urea (and hence of mass M+3), as described previously (Hamadeh and Hoffer, 1998; Hoffer et al, 1997; Matthews and Downey, 1984). This was accounted for in the mole ratio calibration curve prepared according to the method of Tserng and Kalhan (1983). [^{13}C]urea concentration was calculated as:

$$[[^{13}C]\text{urea}] = \frac{TTR \times [\text{urea}]}{1 + TTR}$$

where [[^{13}C]urea] is [^{13}C]urea concentration in μmol/L, TTR the tracer to tracee ratio, and [urea] urea concentration in μmol/L.

Statistical analyses

For the control group, three-way repeated measures ANOVA was used to determine significant differences in serum sulfate and urea concentrations, the factors being diet (high versus low protein), ^{15}N source ([^{15}N]ALA vs. [^{15}N]SPI), and time (h 0 to 9). Two-way repeated measures ANOVA was used to determine significant differences in body weight, and 24 h urea and sulfate excretion, the factors being ^{15}N source and day. Two-way repeated measures ANOVA was used to determine significant differences in post-meal urea-N and sulfate metabolism, post-meal N/S ratio, serum postabsorptive urea and sulfate concentrations, and [^{13}C]urea recovery, the 2 factors being diet and ^{15}N source. Within the same ^{15}N source and diet, serum urea and sulfate concentrations over time (h 0 to 9) were analyzed by one-way repeated measures ANOVA. When significance occurred, Newman-Keuls test was used post hoc to determine the source of difference.

To determine differences between the control and IDDM groups, Student's unpaired t-test and two-way repeated measures ANOVA were used. When significance occurred with ANOVA, Newman-Keuls test was used post hoc to determine the source of difference. Differences between data were considered significant at $P \leq 0.05$. Results are presented as means ± SEM unless otherwise indicated.

RESULTS

Normal subjects

Body weights, 24-h urea and sulfate excretion, and 24-h urinary N/S molar ratio

Daily body weights did not change over either 5-day study period. Twenty-four hour urea excretion decreased significantly and reproducibly over 4 days by 73-75% from a mean of 433 mmol on day 1 to 113 mmol on day 4, indicating the occurrence of adaptation to dietary protein restriction. Sulfate excretion showed a similar temporal pattern (Figure 1). Mean 24-h sulfate excretion obtained on day 1 while subjects were adapted to high protein intake was ~25 mmol, similar to values (20-26 mmol/d) obtained by other researchers in men (Cochetto and Levy, 1981; Hoffman et al, 1990; Lundquist et al, 1980; Morris and Levy, 1983). Twenty-four hour urea and sulfate excretion were closely related ($r = 0.95$, $P < 0.0001$, Figure 2).

Serum postabsorptive and postprandial urea concentrations and postprandial urea-N metabolism

Serum urea concentrations on the LP diet were significantly lower than on the HP diet both at baseline (by 60-62%) and over the entire 9 h observation period (by 50-52%; see Figure 3). With the HP diet, serum urea concentrations significantly and reproducibly decreased following the test meal, while with the LP diet serum urea reproducibly increased following the test meal.

On the LP diet, urea-N production was significantly decreased over 9 h following the test meal by 22-29% (Table 3). This reduction was concomitant with a significant

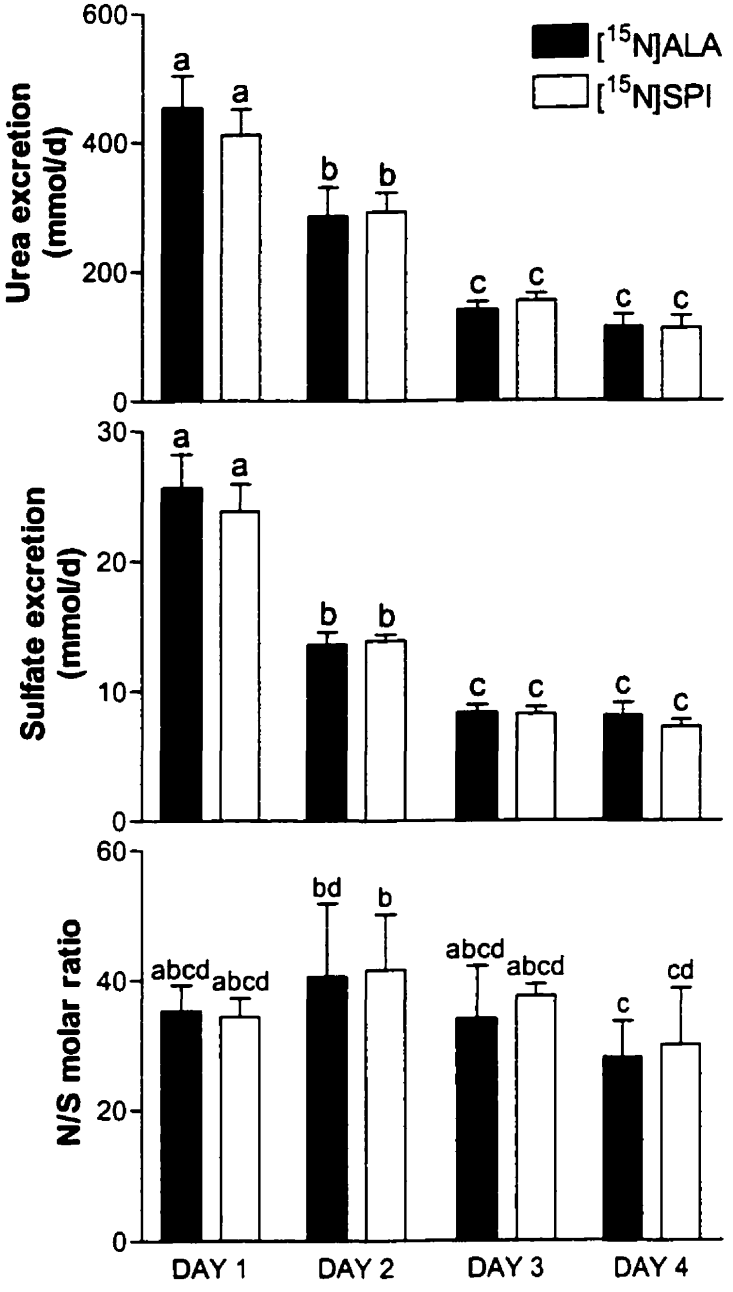

Figure 1. Twenty-four hour urea and sulfate excretion (mmol/d) and N/S molar ratio for 6 healthy men prior to (Day 1) and during protein restriction (Days 2-4).

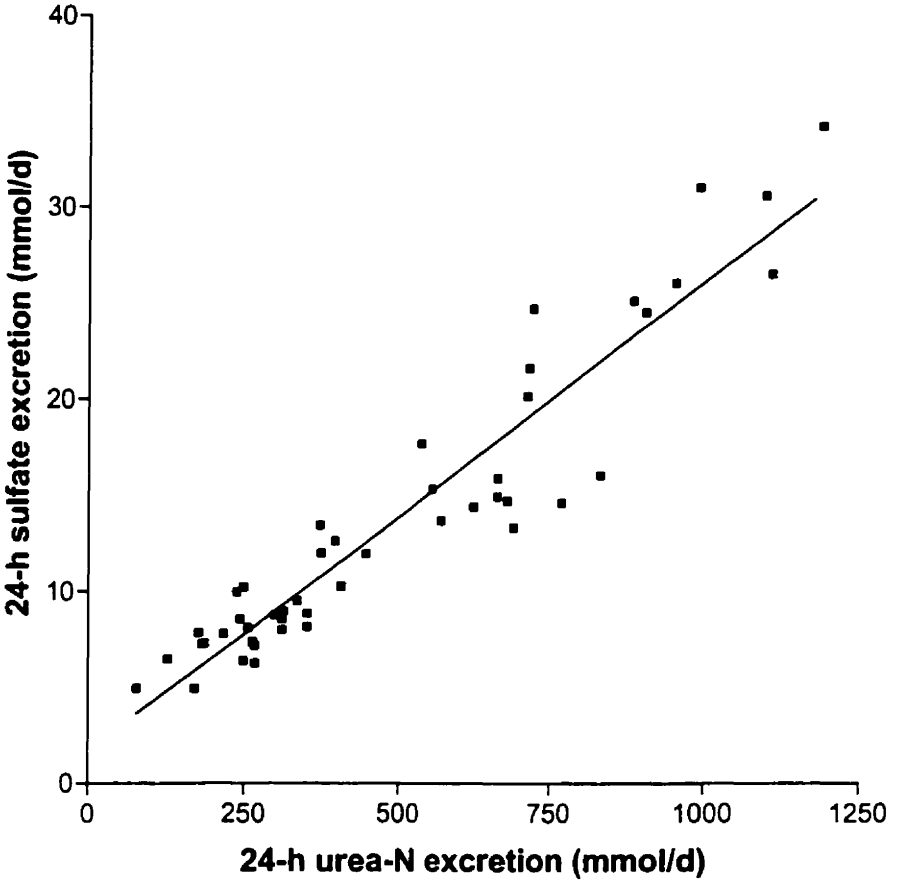

Figure 2. Relationship between 24-h urinary urea-N (mmol/d) and sulfate excretion (mmol/d) ($r = 0.95$, $P < 0.0001$). Sulfate excretion = (0.024 ± 0.001)·urea-N excretion + $(1.68 + 0.70)$. The inverse of the slope is the molar N/S ratio, 41.

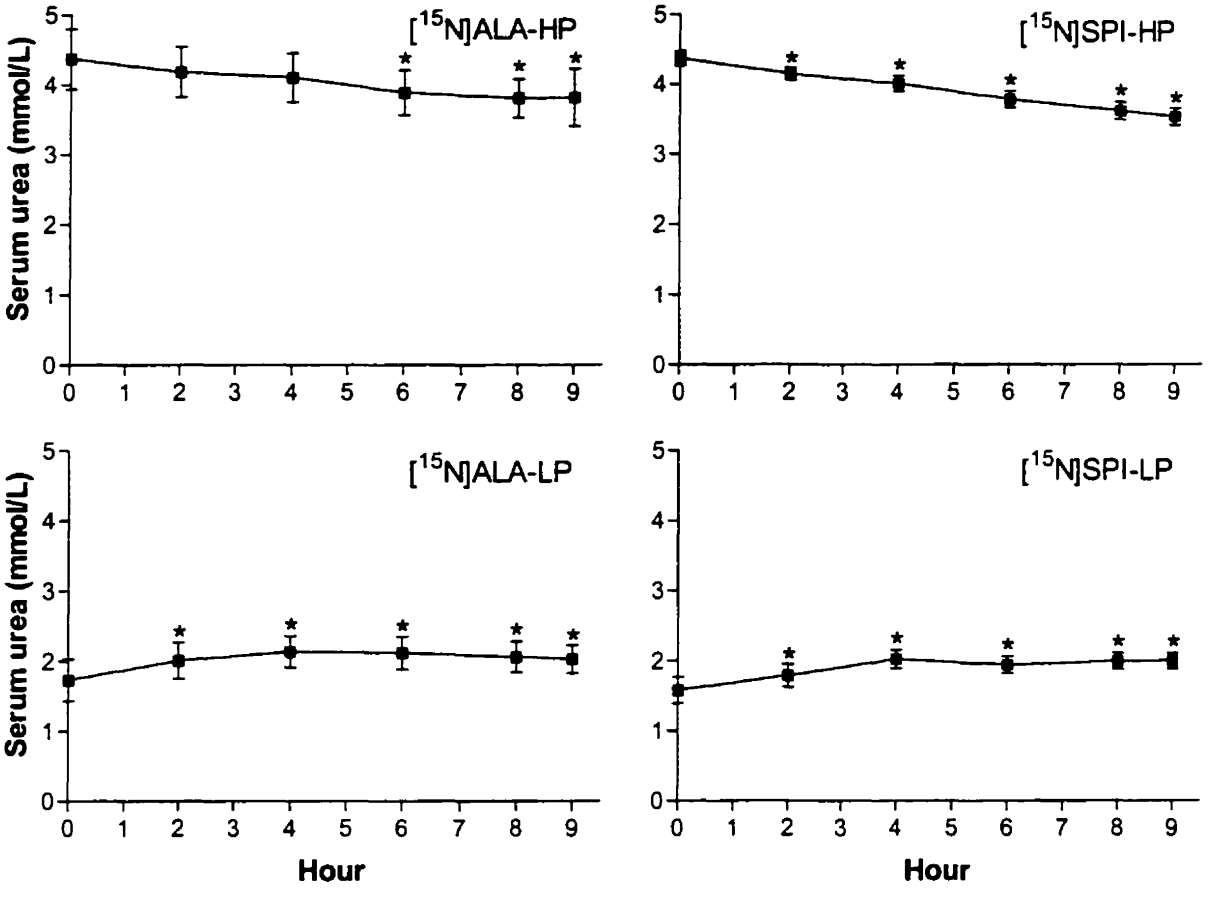

Figure 3. Serum urea concentrations (mmol/L) over 9 h for 6 healthy men prior to ([^{15}N]ALA-HP and [^{15}N]SPI-HP) and following ([^{15}N]ALA-LP and [^{15}N]SPI-LP) protein restriction. The test meal was consumed immediately after the measurement at time zero.

Table 3. Post-meal urea and sulfate metabolism.

Group	Control				IDDM
^{15}N source	[^{15}N]ALA		[^{15}N]SPI		[^{15}N]ALA
Diet	HP	LP	HP	LP	HP
N metabolism (mmol/kg)					
Intake	2.84 ± 0.01	2.84 ± 0.01	3.00 ± 0.02†	3.01 ± 0.01†	2.83 ± 0.00
Production					
per 6 h	2.63 ± 0.16	1.94 ± 0.10*	2.49 ± 0.07	1.85 ± 0.17*	2.25 ± 0.30
per 9 h	3.65 ± 0.16	2.60 ± 0.15*	3.48 ± 0.17	2.72 ± 0.24*	3.38 ± 0.47
Balance					
per 6 h	0.21 ± 0.16	0.91 ± 0.10*	0.52 ± 0.06	1.16 ± 0.16*	0.58 ± 0.30
per 9 h	-0.81 ± 0.17	0.25 ± 0.16*	-0.48 ± 0.17	0.29 ± 0.23*	-0.55 ± 0.47
NPU (%)					
per 6 h	7.4 ± 5.8	31.9 ± 3.6*	17.2 ± 2.2	38.5 ± 5.4*	20.6 ± 10.5
per 9 h	-28.4 ± 6.1	8.6 ± 5.5*	-15.8 ± 5.7	9.9 ± 7.7*	-19.4 ± 16.5
S metabolism (µmol/kg)					
Intake	57.9 ± 0.3	57.9 ± 0.3	57.3 ± 0.2	57.5 ± 0.2	58.1 ± 0.00
Excretion					
per 6 h	66.0 ± 1.8	34.2 ± 4.6*	63.1 ± 1.5	29.5 ± 2.1*	53.3 ± 7.7
per 9 h	89.6 ± 3.2	45.9 ± 4.4*	88.3 ± 2.1	42.3 ± 3.2*	77.3 ± 10.5
Balance					
per 6 h	-8.1 ± 1.8	23.7 ± 4.6*	-5.8 ± 1.4	27.9 ± 2.1*	4.8 ± 7.7
per 9 h	-31.7 ± 3.4	12.2 ± 4.6*	-31.0 ± 2.0	15.2 ± 3.2*	-19.2 ± 10.5
SAA retention (%)					
per 6 h	-14.0 ± 3.1	41.0 ± 8.0*	-10.1 ± 2.5	48.6 ± 3.7*	8.3 ± 13.3
per 9 h	-54.8 ± 6.0	20.7 ± 7.7*	-54.2 ± 3.5	26.4 ± 5.7*	-33.0 ± 18.0
Post-meal production N/S					
per 6 h	40 ± 2	60 ± 6*	40 ± 2	63 ± 5*	42 ± 2
per 9 h	42 ± 1	58 ± 4*	40 ± 1	62 ± 4*	44 ± 2

Data are presented as mean ± SEM. NPU, net protein utilization. SAA, sulfur amino acids. N/S, urea-N to S molar ratio. * Significantly different from HP, and † significantly different from [^{15}N]ALA, two-way repeated measures ANOVA, $P < 0.05$. The IDDM group was not significantly different from the [^{15}N]ALA-HP control group for any of the measurements.

increase in metabolic N balance from negative to slightly positive values. There was no significant difference upon repeat testing ([^{15}N]ALA versus [^{15}N]SPI).

Over 6 h following the test meal, N production significantly decreased while N metabolic balance and NPU increased with the LP diet (Table 3). The decrease in cumulative urea-N production 6 h following the LP test meal (26%) was similar in magnitude to the decrease over 9 h.

Serum postabsorptive and postprandial sulfate concentrations and postprandial S metabolism

The LP diet significantly decreased postabsorptive (11-13%) and mean ([^{15}N]ALA, 9% NS; [^{15}N]SPI, 12% $P < 0.05$) serum sulfate concentrations (Figure 4). Serum sulfate concentrations did not change significantly over 9 h following the test meal upon repeat testing ([^{15}N]ALA versus [^{15}N]SPI) (Figure 4).

On the LP diet, S excretion was reduced over 9 h following the test meal (49-52%) (Table 3). This reduction was concomitant with a significant increase in S metabolic balance from negative to positive. Consequently, SAA retention significantly changed from negative to positive with the LP diet. There was no significant difference upon repeat testing ([^{15}N]ALA versus [^{15}N]SPI).

Over 6 h following the test meal, S excretion significantly decreased while S metabolic balance and SAA retention increased with the LP diet (Table 3). The decrease in cumulative S excretion 6 h following the LP test meal (48-53%) was similar in magnitude to the decrease over 9 h.

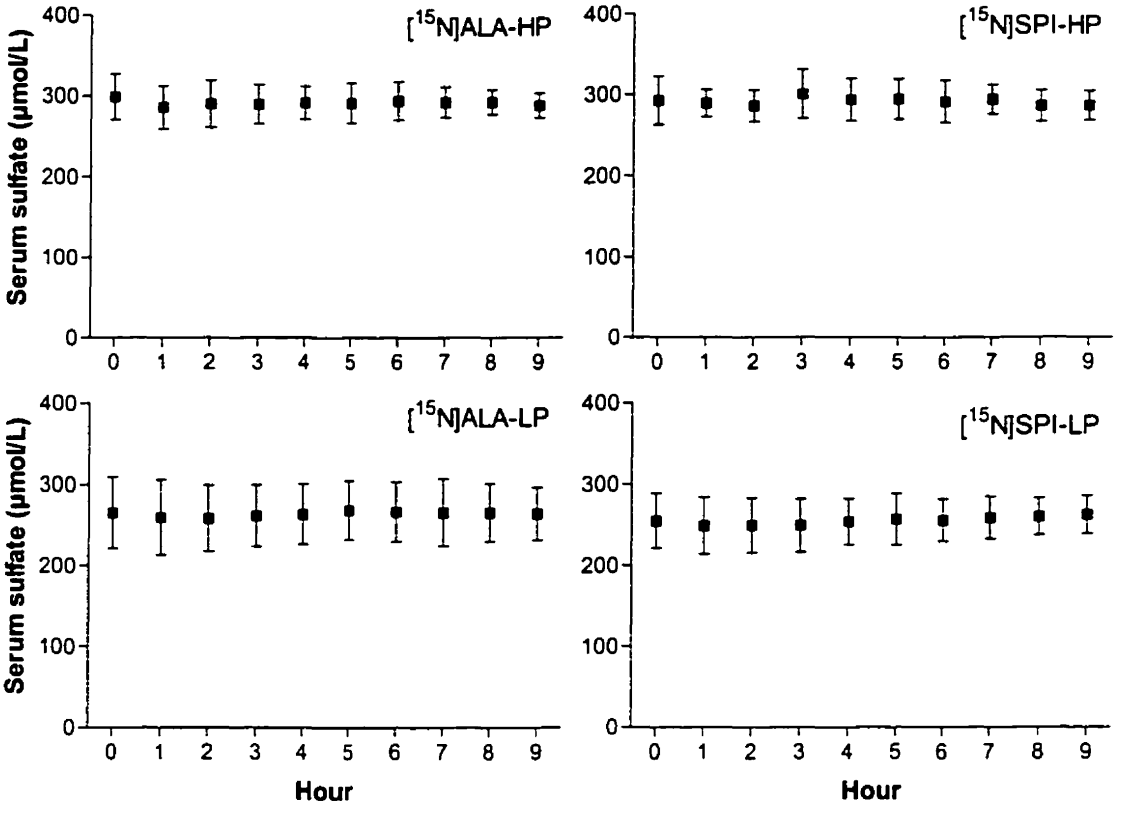

Figure 4. Serum sulfate concentrations (µmol/L) over 9 h for 6 healthy men prior to ([^{15}N]ALA-HP and [^{15}N]SPI-HP) and following ([^{15}N]ALA-LP and [^{15}N]SPI-LP) protein restriction. The test meal was consumed immediately after the measurement at time zero.

Post-meal production N/S molar ratio

Protein restriction significantly increased post-meal production N/S molar ratio, implying a higher proportional retention of SAA than total amino acids over the 6 or 9 h following the test meal (Table 3). There was no significant difference upon repeat testing ([^{15}N]ALA versus [^{15}N]SPI).

Subjects with diabetes mellitus

Six persons with conventionally-treated IDDM consumed the test meal while adapted to their customary, high protein diet. Prior to consuming the test meal, serum glucose concentrations were 9.8 ± 0.9 mmol/l, rising to a maximum of 14.9 ± 2.0 mmol/l two h following the test meal ($P < 0.05$; Figure 5). Postabsorptive serum glucose was 5.3 ± 0.1 mmol/l in the nondiabetic subjects and did not change after the test meal. The subjects with IDDM had a glucose area-under-the-curve (AUC; 95 ± 7 mmol.h/L) twice that of the control group (48 ± 1 mmol.h/L; $P < 0.0001$). Postabsorptive serum urea (4.1 ± 0.7 mmol/l) and sulfate (316 ± 36 μmol/l) concentrations were similar to those of the normal subjects (Figures 6 and 7). Serum urea and sulfate concentrations for the IDDM group decreased over time but were not significantly different from the control group. Urea-N and S metabolism over the 6 and 9 h after the test meal are shown in Table 3. Despite greater variability, there was no indication of excessive fed-state N or sulfate production.

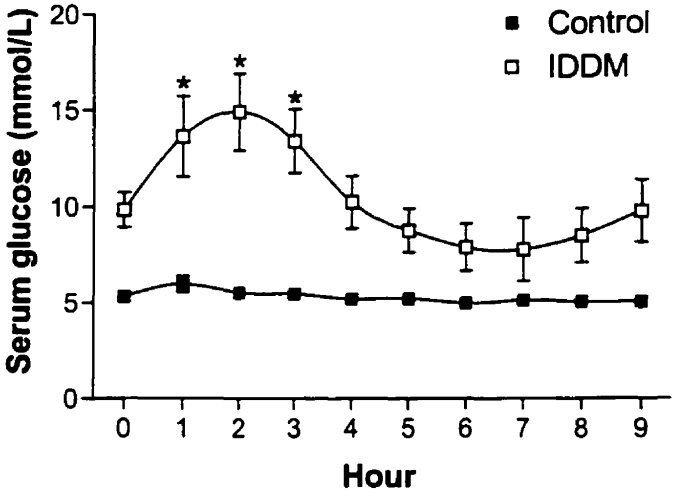

Figure 5. Serum glucose concentrations (mmol/L) over 9 h for 6 control subjects (■) and 6 subjects with IDDM (▫).

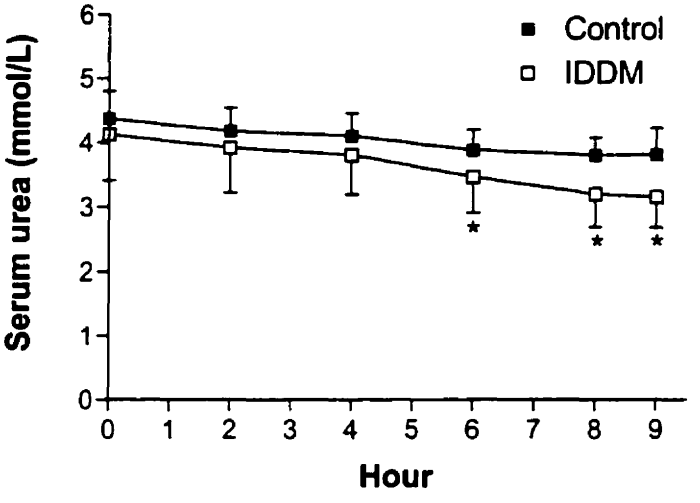

Figure 6. Serum urea concentrations (mmol/L) over 9 h for 6 control subjects (■) and 6 subjects with IDDM (▫).

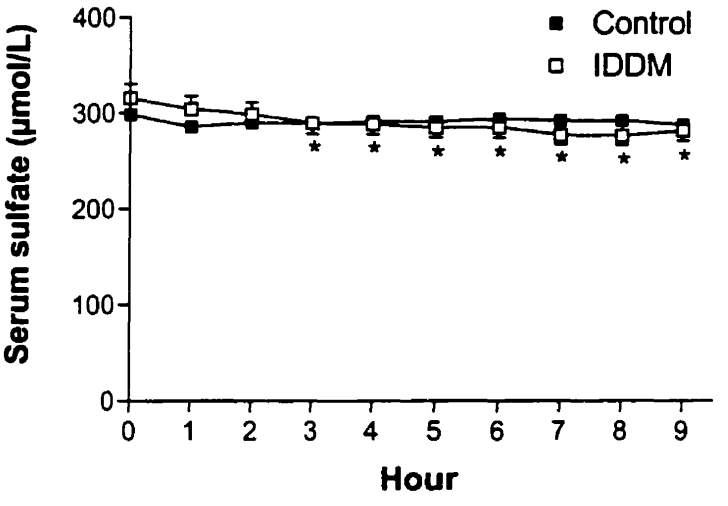

Figure 7. Serum sulfate concentrations (µmol/L) over 9 h for 6 control subjects (■) and 6 subjects with IDDM (▫).

[^{13}C]urea recovery

A trace amount (2.98 mmol) of [^{13}C]urea was added to each test meal with the aim of using its recovery in the serum and urine to indicate the effects of diet or diabetes mellitus on unmeasured extra-urinary urea losses, or altered distribution of urea between excretion and retention in body water. When [^{15}N]alanine was in the test meal ([^{15}N]ALA), post-meal recovery of the urea tracer was 97 ± 6 % and 99 ± 10 % for the normal subjects (high and low prior protein intakes, respectively) and 108 ± 3 % for the IDDM subjects. Recovery was significantly greater ($P < 0.05$) when the ^{15}N-labeled amino-acid tracer in the meal was the intrinsically labeled protein ([^{15}N]SPI), (111 ± 6% and 121 ± 6 % for high and low prior protein intakes, respectively). [^{13}C]urea tracer recoveries did not differ significantly with regard to the adaptation diet or between the normal and IDDM subjects.

DISCUSSION

We tested the reproducibility of postprandial urea and sulfate production upon repeat testing. We also investigated the use of sulfate production as an alternative to urea to measure whole-body amino acid oxidation over 6 and 9 h following the nonsteady state consumption of a protein-restricted test meal in persons previously adapted to high and low protein diets. We also measured N and S metabolic balance, NPU and SAA retention. We found postprandial urea and sulfate production were reproducibly decreased following adaptation to protein restriction. Changes in sulfate production paralleled those of urea-N and changes were evident within 6 h following test meal

consumption. We showed that the low protein (0.25 g/kg) test meal is effective for detecting these changes.

We also tested whether the abnormal protein turnover present in conventionally treated IDDM affects body protein economy. Urea-N and S metabolism in 6 subjects with IDDM on conventional insulin therapy and adapted to a conventional high protein diet was similar to that of control subjects.

Normal subjects

Twenty four hour urea and sulfate excretion

Over 3 days of protein restriction, 24-h urinary urea and sulfate excretion reproducibly decreased by 74% and 70%, respectively (Figure 1). This indicates successful adaptation to protein restriction by decreasing whole-body amino acid oxidation. A significant decrease (80-87%) in 24-h sulfate excretion following consumption of SAA-free diets has been observed previously (Lakshmanan et al, 1976).

It is noteworthy that 24-h sulfate excretion on days 3 and 4 were closely similar (Figure 1), in agreement with previous data showing a leveling off in sulfate excretion after 48 h on a SAA-deficient diet (Lakshmanan et al, 1976). This indicates fast adaptation in sulfate excretion to dietary SAA intake.

An important question is whether 3 days of protein restriction is adequate for full metabolic adaptation to a change in the dietary protein level. The answer is no. It is customary in protein metabolic studies to allow a minimum of 4 days for full metabolic adjustment to a dietary change (FAO/WHO/UNU Expert Consultation, 1985; Hoffer,

1999; Quevedo et al, 1994). In a previous study which demonstrated increased urinary obligatory N excretion in intensively-treated IDDM, we allowed 10 days (Larivière et al, 1992). Notwithstanding this caveat, adaptation is substantially underway within 3 days of a change in protein intake (Motil et al, 1994). Thus, while 3 days of low-protein intake is insufficient for complete adaptation, the adaptation that occurs is large and reproducible.

Twenty-four hour sulfate excretion closely correlated with 24-h urea-N excretion ($r = 0.95$, $P < 0.0001$, Figure 2), in agreement with previous observations showing close correlations between urinary sulfate excretion and urinary urea-N (Jourdan et al, 1980) and total N excretion (Jourdan et al, 1980; Lakshmanan et al, 1976). The slope of the regression line indicates a molar N/S ratio of ~40, consistent with the N/S ratio in mixed body proteins and in high quality dietary proteins (Beach et al, 1942; Cheema-Dhadli and Halperin, 1993; Wilson, 1925).

Serum urea

The postprandial decrease in serum urea concentrations in persons adapted to a HP diet can be attributed to the relative scarcity of protein in the meal (0.25 g/kg), and hence the lower availability of dietary amino acids for oxidation. Relative to a conventional breakfast on a normal high protein diet (1.5 g/kg.d), the test meal represented a low protein breakfast.

The postprandial increase in serum urea concentrations in persons adapted to a LP diet suggests that the test meal of 0.25 g protein/kg provided relatively abundant amino

acids for oxidation when previous daily protein intake did not exceed 0.3 g protein/kg.d. The protein in the test meal was more than twice the amount the subjects would consume for breakfast on the LP diet (0.3 g/kg.d).

Baseline and postprandial urea concentrations were reproducible upon repeat testing ([^{15}N]ALA versus [^{15}N]SPI) (Figure 3).

Serum sulfate

Serum sulfate concentrations did not change significantly over the 9 h following the test meal (Figure 4). This obviated the need to correct sulfate production for any changes in ECF, so that sulfate production was equivalent to its urinary excretion. This ascertains the use of urinary sulfate excretion as a measure of sulfate production postprandially, an advantage when carrying out metabolic studies in the clinical setting.

Baseline and postprandial sulfate concentrations were reproducible upon repeat testing ([^{15}N]ALA versus [^{15}N]SPI) (Figure 4).

Postprandial cumulative urea-N and S metabolism and 6-h collection periods

Cumulative urea-N and S production 9 h following the test meal reproducibly decreased with the LP diet (Table 3). These changes were similar in magnitude to the decrease in cumulative N and S production 6 h following the test meal. The observed increase in conservation of dietary protein was closely reproducible upon repeat testing.

Results obtained over collection periods of 6 h corresponded well with those obtained over 9 h. Therefore, the use of collection periods as short as 6 h can provide

accurate information on changes in whole-body protein conservation under normal fed-state conditions and following adaptation to protein restriction.

We found post-meal urea production to be reproducible despite the potential for problems with this measurement. One problem is the need to adjust urinary excretion for any change in the body's large urea pool. In the present study, serum urea concentrations fell after consumption of the test meal when the previous protein intake had been high, a phenomenon also observed by Owen et al (1980). We attribute this to the relatively low protein content of the test meal in the face of continuing turnover of a large urea pool. Post-meal serum urea rose when the test meal followed protein restriction, for the protein in the test meal was now more than twice what the subjects consumed for breakfast on their protein-restricted diet, and hence sufficient to increase the size of their diminished body urea pool.

There are theoretical advantages to using sulfate production to study the factors governing metabolic adaptation and short-term amino acid catabolism (Cheema-Dhadli and Halperin, 1993). First, from the strictly technical point of view, the sulfate production measurement must be more reliable than urea production in short-term metabolic studies. Unlike urea, which distributes throughout TBW, sulfate distributes in the considerably smaller ECF; moreover, unlike urea, it is not subject to important losses in the gut (Hamadeh and Hoffer, 2001a). An additional technical advantage in the present study was that, unlike urea, serum concentrations remained constant in the post-meal state, entirely obviating the need for a body pool correction, with its attendant potential for error (Figures 3 and 4). An important feature which distinguishes SAA

from total amino acid catabolism is the considerable storage of cysteine in a non-protein reservoir, glutathione, which occurs under certain circumstances (Cheema-Dhadli and Halperin, 1993; Cho et al, 1981; Fukagawa et al, 1996; Tateishi et al, 1981). To the extent that this occurs, sulfate production will not simply indicate whole body net proteolysis; rather, in conjunction with an appropriate measure of whole body urea production, it could provide additional insight into the mechanisms governing metabolic adaptation (Hamadeh and Hoffer, 2001a).

This is illustrated in Table 3, which shows that adaptation to protein restriction was associated with a ~25% reduction in post-meal urea production but a far greater, ~50% reduction in post-meal sulfate production, and a corresponding, marked increase in the post-meal production N/S molar ratio. This simple observation suggests that an important feature of the adaptation to protein restriction is specific replenishment of the non-protein SAA pool (presumably glutathione), in addition to new protein synthesis, after consumption of a protein-containing meal.

Subjects with diabetes mellitus

Post-meal urea and sulfate production by the IDDM subjects was normal. This supports our earlier conclusion that IDDM subjects previously adapted to a high protein intake have normal post-meal urea production (Hoffer et al, 1997). In fact, we predicted that our low-protein test meal might unmask a state of inefficient amino acid conservation created by the mildly insulin-deficient state characteristic of conventionally-treated IDDM, but concealed by the high protein content of the customary diet (Hoffer,

1998). The customary daily protein intake of ~1.5 g/kg corresponds roughly to three 0.5 g/kg protein meals, about twice what was in the test meal. A person adapted to such a high protein intake would be expected to catabolize an excessive amount of the protein in the first of a series of low protein meals, until adaptive mechanisms come into play to reduce amino acid catabolism to a lower level compatible with zero N balance (Hoffer, 1999).

This is illustrated in Table 4, which incorporates data from an earlier study using a 0.5 g protein/kg test meal (Hoffer et al, 1997). When a conventional amount of protein was consumed by persons adapted to a high customary protein intake, their metabolic N balance and NPU were mildly positive, as would be anticipated under such conditions. When similarly conditioned subjects were presented with a test meal that contained half the amount of protein they were habituated to (present study), their post-meal metabolic N balance and NPU were substantially negative. We predicted that IDDM subjects would have an even more profoundly negative N (or S) balance on account of their relative insulin deficiency, but despite basal hyperglycemia and a post-meal rise in plasma glucose consistent with conventional insulin therapy (DCCT Research Group, 1993), this proved not to be the case.

We further predicted that post-meal sulfate production would be even more markedly abnormal in IDDM. Insulin withdrawal in IDDM increases both splanchnic proteolysis and splanchnic protein synthesis, the latter stimulated in part by an influx of amino acids resulting from increased muscle proteolysis (Nair et al, 1995). Such increased splanchnic amino acid turnover should yield abundant cysteine for glutathione

Table 4. Postprandial N metabolism 9 h following test meal consumption in healthy subjects prior to and following protein restriction in two different studies.

Test meal protein content	0.50 g protein/kg		0.25 g protein/kg	
Level of dietary protein	HP	LP	HP	LP
Urea-N production (mg N/kg.9 h)	70 ± 3	48 ± 3*	51 ± 2	36 ± 2†
Metabolic N balance (mg N/kg.9 h)	10 ± 4	32 ± 3*	-11 ± 2	3 ± 2†
NPU (%)	13 ± 4	40 ± 4*	-28 ± 6	9 ± 6‡

Data are presented as mean ± SEM. Total N intake was 80 mg N/kg for the 0.50 g protein/kg test meal (n = 8) and 40 mg N/kg for the 0.25 g protein/kg test meal (n = 6). Data for the 0.50 g protein/kg test meal are from Hoffer et al, 1997.

* Significantly different from the HP diet, paired t test $P < 0.05$.

† Significantly different from the HP diet, paired t test $P = 0.0007$

‡ Significantly different from the HP diet, paired t test $P = 0.0005$.

synthesis, and indeed, unlike the fasting (Hum et al, 1991) or protein deficient rat (Hum et al, 1992), the IDDM rat has a normal hepatic GSH store (McLennan et al, 1991). As a consequence of their mildly increased visceral protein turnover, persons with imperfectly regulated IDDM may have better filled GSH stores prior to consuming a protein meal, and hence promptly convert a greater fraction of incoming dietary SAA to sulfate. We consider possible reasons why this result was not obtained in the accompanying article, in the context of the ^{15}N tracer results.

[^{13}C]urea recovery

A problem with measuring urea production is urea hydrolysis in the gut (Fuller and Reeds, 1998; Long et al, 1978; Walser, 1980; Young et al, 2000). Our calculation of urea production ignores this, and therefore underestimated actual urea synthesis. We added a trace amount of [^{13}C]urea to each test meal with the intention of using the recovery of this isotopic tracer as a whole-body "internal standard" that could reveal effects of diet or IDDM on extra-urinary urea losses. [^{13}C]urea concentrations in serum and urine were calculated as the product of total urea concentration and the fraction of it that was mass M+1, while subtracting the contribution to this mass of the [^{15}N]urea synthesized in the body from the ^{15}N-labeled amino acid tracer in the test meals (See Methods). Recovery of the ^{13}C-labeled urea tracer in serum and urine was ~98% with [^{15}N]ALA (in which [^{15}N]alanine was added to the test meal) whether the adaptation diet was high or low in protein, and ~116% with [^{15}N]SPI (in which [^{15}N]*Spirulina platensis* was added to the test meal). Both recoveries are substantially greater than the ~80% that

was anticipated with a non-recycling urea tracer (Long et al, 1978). More [^{15}N]urea was produced with [^{15}N]SPI, which was associated with a much greater apparent [^{13}C]urea recovery. This suggests that our mathematical correction for the [^{15}N]urea contribution to total [M+1]urea was inadequate, probably owing to the inequivalence of enrichment values measured by GCMS (for total [M+1]urea) and IRMS (for [^{15}N]urea). In principle, the technique of using the recovery of a non-recycling urea tracer to correct for hydrolysis and other nonurinary losses should be sound. We are undertaking experiments to elucidate the reasons for these unexpectedly high apparent [^{13}C]urea recoveries.

Despite these concerns, the physiologically important observation is that there was no significant difference in recovery of the urea tracer added to the test meal after high or low protein diets, or between the normal and IDDM subjects. This permits the conclusion that post-meal changes in urea production ascribed to protein restriction or IDDM were not confounded by alterations in non-renal urea elimination or renal clearance. In a previous study using this method with [^{15}N]ALA we reported [^{13}C]urea recoveries of 71-75% (Hoffer et al, 1997). In reviewing those results in light of the present findings, we discovered a calculation error in the earlier data. The correct values are 91 ± 4% (first test meal) and 96 ± 2% (second test meal) for the normal subjects, and 92 ± 3% (first test meal) and 94 ± 3% (second test meal) for the subjects with diabetes mellitus.

Conclusions

Normal adaptation to protein restriction is associated with a reduction in postprandial urea and sulfate production that is highly reproducible on repeat testing. A protein level of 0.25 g/kg in the test meal detects adaptation and could prove to be more sensitive for detecting metabolic dysregulation than a test meal with a higher protein level. Sulfate production effectively depicts fed-state adaptation to protein restriction. The sulfate production measurement is less prone to error than urea since sulfate distributes in a smaller volume than urea, and under different dietary intakes serum sulfate levels do not change significantly 9 h following the test meal, obviating the need, in some cases, for any correction to account for change in its ECF pool size. Postprandial adaptive changes in urea and sulfate production can be accurately depicted over observation periods as short as 6 h. Whole-body protein economy on standard high protein intake is not different between healthy controls and persons with IDDM on conventional insulin therapy.

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CONNECTING TEXT V

Our results from chapter 7 determined that normal adaptation to protein restriction is associated with a reduction in postprandial urea and sulfate production that is highly reproducible on repeat testing. Sulfate production effectively depicts fed-state adaptation to protein restriction. Postprandial adaptive changes in urea and sulfate production can be accurately depicted over observation periods as short as 6 h. Whole-body protein economy on standard high protein intake is not different between healthy controls and persons with IDDM on conventional insulin therapy.

Our next objective was to determine the validity of using the fate of [^{15}N]ALA as a marker of dietary amino acid N. To do this, we tested whether the reduction in the transfer of ^{15}N added to a meal as [^{15}N]ALA into urea, a measure of adaptive increase in first pass dietary protein conservation, was comparable to that for [^{15}N]Spirulina platensis ([^{15}N]SPI), a ^{15}N-labeled intact protein tracer. We observed the distribution of ^{15}N labels in serum amino acids and the time course of serum amino acid concentration over 9 h following a mixed test meal for the two ^{15}N tracer sources ([^{15}N]ALA versus [^{15}N]SPI).

We also tested whether subjects with IDDM on conventional therapy exhibit first pass dietary protein conservation similar to that of controls when tested with a low-protein meal and [^{15}N]ALA.

CHAPTER VIII

Effect of Protein Restriction on ^{15}N Transfer From Dietary [^{15}N]alanine and [^{15}N]*Spirulina platensis* Into Urea

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Effect of Protein Restriction on ^{15}N Transfer From Dietary [^{15}N]alanine and in [^{15}N]*Spirulina platensis* Into Urea

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Running head: Transfer of ^{15}N from [^{15}N]alanine and [^{15}N]*Spirulina platensis*

Key Words: humans, [^{15}N]*Spirulina platensis*, [^{15}N]alanine, stable isotope, fed state, amino acid oxidation.

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ABSTRACT

We have suggested that increased first pass conservation of dietary amino acids is an important adaptation to protein restriction, since protein restriction is associated with a marked reduction in the transfer of the label in [^{15}N]alanine ([^{15}N]ALA) added to a test meal into urea. However, [^{15}N]ALA is a free amino acid and its metabolic fate may not reflect that of whole dietary proteins. In this study, we fed 6 healthy men a mixed test meal containing 0.25 g protein/kg and 10 kcal/kg plus either 3 mg/kg of [^{15}N]ALA or 30 mg/kg of fully ^{15}N-labeled [^{15}N]*Spirulina platensis* ([^{15}N]SPI) while adapted to high (HP, 1.5 g/kg.d) and low (LP, 0.3 g/kg.d) protein intakes, in a randomized cross-over design. Also, 6 subjects with insulin-dependent diabetes mellitus (IDDM) on conventional insulin therapy consumed this test meal and [^{15}N]ALA while adapted to a high protein intake. Serum [^{15}N]urea enrichment and ^{15}N recovery in serum and urinary urea were measured over the 9 h following the test meal as was the pattern of enrichment in circulating amino acids. The LP diet increased postabsorptive, area under the curve and mean serum alanine, glycine, and methionine concentrations, but amino acid enrichments over 9 h following the test meal were similar under high and low protein adaptation for a given ^{15}N tracer source. With [^{15}N]ALA, there was a rapid transfer of the tracer into serum leucine, isoleucine and glutamine. With [^{15}N]SPI, enrichments increased in serum amino acids in general following the test meal. Despite these differences, protein restriction reduced transfer of dietary ^{15}N in [^{15}N]ALA and [^{15}N]SPI to [^{15}N]urea by comparable amounts (38% and 43%, respectively, NS). Postprandial ^{15}N enrichment and recovery in subjects with IDDM were similar to those with the control group. We

conclude that [^{15}N]ALA in a test meal provides information about adaptive changes in dietary amino acid catabolism equivalent to provision of a ^{15}N-labeled intact protein tracer.

INTRODUCTION

Dietary protein restriction induces an adaptive reduction in urea production over the hours following consumption of a constant-composition test meal (Hoffer et al, 1997). There is also reduced transfer of the ^{15}N in a tracer dose of [^{15}N]alanine included in the test meal into urea, suggesting that first-pass splanchnic amino acid retention is involved in the adaptative process (Hoffer et al, 1997). This reduction in ^{15}N transfer into urea was less successful in protein-restricted persons with conventionally-treated insulin-dependent diabetes mellitus (IDDM), raising the possibility that dietary protein requirements are increased for some persons with IDDM (Hoffer, 1998; Hoffer et al, 1997).

However, tracer [^{15}N]ALA is not protein bound and its metabolism may not represent the extent to which the amino acids consumed as dietary proteins are oxidized or conserved for protein synthesis upon their first passage through the splanchnic bed (Metges et al, 2000). To evaluate the use of [^{15}N]ALA as a measure of first pass dietary protein metabolism, we conducted a controlled trial of feeding our subjects a mixed test meal containing either [^{15}N]ALA or [^{15}N]*Spirulina platensis* ([^{15}N]SPI), a ^{15}N-labeled intact protein tracer (Berthold et al, 1991), prior to and following protein restriction.

Previously, we used a high protein (0.5 g protein/kg) test meal in our studies to measure whole-body protein economy prior to and following adaptation to protein restriction (Hoffer et al, 1997; Taveroff et al, 1994). Postprandial cumulative urea production decreased following adaptation to protein restriction, with an increase in metabolic N balance and net protein utilization. This high protein test meal was adequate

to depict adaptive changes in dietary protein conservation, yet a test meal that is limiting in protein content may be preferable for demonstrating subtle changes in dietary protein retention. In the present study, we used a low protein (0.25 g protein/kg) test meal to study whole body protein economy following adaptation to protein restriction, as discussed in the accompanying paper which describes different results from the same research protocol.

The present measurements were made, first, to test whether the reduction in the transfer of ^{15}N added to a meal as [^{15}N]ALA into urea following protein restriction is comparable to what occurs after ingestion of [^{15}N]SPI, an intrinsically labeled protein and, second, to compare the distribution of the different ^{15}N labeling vectors in serum amino acids in order to gain insight into the validity of using the fate of [^{15}N]ALA as a marker for dietary free amino N in future studies. To do this, we studied the transfer of ^{15}N from a mixed test meal into urea in 6 normal men prior to and following adaptation to low protein intake. The subjects underwent the same protocol twice, the replicate protocols differing only in that a tracer dose of [^{15}N]ALA was included in the test meals for three subjects the first time they followed the protocol, and a tracer dose of fully ^{15}N-labeled whole protein, [^{15}N]SPI the second time, with the order reversed for the other three subjects. We also measured ^{15}N enrichment and recoveries 9 h following consumption of the test meal. Finally, we compared ^{15}N tracer results following a low protein test meal in normal subjects and persons with IDDM on conventional insulin therapy adapted to high protein intake.

METHODS

Normal subjects

Subjects and protocols

Six healthy, non-smoking men (age, 27 ± 2 y; weight, 70 ± 9 kg; body mass index (BMI), 23 ± 2 kg/m^2; total body water (TBW), 59 ± 3% of body weight; fat free mass (FFM), 81 ± 4% of body weight; corrected bromide space (CBS), 202 ± 14 mL/kg) with normal biochemistries and taking no medications were randomly assigned to a 5-day cross-over study. Written consent was given for the study, which was approved by the Research and Ethics Committee of the Jewish General Hospital in Montreal.

On day 1, subjects were admitted to the clinical research unit at 0700 h and assigned a high protein diet (1.5 g/kg body weight.d) following anthropometric measurements. On day 2 (test day), a mixed test meal containing either [^{15}N]alanine ([^{15}N]ALA) or processed [^{15}N]*Spirulina platensis* ([^{15}N]SPI) was orally administered after the overnight fast. Upon completion of the study 9 h following test meal intake, subjects consumed 2 low protein meals. On days 3 and 4, subjects consumed a low protein diet (0.3 g/kg.body weight.d). On day 5 (test day), the identical study as on day 2 was repeated. Subjects returned 10 days later to undergo the same 5-day protocol but using the alternate ^{15}N tracer. Half the subjects used [^{15}N]ALA first, and half used [^{15}N]SPI first. On study days 2 and 5, blood and urine were collected prior to and over 9 h following consumption of the test meal. More details are provided in the accompanying paper.

Diet

On day 1, subjects consumed high protein (1.5 g/kg body weight.d) breakfast (0830 h), lunch (1230 h) and dinner (1730 h) at maintenance energy. On day 2 (test day), upon completion of the study 9 h following the test meal subjects consumed 2 low protein meals, thus complementing their daily calories; average protein intake 27.2 ± 1.2 g/day (0.39 ± 0.01 g/kg.d). Day 2 was considered the first day of protein restriction. On days 3 and 4, subjects consumed a low protein diet (0.3 g/kg.d). On day 5, only the test meal was consumed. Total daily energy provision was 38 kcal/kg body weight.

Test meal

The test meal consisted of Glucerna (Ross Laboratories, St. Laurent, QC) mixed with sufficient beet sugar (Rogers Sugar Limited, Winnipeg, MB) to provide per kg of body weight 0.25 g of protein and 10 kcal of energy (10% protein, 30% fat and 60% carbohydrate). Together with the test meal was given either 3 mg [^{15}N]ALA/kg.body weight (99%; MSD Isotopes, Montreal, QC) or 30 mg of processed dried [^{15}N]SPI/kg.body weight (99% ^{15}N; Martek, Columbia, MD). To each meal was also added 200 mg [^{13}C]urea (99%; MSD Isotopes, Montreal, QC) with the aim of using the difference between the amount of tracer administered and the amount of it found in the serum and urine after the meal as a measure of extra-urinary urea losses. The ^{15}N dose ingested with [^{15}N]ALA was 0.47 ± 0.002 mg N/kg body weight and with [^{15}N]SPI was 3.09 ± 0.08 mg N/kg of which ~0.55 mg/kg was in Spirulina alanine (Berthold et al, 1991).

Subjects with diabetes mellitus

Six non-obese subjects (4 men and 2 women; age, 33 ± 5 y; weight, 66 ± 5 kg; BMI, 22 ± 1 kg/m^2; TBW, 58 ± 2% of body weight; FFM, 79 ± 3% of body weight; CBS, 221 ± 8 mL/kg) with IDDM presented to the research unit at 0700 h on the day of the study. Their blood hemoglobin A1C concentrations were 6.2 ± 0.7% (mean ± SD; normal range 3.5-5.5%) and serum insulin C-peptide concentrations were 0.24 ± 0.04 nmol/L (normal range 0.46-0.72 nmol/L). Their diet was the typical Canadian diet, which provides ~1.5 g protein/kg.day (Gray-Donald et al, 2000); they did not change their dietary habit in the days prior to the test meal.

The test meal consisted of Glucerna mixed with sufficient beet sugar to provide per kg of body weight 0.25 g of protein and 10 kcal of energy and included 200 mg [^{13}C]urea and 3 mg [^{15}N]ALA/kg.body weight.

To obtain a fasting blood glucose level akin to that found in conventionally treated diabetes mellitus (10-12 mmol/L) (DCCT Research Group, 1986), subjects reduced their self-administered short-acting insulin dose as described in the accompanying paper (Hamadeh et al, 2001).

Blood and urine were collected prior to and over 9 h following consumption of the test meal. Blood samples were drawn hourly from an arterialized arm vein kept patent with 77 mmol/L NaCl solution at 80 mL/h.

Spirulina platensis

Fully ^{15}N-labeled *Spirulina platensis* was purchased from Martek Biosciences

Corp (Columbia, MD) and confirmed to be 99% ^{15}N by isotope ratio-mass spectrometry (IRMS) on a Kjeldahl digestate. One gram of untreated algae contained 320 mg glycogen (Lo et al, 1970). *Spirulina platensis* was treated in the following manner to remove non-protein N (free nucleotides, free amino acids, and nucleic acids) (Hutchison and Munro, 1961). Perchloric acid (30-35 mL of 0.5 N in saline) was added to each 3 g batch of [^{15}N]SPI, thoroughly mixed and incubated in a 70°C water bath for 20 min. The mixture was centrifuged at 200 *g* for 10 min at room temperature. Perchloric acid supernatant was decanted and the procedure repeated. Following the addition of 40-45 mL of ethanol, the mixture was centrifuged at 200 *g* for 10 min at room temperature. Ethanol was decanted and the procedure repeated 5 times to remove the perchlorate and cholorphyll, after which the resulting product was oven dried at 60°C. Of this dried product, 30 mg/kg body weight were added to the test meal when required.

To analyze for protein N, 30 mg of the dried algal protein residue and 10 mg of catalyst (9 g K_2SO_4 + 1 g $CuSO_4$) were digested with 1mL acid mixture (7 g SeO_2 in 1 L of 18M H_2SO_4) and 1 mL H_2O at 125°C for 30 min, then at 250°C for the next 17 min and at 450°C for 45 min (Munro and Fleck, 1969). To the digestate, 0.1 M sodium phosphate buffer was added for a total volume of 40 mL and thoroughly mixed. The mixture (1 mL) was diluted with 0.8 mL 1 N NaOH and 0.2 mL H_2O and measured on the Antek 7000 elemental analyzer (Antek Instuments Inc., Houston, TX). A typical lot of dried algal protein residue contained 98-118 mg N/g weight.

Analytical methods

Clotted arterialized venous blood was centrifuged at 1400 *g* for 30 min at room temperature and the resulting serum stored at -30°C until analysis. Urea and amino acid concentration and enrichment were analyzed using gas chromatograph-mass spectrometer (GCMS), as previously described (Hamadeh and Hoffer, 1998). To do this, 0.1 mL of serum was mixed with 20 μg of [^{13}C,$^{15}N_2$]urea (99% ^{13}C, 99% ^{15}N, MSD Isotopes, Montreal, QC), 2.5 μg of norleucine (Sigma Chemical Co., St. Louis, MO), 2.5 μg of L-[3,3,3-$^{2}H_3$]alanine (99.4% ^{2}H, MSD Isotopes, Montreal, QC), 2 μg of [2,2-$^{2}H_2$]glycine (CDN Isotopes, Pointe-Claire, QC), 0.5 μg of L-[s-methyl-$^{2}H_3$]methionine (CDN Isotopes, Pointe-Claire, QC), 0.1 μg of L-[3,4-$^{13}C_2$]aspartate (Masstrace Inc., Woburn, MA), and 10 μg of L-[3,3,4,4-$^{2}H_4$]glutamine (Tracer Technologies Inc., Somerville, MA) internal standards, acidified with 1.5 mL of 1 M acetic acid, and applied to 1 mL columns of cation exchange resin (Dowex 50W-X8, 100-200 mesh hydrogen form, Bio-Rad Laboratories, Richmond, CA). Urea and amino acids were eluted into 3.7 mL flat-bottomed vials equipped with Teflon-lined caps (E.I. du Pont de Nemours, Wilmingont, DE) with four sequential 1-mL additions of 3M NH_4OH. The NH_4OH fraction was evaporated under a gentle stream of N_2. Tert-butyldimethylsilyl (TBDMS) derivatives of urea and amino acids were prepared as described by Patterson et al (1993). Urinary urea enrichment was measured similarly by mixing 0.01 mL of urine with 20 μg of [^{13}C,$^{15}N_2$]urea internal standard, bypassing the resin-treatment step.

GCMS analyses were performed using an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) directly coupled to an HP 5988A quadrupole mass spectrometer.

Samples were introduced by splitless injection (1.0 μL) from an HP 7673 autoinjector onto a fused silica DB-1 capillary column (30 x 0.25 mm, 0.25-μm film thickness, J&W Scientific, Folsom, CA) under the following GC conditions: initial column temperature. 110°C (maintained for 2 min); program rate, 6°C/min until 200°C then 10°C/min to a final column temperature of 200°C; helium carrier gas column head pressure, 70 kPa; and injector port and transfer line temperatures, 250°C. The electron impact (EI) MS conditions were as follows: ionizing energy, 70eV; emission current, 300 μA; and source temperature, 200°C. The following ions were monitored by selected ion monitoring (SIM): TBDMS-$CO(NH_2)_2$ (m/z 231.1), TBDMS-[M+1]urea (m/z 232.1), TBDMS-$^{13}CO(^{15}NH_2)_2$ (m/z 234.1), TBDMS-alanine (m/z 158.1), TBDMS-[^{15}N]alanine (m/z 159.1), TBDMS-L-[3,3,3-$^2H_3$]alanine (m/z 161.1), TBDMS-glycine (m/z 218.1), TBDMS-[^{15}N]glycine (m/z 219.1), TBDMS-[2,2-$^2H_2$]glycine (m/z 220.1), TBDMS-leucine (m/z 200.2), TBDMS-[^{15}N]leucine (m/z 201.2), TBDMS-isoleucine (m/z 200.2), TBDMS-[^{15}N]isoleucine (m/z 201.2), TBDMS-norleucine (m/z 200.2), TBDMS-methionine (m/z 218.1), TBDMS-[^{15}N]methionine (m/z 219.1), TBDMS-[s-methyl-$^2H_3$]methionine (m/z 221.1), TBDMS-serine (m/z 390.3), TBDMS-[^{15}N]serine (m/z 391.3), TBDMS-aspartate (m/z 418.2), TBDMS-[^{15}N]aspartate (m/z 419.2), TBDMS-[3,4-$^{13}C_2$]aspartate (m/z 420.2), TBDMS-glutamine (m/z 432.3), TBDMS-[^{15}N]glutamine (m/z 433.3), TBDMS-[^{15}N,^{15}N]glutamine (m/z 434.3), and TBDMS-[3,3,4,4-$^2H_4$]glutamine (m/z 436.3). TBDMS-leucine eluted at 11.5 min, TBDMS-isoleucine at 12.0 min, TBDMS-norleucine at 12.5 min, TBDMS-glycine at 9 min and TBDMS-methionine at 16.0 min.

IRMS was used to measure ^{15}N-urea enrichment. Serum and urinary [^{15}N]urea enrichments were determined by Metabolic Solutions Inc., Merrimack, NH, using a Europa Tracer-mass IRMS (Europa Scientific, Crewe, England), with the N_2 generated from ammonium sulfate used as the reference gas, according to the method of Read et al (1982). This method converts protein nitrogen into NH_3, eliminating any contribution from ^{13}C and ^{18}O.

GCMS was used to measure urea and [M+1]urea enrichments in serum and urine. Standard curves were constructed to measure the concentration of [^{12}C]urea and the mole ratio of [^{13}C]urea to [^{12}C]urea (M+1/M), as described previously (Hamadeh and Hoffer, 1998). To measure [^{12}C]urea concentration, varying amounts of [^{12}C]urea were added to tubes containing 20 μg of [^{13}C,$^{15}N_2$]urea internal standard, which were then derivatized and analyzed by GCMS, and an areas ratio standard curve relating the intensity ratio 231.1/234.1 (M/M+3) and urea was constructed. Serum and urine [^{12}C]urea concentrations were determined from their corresponding M/(M+3) intensity ratios using the areas ratio standard curve. Total urea was calculated as the sum of [^{12}C]urea and [M+1]urea.

Total amino acid concentration was calculated as the sum of [^{14}N]amino acid and its corresponding ^{15}N tracer concentration. ^{15}N recovery in urea was calculated as the cumulative [^{15}N]urea excretion over 9 h following the test meal plus the amount of [^{15}N]urea in TBW at hour 9, divided by total ^{15}N intake.

Statistical analyses

Three-way repeated measures ANOVA was used to determine significant differences in serum amino acid and urea concentrations with the 3 factors being diet (HP vs. LP), ^{15}N source ([^{15}N]ALA vs. [^{15}N]SPI), and time (h 0, 2, 4, 6, 8, and 9). Two-way repeated measures ANOVA was used to determine significant differences in ^{15}N enrichment and recovery, [^{13}C]urea recovery, serum baseline and mean amino acid and urea concentrations, and amino acid area-under-curve (AUC) with the 2 factors being diet (HP vs. LP) and ^{15}N source ([^{15}N]ALA vs. [^{15}N]SPI). Because the absolute amount of ^{15}N in the test meals differed due to the ^{15}N source ([^{15}N]ALA, 0.47 ± 0.00 mg N/kg vs. [^{15}N]SPI, 3.09 ± 0.08 mg N/kg), urea and amino acid enrichments were tested for the two factors, diet (HP vs. LP) and time (h 0, 2, 4, 6, 8, and 9) within the same ^{15}N source. Within the same ^{15}N source and protein level, serum urea concentration over time (h 0 to 9) was subjected to one-way repeated measures ANOVA. When significance occurred, Newman-Keuls test was used post hoc to determine the source of difference. Paired t test was used to determine significant differences in pooled mean AUC [M+1]urea concentrations between the HP and LP diets, and in the decrease in ^{15}N recovery due to the LP diet between [^{15}N]ALA and [^{15}N]SPI.

To determine differences between the control and IDDM groups, Student's unpaired t-test and two-way repeated measures ANOVA were used. When significance occurred with ANOVA, Newman-Keuls test was used post hoc to determine the source of difference. Differences between data were considered significant at $P \leq 0.05$. Results are presented as means ± SEM unless otherwise indicated.

RESULTS

Normal subjects

Serum amino acid concentrations

Postabsorptive serum alanine concentrations were significantly increased on the LP diet by 81-82% (HP, 294-300 μmol/L; LP, 534-542 μmol/L) for both [^{15}N]ALA and [^{15}N]SPI. Serum alanine concentration reached a maximum 2 h following the test meal for both [^{15}N]ALA and [^{15}N]SPI, falling gradually thereafter to baseline levels on the HP diet but lower on the LP diet (Figure 1). With [^{15}N]ALA, h 2 levels (477-544 μmol/L) were 62-81% higher than baseline values (294-300 μmol/L). With [^{15}N]SPI, h 2 levels (667-690 μmol/L) were 25-27% higher than baseline values (534-542 μmol/L). Prior to and 9 h following the test meal, mean serum alanine concentrations significantly increased with the LP diet by 54% for both [^{15}N]ALA and [^{15}N]SPI compared to the HP diet. Serum alanine AUC on the LP diet was significantly higher by 45% with [^{15}N]ALA (HP, 3.29 mmol.h/L; LP, 4.78 mmol.h/L) and 58% with [^{15}N]SPI (HP, 3.07 mmol.h/L; LP, 4.84 mmol.h/L). For the same protein intake, serum alanine concentrations were similar for [^{15}N]ALA and [^{15}N]SPI.

Postabsorptive serum glycine concentrations were increased on the LP diet by 32% with [^{15}N]ALA (HP, 228 ± 13 μmol/L; LP, 300 ± 20 μmol/L: $P < 0.05$) and 26% with [^{15}N]SPI (HP, 226 ± 11 μmol/L; LP, 285 ± 22 μmol/L; N/S). With [^{15}N]ALA, glycine concentration decreased below baseline values over 9 h following the test meal on the LP diet, but virtually did not vary on the HP diet (Figure 2). With [^{15}N]SPI, glycine concentration did not change significantly following the test meal. Prior to and 9

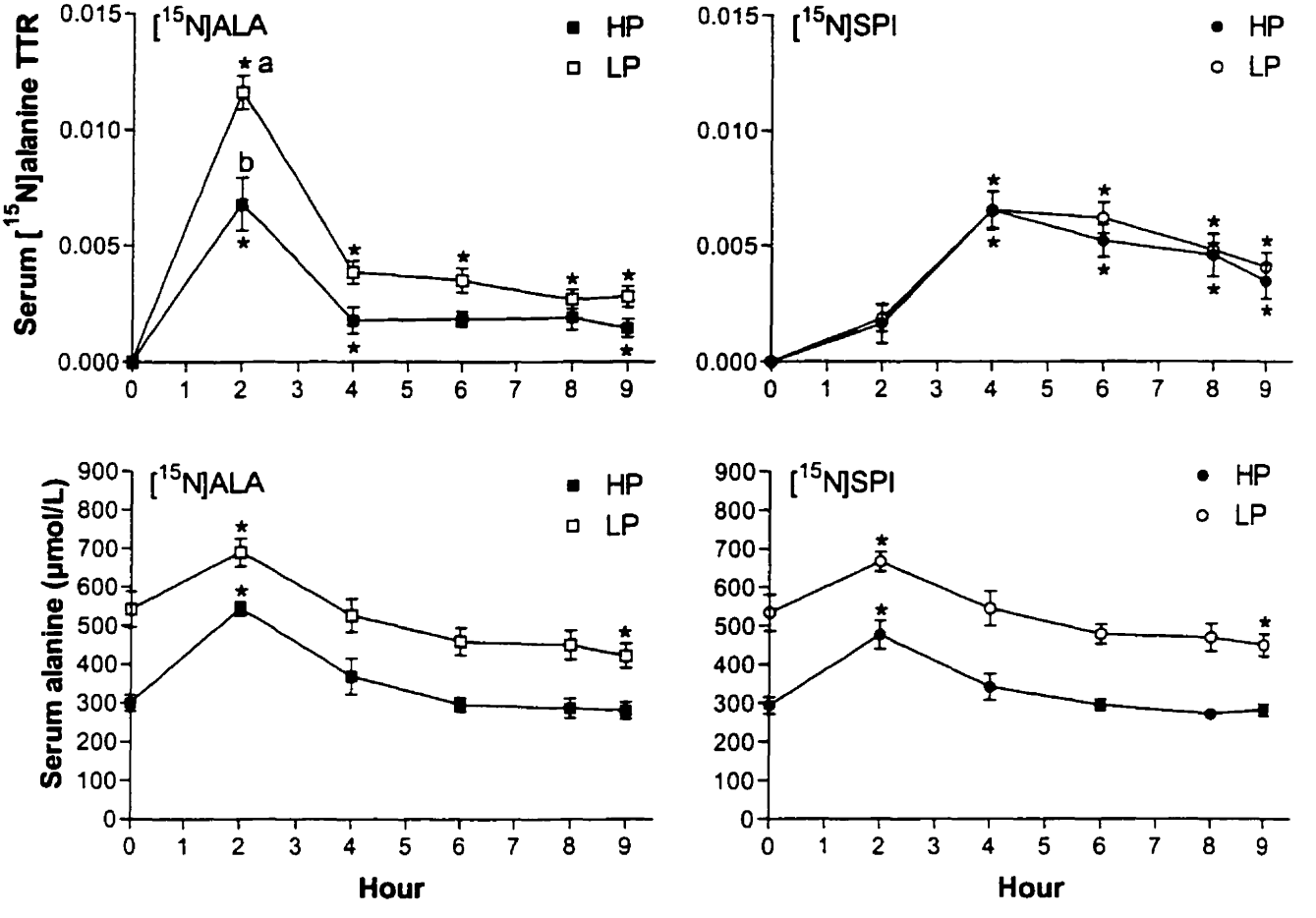

Figure 1. Serum alanine enrichment (TTR) and concentration (μmol/L) over 9 h for 6 healthy men prior to ([^{15}N]ALA-HP, ■; [^{15}N]SPI-HP, ●) and following ([^{15}N]ALA-LP, □; [^{15}N]SPI-LP, ○) protein restriction.

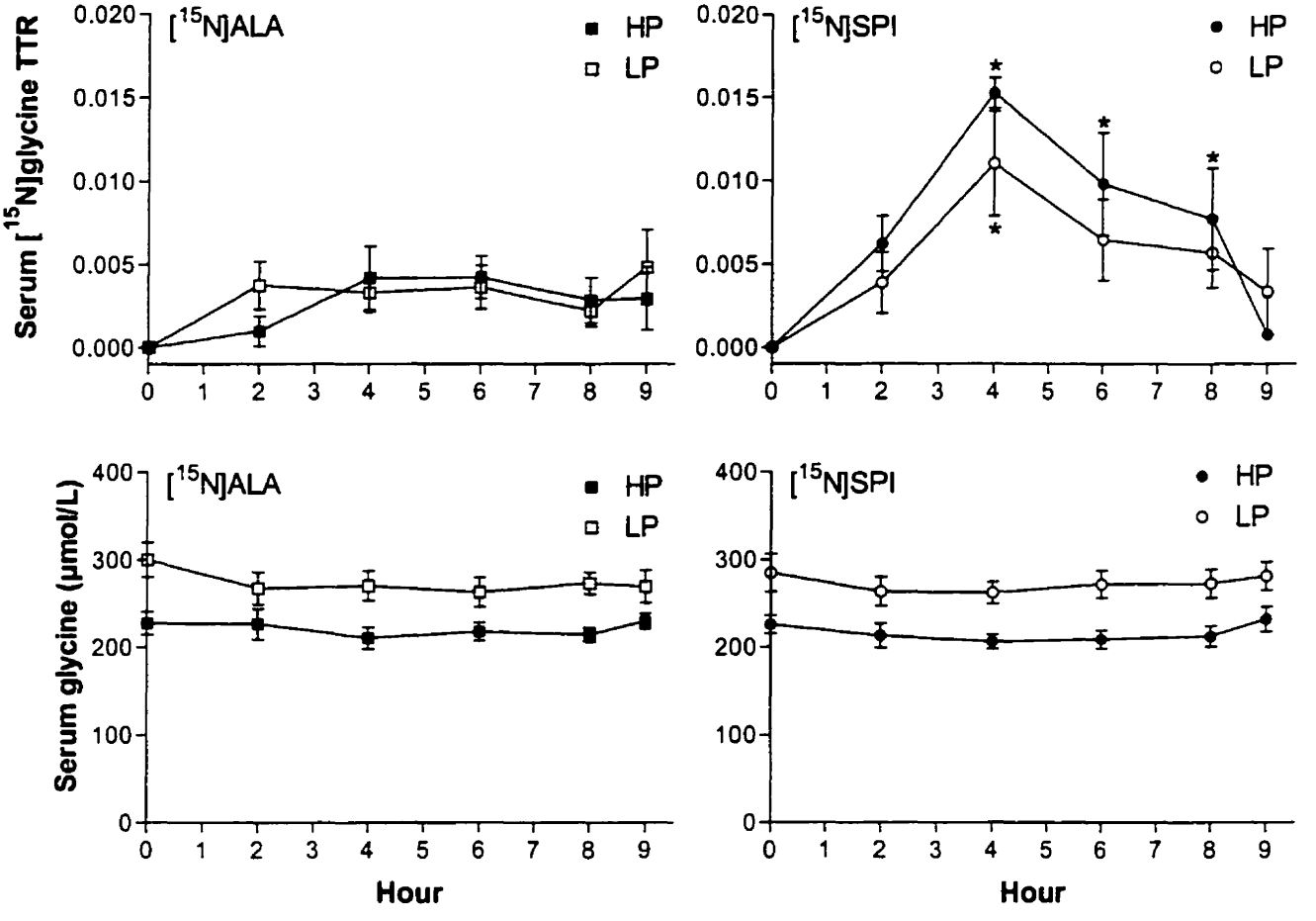

Figure 2. Serum glycine enrichment (TTR) and concentration (µmol/L) over 9 h for 6 healthy men prior to ([^{15}N]ALA-HP, ■; [^{15}N]SPI-HP, ●) and following ([^{15}N]ALA-LP, □; [^{15}N]SPI-LP, ○) protein restriction.

h following the test meal, mean serum glycine concentrations on the HP diet (216-221 μmol/L) were 80% of the LP diet (273-274 μmol/L) ($P < 0.05$). For the same protein intake, serum glycine concentrations were similar for [^{15}N]ALA and [^{15}N]SPI.

Postabsorptive serum leucine concentrations were lower on the LP diet by 10-16% with borderline statistical significance ($P = 0.068$). Serum leucine concentrations reached a maximum 2 h following the test meal, falling to baseline values thereafter (Figure 3). With the HP diet, h 2 concentrations (161-166 μmol/L) were 14-20% higher than baseline values (138-141 μmol/L). With the LP diet, h 2 concentrations (185-186 μmol/L) were 50-55% higher than baseline values (119-124 μmol/L). Prior to and 9 h following the test meal, mean serum leucine concentrations on the LP diet were lower by 7% ($P = 0.062$) compared to the HP diet. Serum AUC were similar between the different treatments. For the same protein intake, serum leucine concentrations were similar for [^{15}N]ALA and [^{15}N]SPI. Closely similar findings were obtained for isoleucine (Figure 4).

Postabsorptive serum methionine concentrations were significantly increased on the LP diet by 18-24%. Serum methionine concentrations reached a maximum 2 h following the test meal, decreasing to below baseline values thereafter (Figure 5). Hour 2 concentrations (33-42 μmol/L) were 42-48% higher than baseline values (23-29 μmol/L). Prior to and over 9 h following the test meal, mean serum methionine concentrations on the HP diet were 81-85% of the LP diet ($P = 0.011$). Serum methionine AUC were significantly increased on the LP diet by 17-24%. For the same protein intake, serum methionine concentrations were similar for [^{15}N]ALA and [^{15}N]SPI.

Postabsorptive serum glutamine concentrations were increased on the LP diet by

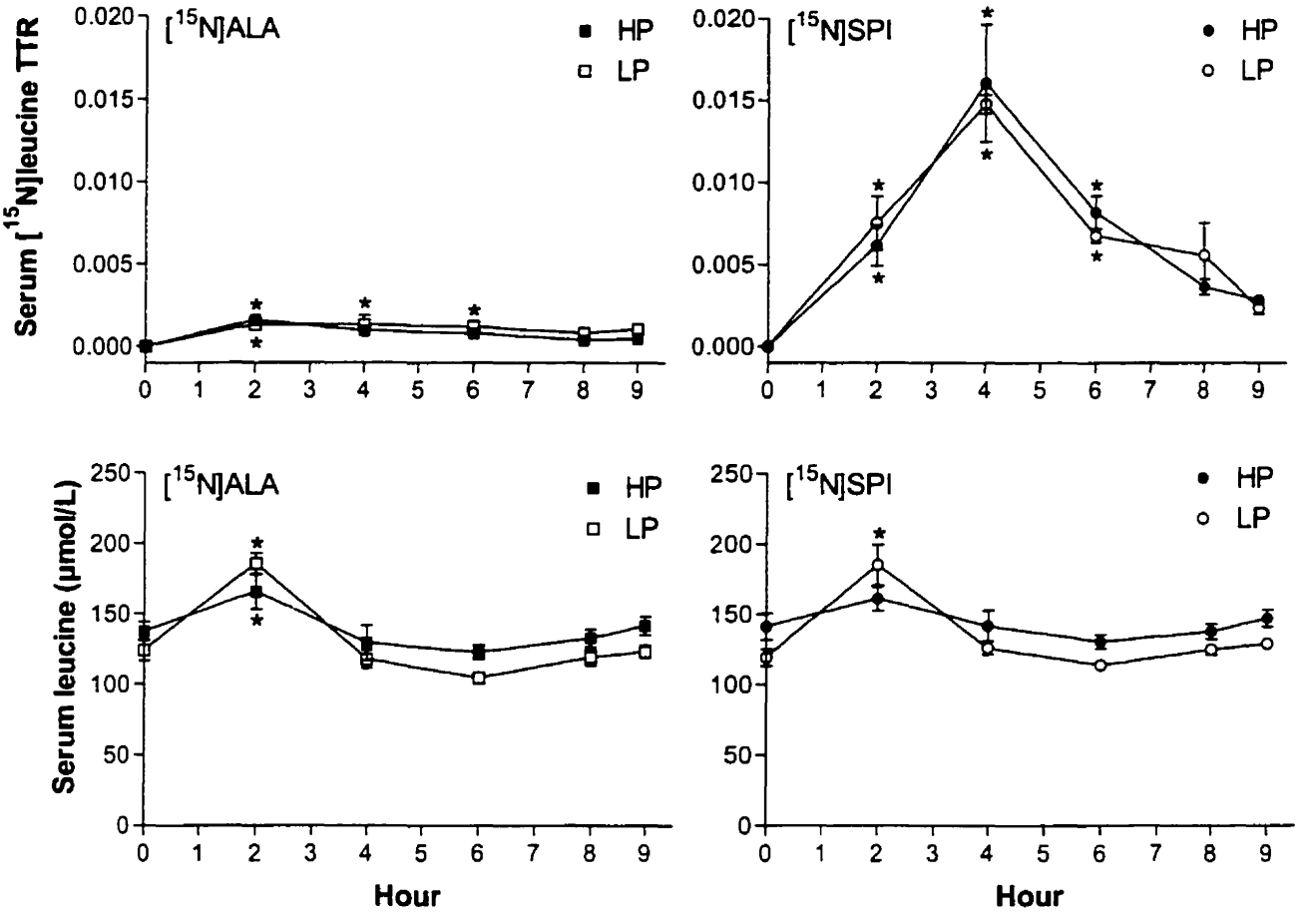

Figure 3. Serum leucine enrichment (TTR) and concentration (μmol/L) over 9 h for 6 healthy men prior to ([^{15}N]ALA-HP, ■; [^{15}N]SPI-HP, ●) and following ([^{15}N]ALA-LP, □; [^{15}N]SPI-LP, ○) protein restriction.

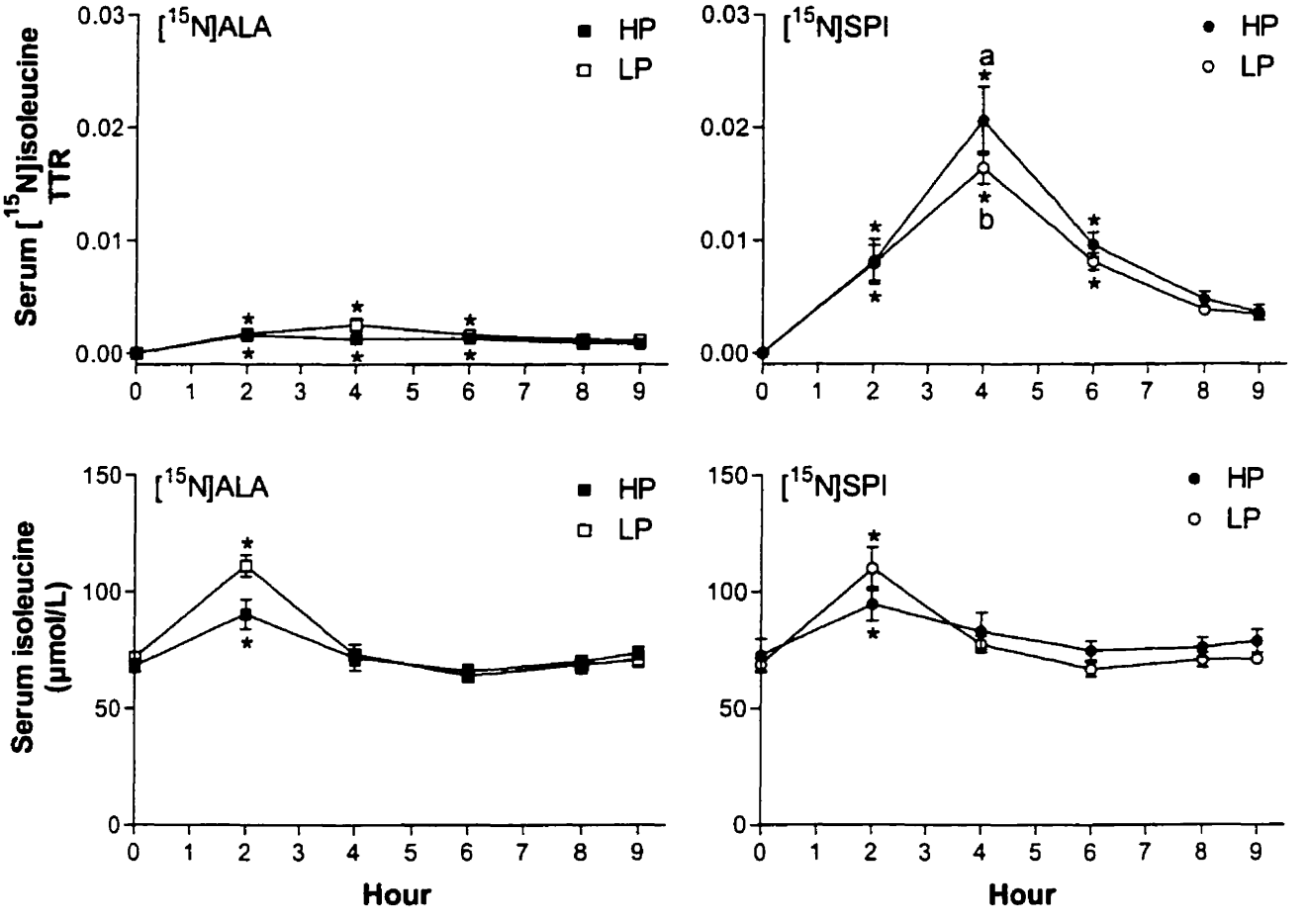

Figure 4. Serum isoleucine enrichment (TTR) and concentration (µmol/L) over 9 h for 6 healthy men prior to ([^{15}N]ALA-HP, ■; [^{15}N]SPI-HP, ●) and following ([^{15}N]ALA-LP, □; [^{15}N]SPI-LP, ○) protein restriction.

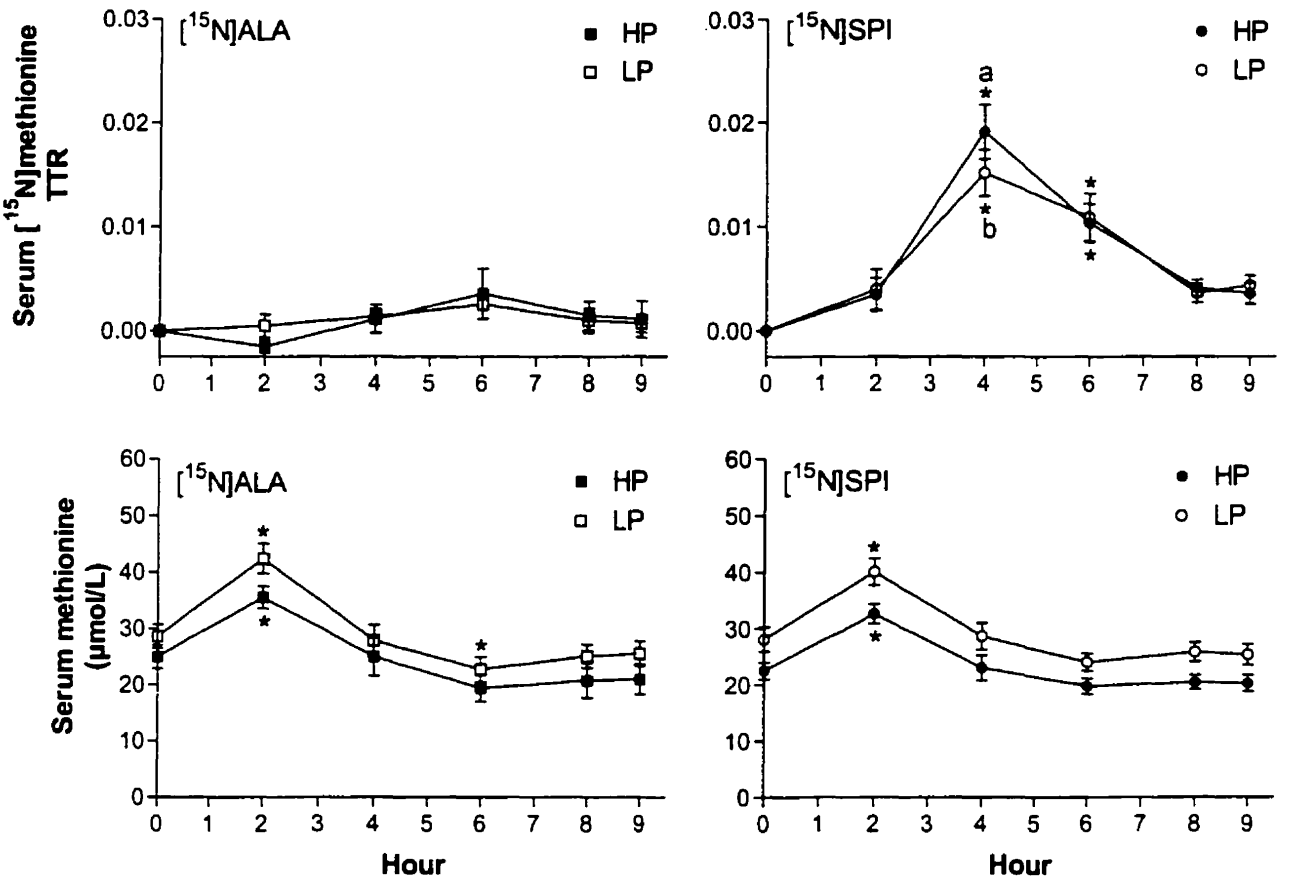

Figure 5. Serum methionine enrichment (TTR) and concentration (μmol/L) over 9 h for 6 healthy men prior to ([^{15}N]ALA-HP, ■; [^{15}N]SPI-HP, ●) and following ([^{15}N]ALA-LP, □; [^{15}N]SPI-LP, ○) protein restriction.

21-22% (Figure 6). Serum glutamine concentrations over 9 h following the test meal were similar to baseline values. Prior to and over 9 h following the test meal, mean serum glutamine concentrations were significantly increased on the LP diet by 14%. Serum glutamine concentrations were significantly higher with [^{15}N]ALA compared to [^{15}N]SPI.

Postabsorptive serum aspartate concentrations were not significantly different between the different treatments. Serum aspartate concentrations over 9 h following the test meal were not significantly different from baseline values (Figure 7).

Serum ^{15}N amino acid enrichments

Serum [^{15}N]alanine enrichment with [^{15}N]ALA reached a maximum (TTR, 0.0068-0.012) 2 h following the test meal, falling precipitously thereafter to levels 26-33% of maximum (Figure 1) . On the LP diet, enrichment at h 2 was significantly higher than with the HP diet. Serum [^{15}N]alanine enrichment with [^{15}N]SPI reached a maximum (TTR, 0.0016-0.0019) 4 h following the test meal, falling gradually thereafter. Enrichments over 9 h following the test meal were similar for both the HP and LP diets.

Serum [^{15}N]glycine enrichment with [^{15}N]ALA did not change significantly following the test meal for both the HP and LP diets (Figure 2). With [^{15}N]SPI, [^{15}N]glycine enrichment reached a maximum (TTR, 0.011-0.015) 4 h following the test meal, falling to almost baseline values by h 9. For the same ^{15}N source, [^{15}N]glycine enrichments were not significantly different between the HP and LP diets. A precisely similar pattern was obtained for serine (data not shown).

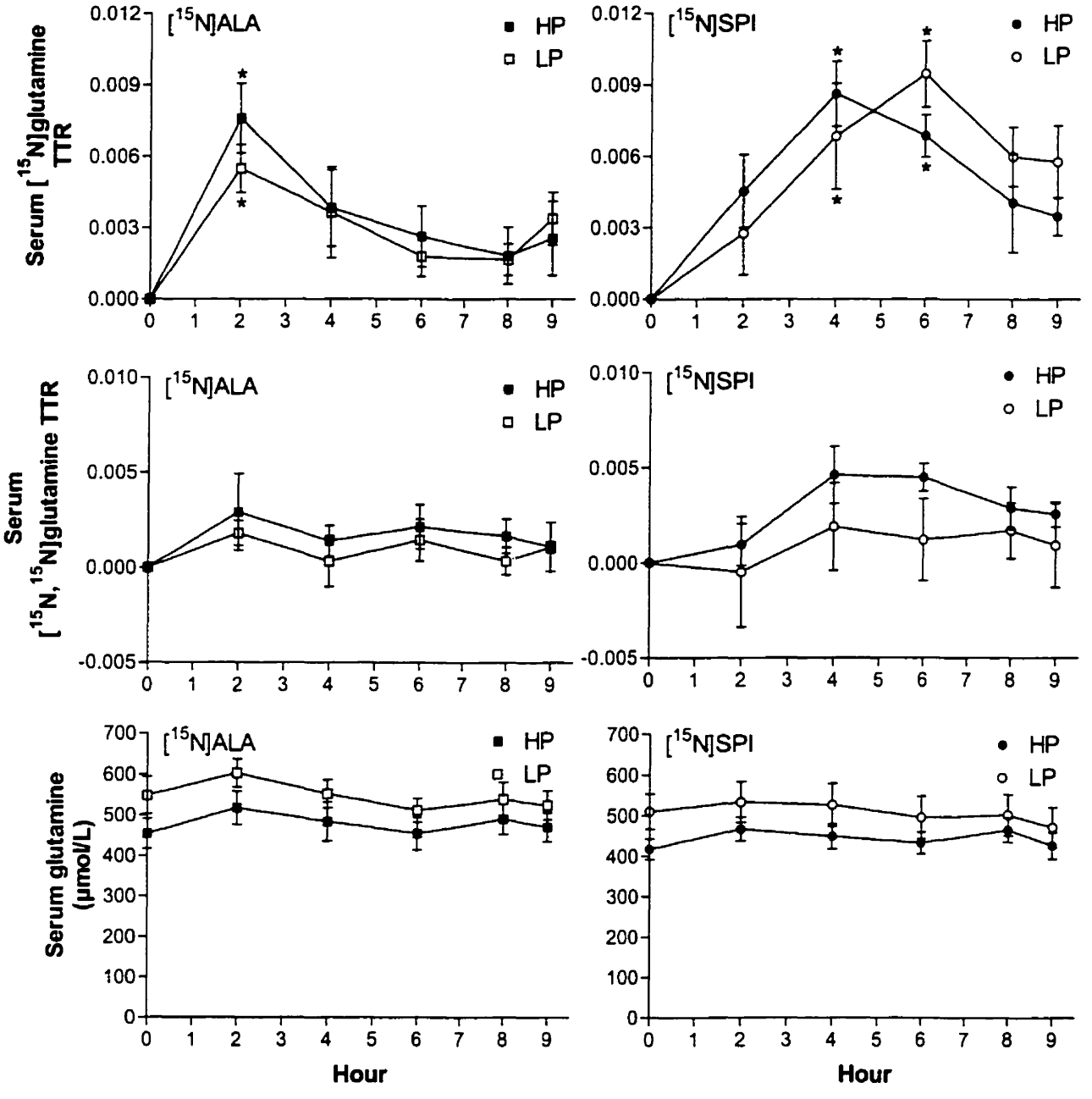

Figure 6. Serum glutamine enrichment ([^{15}N]glutamine and [^{15}N,^{15}N]glutamine, TTR) and concentration (μmol/L) over 9 h for 6 healthy men prior to ([^{15}N]ALA-HP, ■; [^{15}N]SPI-HP, ●) and following ([^{15}N]ALA-LP, □; [^{15}N]SPI-LP, ○) protein restriction.

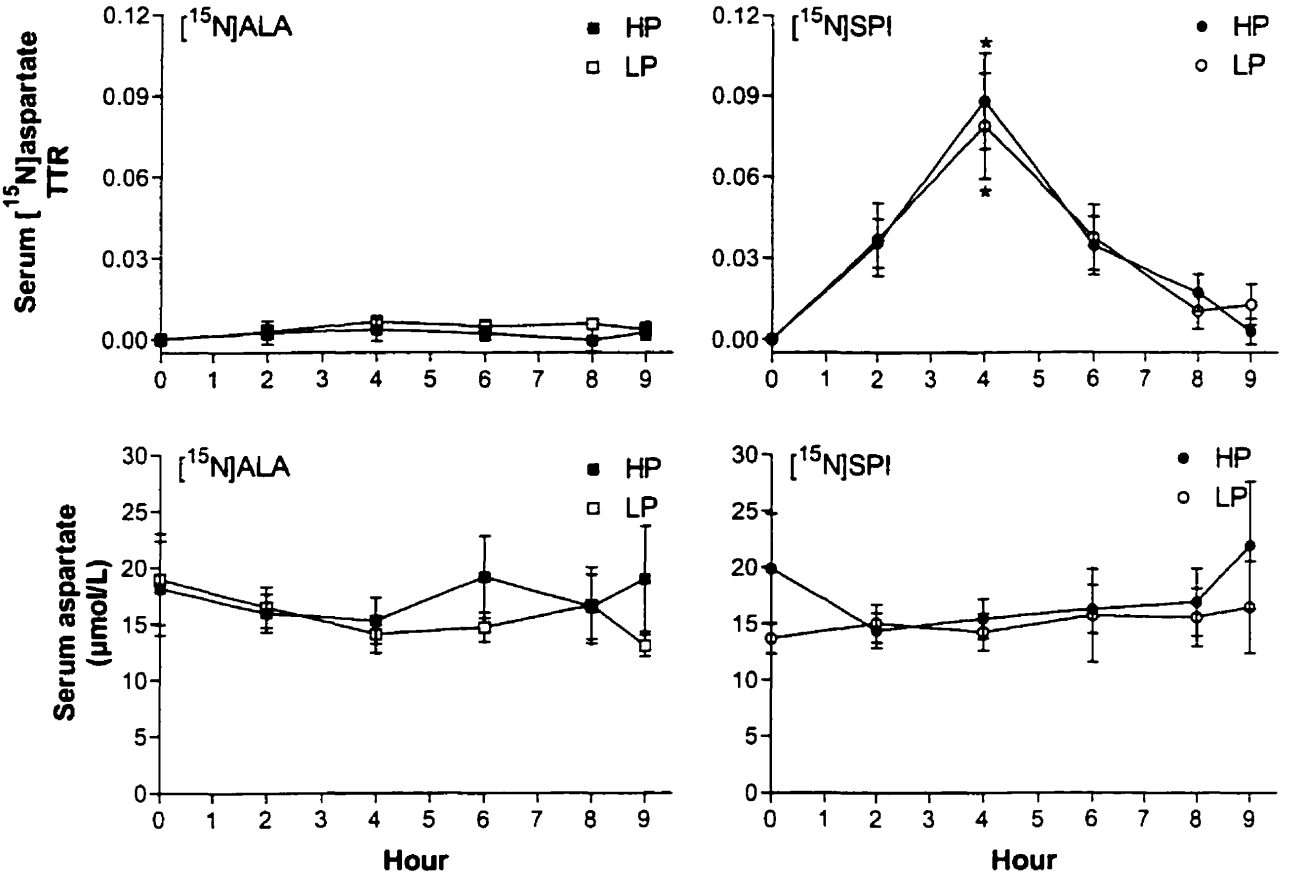

Figure 7. Serum aspartate enrichment (TTR) and concentration (µmol/L) over 9 h for 6 healthy men prior to ([^{15}N]ALA-HP, ■; [^{15}N]SPI-HP, ●) and following ([^{15}N]ALA-LP, □; [^{15}N]SPI-LP, ○) protein restriction.

Serum [^{15}N]leucine enrichment with [^{15}N]ALA increased slightly, but significantly, reaching a maximum (TTR, 0.0013-0.0016) 2 h following the test meal (Figure 3). By h 8, enrichment was down almost to baseline values. With [^{15}N]SPI, [^{15}N]leucine enrichment reached a maximum (TTR, 0.014-0.016) 4 h following the test meal, falling to almost baseline values by h 9. For the same ^{15}N source, [^{15}N]leucine enrichments were similar for the HP and LP diets.

Serum [^{15}N]isoleucine enrichment with [^{15}N]ALA reached a maximum (TTR, 0.0016-0.0025) 2-4 h following the test meal, falling gradually thereafter, being similar for the HP and LP diets (Figure 4). With [^{15}N]SPI, [^{15}N]isoleucine enrichment reached a maximum (TTR, 0.016-0.021) 4 h following the test meal, being significantly higher at h 4 with the HP than the LP diet.

Serum [^{15}N]methionine enrichment with [^{15}N]ALA did not change significantly following the test meal, being similar for the HP and LP diets. (Figure 5). With [^{15}N]SPI, [^{15}N]methionine enrichment reached a maximum (TTR, 0.015-0.019) 4 h following the test meal, being significantly higher at h 4 with the HP than the LP diet.

Serum [^{15}N]glutamine enrichment with [^{15}N]ALA reached a maximum (TTR, 0.0055-0.0076) 2 h following the test meal (Figure 6). With [^{15}N]SPI, [^{15}N]glutamine enrichment reached a maximum (0.0086-0.0094) 4-6 h following the test meal. Serum [^{15}N,^{15}N]glutamine enrichment did not change significantly following the test meal for both [^{15}N]SPI and [^{15}N]ALA (Figure 6).

Serum [^{15}N]aspartate enrichment with [^{15}N]ALA did not change significantly following the test meal (Figure 7). With [^{15}N]SPI, [^{15}N]aspartate enrichment reached a

maximum (TTR, 0.079-0.088) 4 h following the test meal. For the same ^{15}N source, [^{15}N]aspartate enrichments were similar for the HP and LP diets.

Serum [^{15}N]urea enrichments and ^{15}N recovery in urea

[^{15}N]urea enrichment was measured by IRMS which is specific for ^{15}N. Serum [^{15}N]urea enrichment at h 9 following the test meal significantly decreased with the LP diet ([^{15}N]ALA, 48%; [^{15}N]SPI, 68%). It was also significantly higher with [^{15}N]SPI-HP compared to [^{15}N]ALA-HP commensurate with the greater dose of ^{15}N administered ([^{15}N]ALA, 0.47 ± 0.002 mg N/kg; [^{15}N]SPI, 3.09 ± 0.08 mg N/kg).

Recovery of the ^{15}N added to the test meal in body water and urinary urea over 9 h following the test meal was markedly reduced following adaptation to the LP diet (Table 1). ^{15}N recovery in urea was also significantly lower with [^{15}N]SPI than with [^{15}N]ALA. The reduction in the transfer of ^{15}N added to the meal into urea due to protein restriction was 38% for [^{15}N]ALA and 43% for [^{15}N]SPI (NS).

Serum [M+1]urea and total urea concentrations

[M+1]urea enrichment was measured by GCMS, which does not distinguish whether the enrichment is due to ^{15}N or ^{13}C. With the HP diet, [M+1]urea enrichments 2 h following the test meal were similar for both [^{15}N]ALA and [^{15}N]SPI (TTR, 0.013-0.014), thereafter falling gradually with [^{15}N]ALA but remaining high with [^{15}N]SPI. With the LP diet, [M+1]urea enrichment reached a maximum (TTR, 0.032-0.033) 2 h following the test meal for both [^{15}N]ALA and [^{15}N]SPI falling gradually thereafter.

Table 1. Post-meal ^{15}N transfer into urea.

Subjects	Control				IDDM
^{15}N-amino acid source	[^{15}N]ALA		[^{15}N]SPI		[^{15}N]ALA
Prior protein intake	HP	LP	HP	LP	HP
^{15}N produced (mmol)	0.475 ± 0.041	0.296 ± 0.045	0.978 ± 0.122	0.542 ± 0.104	0.330 ± 0.053
^{15}N recovery in urea (%)	20.1 ± 1.4	12.5 ± 1.8*	6.3 ± 1.0†	3.6 ± 0.8*†	15.6 ± 3.0

Data are presented as mean ± SEM. HP, high protein; LP, low protein.

* Significantly different from the HP diet, two-way repeated measures ANOVA $P < 0.05$.

† Significantly different from [^{15}N]ALA, two-way repeated measures ANOVA $P < 0.05$.

[M+1]urea enrichment between h 2 and 9 was significantly higher with the LP diet compared to the HP diet.

With [^{15}N]ALA, [M+1]urea concentration reached a maximum 2 h following the test meal falling gradually thereafter (Figure 8). The levels for the HP and LP diets were similar. With [^{15}N]SPI, [M+1]urea concentrations reached a maximum 4 h following the test meal. With the HP diet, concentrations at h 8 and 9 were significantly higher than with the LP diet.

Postabsorptive and serum urea concentrations 9 h following test meal significantly decreased with the LP diet compared to the HP diet (Figure 8). Overall serum urea concentrations with the LP diet were about half those with the HP diet.

Postprandial cumulative production of [^{15}N]urea as specifically measured by IRMS over 9 h following the test meal was 9-20% that of [M+1]urea. Serum [^{15}N]urea enrichments at h 9 were 0.7-4.7% that of [M+1]urea. Thus, the great majority of the [M+1]urea measured in serum and urine was attributable to the [^{13}C]urea included in the test meal.

Subjects with diabetes mellitus

Subjects with IDDM were studied only under conditions of adaptation to a HP diet. Serum amino acid concentrations followed time patterns similar to the normal subjects although serum glycine and glutamine concentrations were significantly higher while serum leucine and isoleucine concentrations were significantly lower (Figure 9). Serum alanine, aspartate and methionine concentrations were similar for both groups.

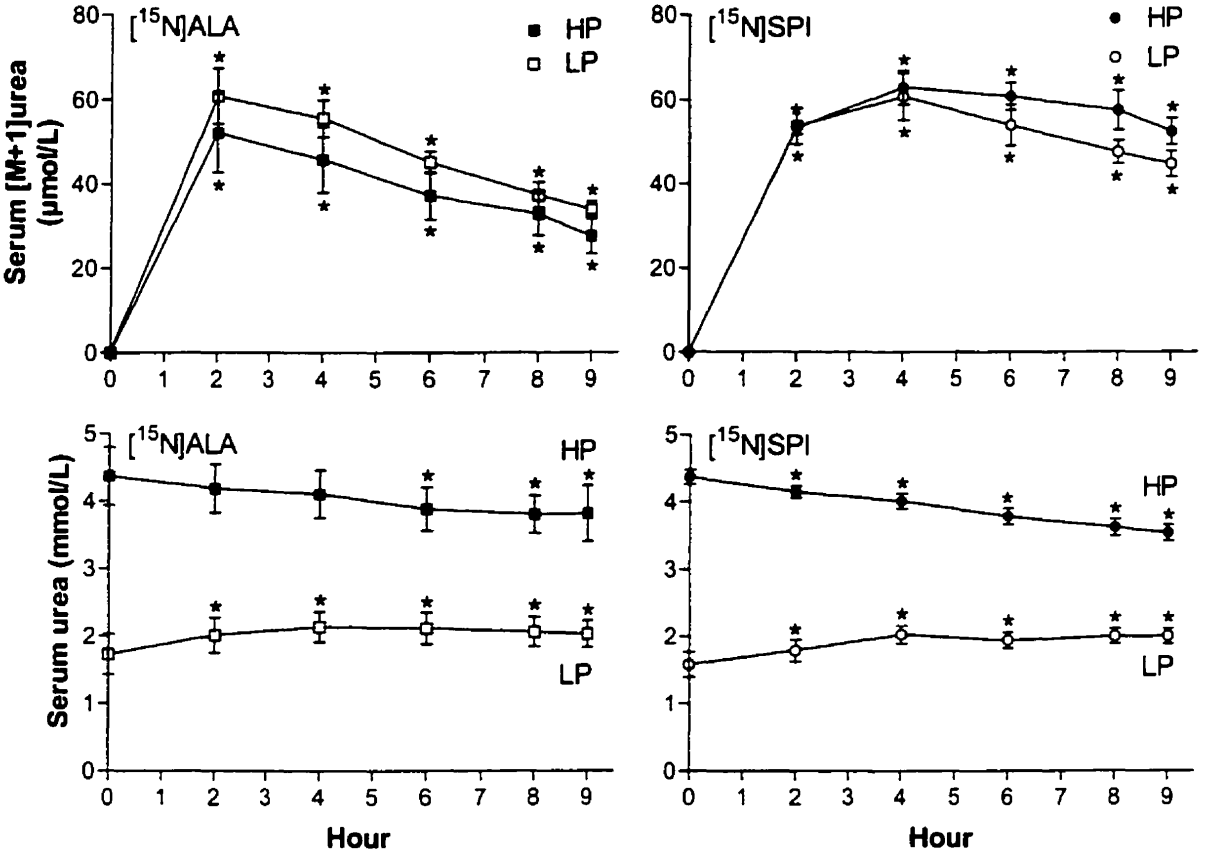

Figure 8. Serum [M+1]urea (μmol/L) and urea concentrations (mmol/L) over 9 h for 6 healthy men prior to ([^{15}N]ALA-HP, ■; [^{15}N]SPI-HP, ●) and following ([^{15}N]ALA-LP, □; [^{15}N]SPI-LP, ○) protein restriction.

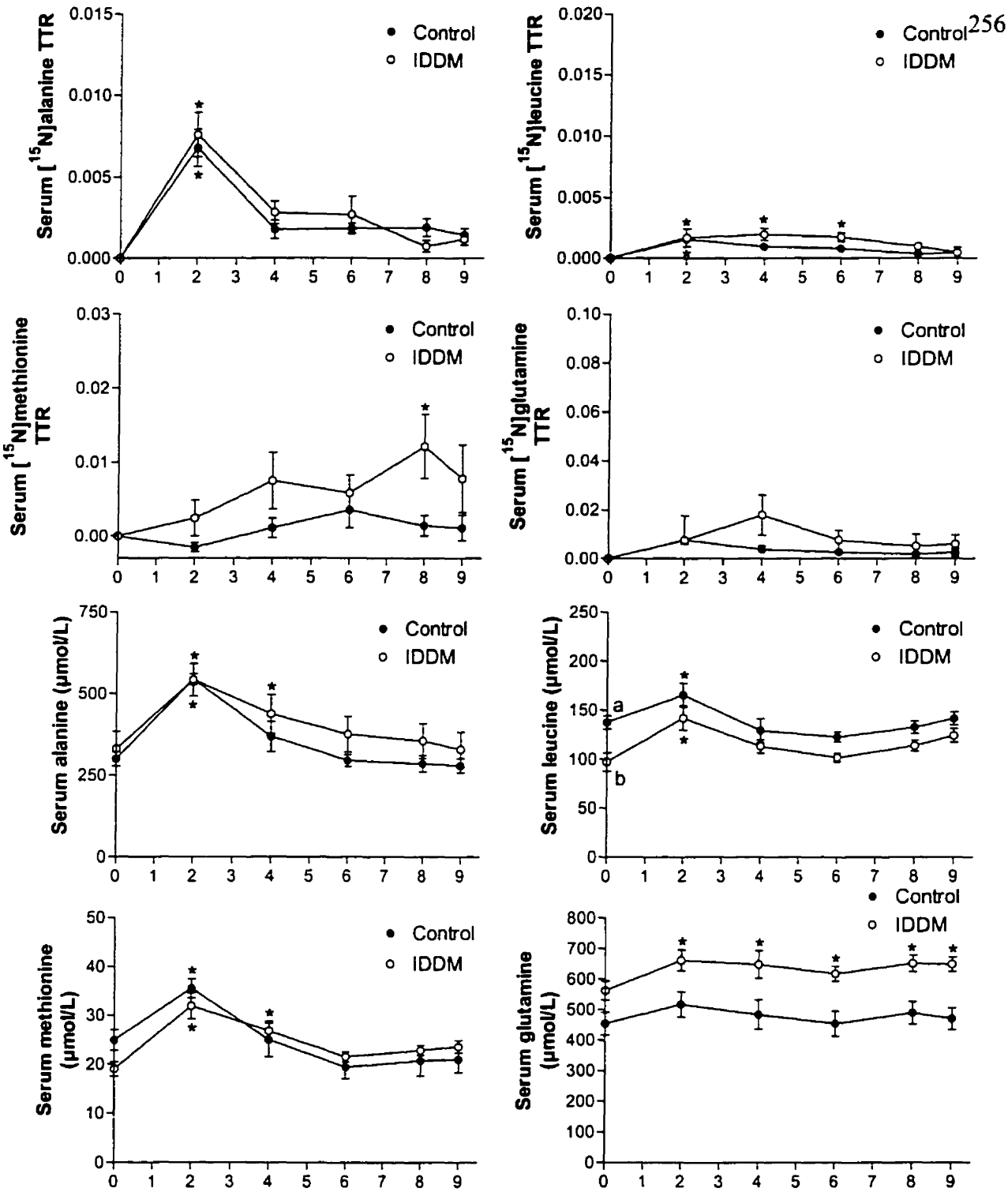

Figure 9. Serum amino acid enrichments (TTR) and concentrations (µmol/L) over 9 h for 6 normal subjects and 6 subjects with IDDM on conventional insulin therapy.

Serum ^{15}N amino acid enrichments were not significantly different between the two groups. Serum [M+1]urea enrichment and serum [M+1]urea and urea concentrations were similar for the IDDM and control groups (data not shown). Post-meal ^{15}N-amino acid enrichments (including those in alanine; data not shown) and ^{15}N transfer into urea (Table 1) was not significantly different between the two groups. ^{15}N recovery in serum and urinary urea over 9 h following consumption of the test meal was similar for the IDDM and control groups (Table 1).

DISCUSSION

We previously concluded that there is an adaptive increase in first pass dietary protein conservation in normal subjects adapted to a low protein intake, because we observed a marked reduction in the transfer of ^{15}N added to a mixed meal as [^{15}N]ALA into plasma and urinary urea. To determine the validity of using the fate of [^{15}N]ALA as a marker of dietary amino acid N, we tested whether the reduction in the transfer of ^{15}N added to a meal as [^{15}N]ALA into urea was comparable to that for [^{15}N]SPI and observed the distribution of ^{15}N labels in serum amino acids and the time course of serum amino acid concentration over 9 h following a mixed test meal for the two ^{15}N tracer sources ([^{15}N]ALA versus [^{15}N]SPI). As was anticipated, far less of the ^{15}N in the whole protein source made it into urea over the time course of the study. The important finding was that the reduction in the transfer of ^{15}N added to the test meal as [^{15}N]ALA following protein restriction was comparable to that for [^{15}N]SPI. This occurred despite quite different patterns of postprandial serum amino acid enrichments with the two tracers.

We also tested whether subjects with IDDM on conventional therapy exhibit first pass dietary protein conservation similar to that of controls when tested with a low-protein meal and [^{15}N]ALA. ^{15}N recoveries in serum and urinary urea and serum ^{15}N amino acids over the 9 h following consumption of the test meal were similar for the IDDM and control groups. This agrees with our previous conclusion, based on a study using a high-protein test meal, that conventionally treated IDDM is associated with normal postprandial amino acid handling as long as the prior diet is high in protein.

Normal subjects

Amino acid concentrations and enrichments

Figures 1-7 illustrate that serum amino acid concentration profiles were closely similar when the dietary protocol was repeated, and that post-meal serum amino acid ^{15}N-labeling pattern was similar for a given ^{15}N tracer whether the preceding diet was high or low in protein. However, the labeling patterns were very different with the different tracers. The observation that serum alanine enrichment reaches a maximum 2 h following consumption of the test meal, rapidly decreasing thereafter with [^{15}N]ALA, but reaches a maximum at h 4 gradually decreasing thereafter with [^{15}N]SPI (Figure 1) is primarily due to the source of ^{15}N tracer. With [^{15}N]ALA, the ^{15}N tracer can be assumed to have been rapidly absorbed and a large amount of it deaminated (Battezzati et al, 1999; Kay et al, 1986; Wolfe et al, 1987). With [^{15}N]SPI, ^{15}N-labeled amino acids were released into the circulation more slowly as the individually pre-labeled amino acids. Despite the difference in serum amino acid enrichment profiles between [^{15}N]ALA and

[^{15}N]SPI, there was a similar reduction in the transfer of the ^{15}N added to the meal into urea following adaptation to protein restriction.

The difference in the time profile distribution of the ^{15}N amino acid enrichments between the [^{15}N]ALA and [^{15}N]SPI treatments is due to the different ^{15}N tracer sources, and was not evident between the HP and LP diets for the same ^{15}N tracer source. The rapid appearance of ^{15}N in serum glutamine, leucine and isoleucine within 2-4 h following consumption of the test meal with [^{15}N]ALA indicates rapid interchange of the amino group between these amino acids. However, the typical increase in serum ^{15}N amino acid enrichments following consumption of the test meal with [^{15}N]SPI is mainly due to tracer dietary amino acids. However, the splanchnic processes underlying the different patterns of amino acid enrichments are, nevertheless, reproducible upon repeat testing within the same ^{15}N tracer source. Thus, the similar decrease in ^{15}N recovery observed following protein restriction for both [^{15}N]ALA and [^{15}N]SPI indicates true reduction in first pass amino acid oxidation.

There was greater total [^{15}N]urea production after [^{15}N]SPI; this can be attributed to its larger ^{15}N dose. The greater *recovery* of ^{15}N from [^{15}N]ALA in urea is a consequence of its rapid deamination in comparison with other amino acids (Battezzati et al, 1999; Kay et al, 1986; Wolfe et al, 1987). The same phenomenon is observed when whole-body N flux is measured using the single-dose end-product model, which calculates flux as the ^{15}N dose divided by the ^{15}N enrichment in urea produced over the subsequent 12 hours (Fern et al, 1984). The flux obtained when the dose is administered as an intrinsically labeled protein is approximately twice that obtained using [^{15}N]ALA

(Fern et al, 1985). We presume that very rapid mixing of ^{15}N from [^{15}N]ALA in the urea precursor pool is the reason why our main outcome measure, fractional change tracer appearance in urea, was closely similar for [^{15}N]ALA and [^{15}N]SPI.

The notion that splanchnic first-pass amino acid conservation or catabolism is important in nutritional adaptation is supported by several studies that have used sophisticated tracer approaches in humans and piglets (Berthold et al, 1995; Cayol et al, 1997; Hunter et al, 1995; Metges et al, 2000; Stoll et al, 1998a; Stoll et al, 1998b). Notwithstanding the complex nature of splanchnic amino acid metabolism indicated by these studies, and the important effects on model parameters when individual tracer amino acids or intrinsically-labeled protein tracers are used (Boirie et al, 1996; Metges et al, 2000), it may be useful in certain situations to bypass the details of how the body achieves its homeostatic aims, and, as in the present study, simply measure the system's regulated output: short-term amino acid catabolism after a metabolic or nutritional challenge.

The present results support such a conceptual approach. They suggest that despite serum amino-acid ^{15}N-labeling that differed greatly from that produced by an intrinsically labeled protein, [^{15}N]ALA gave a similar system output, i.e, an equivalent reduction in ^{15}N-tracer incorporation into urea. The advantages of this method are simplicity and robustness. These render it amenable for use in a variety of clinical settings, with the potential for insights that can be coupled with the results of more sophisticated tracer methodologies.

Serum [M+1]urea and total urea concentrations

The [M+1]urea concentrations measured are almost entirely due to the [^{13}C]urea added to each test meal as a means of verifying equivalent urea handling under conditions of adaptation to high or low protein intake. Enrichment specifically due to [^{15}N]urea (as measured by IRMS) makes a minor contribution (0.7-4.7%) to the [M+1]urea.

It is noteworthy that there was a difference in the urea pool size between the HP and LP diets, as depicted by significantly lower serum urea concentrations on the LP diet (Figure 8). However, serum [M+1]urea concentrations were similar for both diets. This argues that changes in the renal handling of urea following protein restriction could not have accounted for the effects we are attributing to reduced postprandial urea synthesis.

Protein content of the test meal

In our previous study, ^{15}N recovery decreased by 43% following consumption of a high protein test meal (0.50 g protein/kg) in normal subjects adapted to protein restriction (0.05 g protein/kg.d) (Hoffer et al, 1997). In the present study, the decrease in ^{15}N recovery following protein restriction was similar in magnitude (38%; NS). The low protein (0.25 g protein/kg) test meal was equally effective in showing a reduction in the transfer of dietary ^{15}N from the meal into urea but may be more useful in depicting subtle increases in the efficiency to conserve dietary amino acids.

Subjects with diabetes mellitus

We found no indication of excess post-meal transfer of ^{15}N from [^{15}N]ALA into urea in persons with mildly hyperglycemic IDDM. This observation is consistent with their normal post-meal sulfate and total urea production (Hamadeh et al, 2001) and with our earlier finding in conventionally-treated IDDM using a high-protein test meal (Hoffer et al, 1997). Insulin withdrawal increases urea production (Almdal et al, 1990; Freyse et al, 1996) and leucine plasma concentration, oxidation and turnover in IDDM (Nair et al, 1995; Nair et al, 1987). Conventional IDDM therapy is also commonly associated with increased circulating branched-chain amino acid concentrations (Devlin et al, 1994; Luzi et al, 1990; Tamborlane et al, 1979; Trevisan et al, 1989) but, as in the present study, this is not always the case (Borghi et al, 1985; Tuttle et al, 1991; Vannini et al, 1982). Where and how does insulin therapy of IDDM regulate amino acid incorporation into body proteins? At one level, it restrains muscle proteolysis, a restraint which is released when insulin provision is grossly inadequate (Nair et al, 1995; Nair et al, 1987). Tracer studies of post-meal (Tessari et al, 1988) or fed-state (Biolo et al, 1995) amino acid kinetics in insulin-deprived IDDM indicate defective suppression of whole-body proteolysis prior to the meal, with persistence to a varying extent into the fed state. Milder insulin-deficient states may yet suffice to restrain proteolysis, while exerting anabolic and regulatory effects when injected insulin, together with dietary amino acids, reach the liver (De Feo, 1998) and the periphery to stimulate muscle protein synthesis (Fryburg and Barrett, 1995). We suggest that the low serum leucine concentrations measured in our IDDM subjects are evidence that they had sufficient exogenous insulin in their tissues to prevent

a protein-catabolic state, despite their hyperglcyemia. The results would presumably have been different had their insulin deficiency been more severe.

Accuracy of [^{15}N]urea recovery

A trace amount of [^{13}C]urea was included in the test meals to verify the accuracy of the [^{15}N]urea recovery results. Measurement of [^{15}N]urea production involves adding the amount of the tracer in urine collected over the 9 h post-meal observation period to that in TBW at the final time point, as described by Fern et al (1984) in their single-dose [^{15}N]glycine end-product model for whole-body N turnover. The calculation ignores any loss of urea (and urea tracer) in the gut or elsewhere (Fuller and Reeds, 1998; Long et al, 1978; Taveroff and Hoffer, 1993; Walser, 1980; Young et al, 2000). It also ignores the possibility that diet or disease could change renal urea clearance, which in turn could introduce systematic error by changing the distribution of urea between TBW and urine (Goldstein et al, 1969). Our finding that [^{13}C]urea recovery was unaffected by diet or IDDM allays concern that these may have had a distorting effect on [^{15}N]urea recovery. As was noted in the accompanying article (Hamadeh et al, 2001), the [^{13}C]urea recoveries were higher than anticipated, perhaps due to the imprecision in our mathematical correction for the contribution of [^{15}N]urea to the [M+1]urea mass. While it is possible that [^{15}N]urea synthesis partially distorted our [^{13}C]urea recovery measurement, the reverse could not occur since the IRMS analysis converts urea to ammonia, eliminating any contribution from ^{13}C or ^{18}O.

Conclusions

We conclude that [^{15}N]ALA in a test meal provides information about adaptive changes in dietary amino acid catabolism equivalent to provision of a ^{15}N-labeled intact protein tracer. Postprandial serum amino acid concentrations following normal adaptation to protein restriction are highly reproducible on repeat testing irrespective of the ^{15}N tracer source. Postprandial amino acid enrichments are reproducible for the same ^{15}N tracer source irrespective of prior adaptation. In conditions of abundant dietary protein, persons with IDDM on conventional insulin therapy exhibit first pass dietary protein retention similar to that found with nondiabetic controls.

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CHAPTER IX

Discussion and Summary

The objective of this thesis was to develop and study sensitive and robust methods that can detect abnormal efficiency to retain dietary amino acids normally masked by high protein intake, and that are convenient and simple enough to be used in the clinical setting. We, first, developed and studied clinical tools for measuring postprandial whole body amino acid catabolism in normal subjects prior to and following adaptation to protein restriction, and, second, we tested whether a test meal low in protein (0.25 g protein/kg body weight) can detect successful adaptation to protein restriction in normal subjects. We also measured postprandial response following a test meal low in protein in persons with IDDM on conventional insulin therapy previously adapted to high protein intake and compared the results with those for normal subjects.

SHORT-TERM VARIATIONS IN UREA PRODUCTION

We tested the accuracy of measuring steady-state urea rate of appearance (Ra) after a 4-h primed continuous tracer infusion as well as the accuracy of this method to determine changes in whole body urea appearance produced by intravenous urea infusions that simulated postprandial changes in urea production. We also measured the short-term effect on urea Ra of infusing alanine, a precursor for urea synthesis.

When only tracer urea was infused, there was no difference between the steady and nonsteady tracer calculations of urea Ra as observed over 12 h. The steady-state Ra at hour 4 was therefore accepted as a valid measure of endogenous urea Ra when no perturbations in urea metabolism occur, in agreement with an earlier conclusion by Jahoor and Wolfe (1987). The nonisotopic Ra was 28% less than the true Ra, also as

reported by other researchers (Jones et al, 1969; Long et al, 1978).

When urea was infused to simulate a single meal (pulse infusion) or nibbling for 8 hours (step infusion), i/TTR increased only slowly so that the Ra indicated by the steady-state calculation was only 61% and 54% of the true Ra after 8 h (step) and 4 h (pulse) of true urea input, respectively (both $P < 0.05$). This failure to observe an isotopic steady state even 8 h after urea input increased can be explained by urea's large pool size and slow fractional turnover rate, approximately 9%/h (Coleman et al, 1972; Walser and Bodenlos, 1959). However, Steele's non-steady-state equation and the nontracer method also underestimated the true urea rate of appearance, although to a lesser degree (Chapter 3, Table 2).

Cumulative urea production, measured nonisotopically as urinary excretion plus increase in body urea, was 73% ($P < 0.05$) of the true urea input over the 8-h step urea infusion and 83% ($P < 0.05$) of true urea input over the 4-h pulse urea infusion. However, this calculation ignores non-urinary urea losses. Correcting cumulative urea productions for their corresponding tracer urea recoveries improved these estimates to 85% ($P < 0.05$) and 93% (NS) of true urea input for the step and pulse infusions, respectively (Chapter 3, Table 3).

Urea production following alanine administration is also underestimated when both isotopic and nonisotopic approaches are used. In an earlier study in which alanine was infused at the same rate as in this study, urea Ra accounted for an even smaller fraction of the total alanine N infused than we found (Wolfe et al, 1987). We therefore conclude that true urea Ra is significantly underestimated even by the non-steady-state

tracer method during acute administration of a urea precursor.

The present conclusions are important for interpreting the results of studies in which urea Ra was measured to determine the effect of an acute intervention, such as exercise, feeding, or hormone administration (Beaumier et al, 1995; Carraro et al, 1993; El-Khoury et al, 1996; El-Khoury et al, 1994; Ensinger et al, 1994; Wolfe et al, 1982; Wolfe et al, 1987). The failure to observe an increase in urea Ra during acute exercise, despite increased amino acid oxidation, is consistent with insensitivity of the model to detect acute increases in urea Ra (Carraro et al, 1993; Wolfe et al, 1982). In feeding studies (El-Khoury et al, 1996; El-Khoury et al, 1994), the increase in urea Ra reported to occur in the fed state using continuous [$^{15}N_2$]urea infusion might have underestimated a true fed-state increase in urea Ra, resulting in an underestimate of urea recycling.

We conclude that the primed continuous tracer urea infusion method can provide a valid measure of urea Ra in a 4-h study, but only under basal conditions. The method is insensitive for detecting changes in urea Ra, even 8 h after urea Ra increases, when either non-steady-state kinetics or nonisotopically measured urea excretion is used to measure it. The accuracy of the nonisotopic measurement is improved if corrections are made to account for non-urinary urea losses by measuring recovery in urine and plasma of the dose of tracer urea introduced into the body over the period of analysis.

USING ELECTROSPRAY TANDEM MASS SPECTROMETRY TO MEASURE SULFATE CONCENTRATIONS AND STABLE ISOTOPE $^{34}SO_4/^{32}SO_4$ TTR

We demonstrated the use of ESI-MS/MS to measure serum and urine sulfate

concentrations and isotopic enrichments. For the tracer enrichment measurement, a sample aliquot without internal standard is analyzed. To measure sulfate concentration, $Na_2{}^{34}SO_4$ internal standard is added to a second aliquot of the same sample which is then analyzed in the manner but with correction for the presence of the small amount of ^{34}S contributed by the tracer.

The ESI-MS/MS method to measure sulfate concentration is far more sensitive than the IEC-CD method which cannot be used for tracer studies. An important advantage is that it may do away with the need to administer sulfate labeled with the radioactive isotope ^{35}S, currently a standard technique for measuring extracellular fluid volumes in humans and animals (Pierson et al, 1982). Since the ^{34}S isotope of sulfur is stable, it eliminates the requirement for difficult and expensive radiosyntheses of the short half-life ^{35}S isotope-containing substrates shortly before use in metabolic studies.

USING STABLE ISOTOPE $^{34}SO_4$ TO MEASURE ECF

We demonstrated the use of an orally administered stable isotope of sulfate to determine ECF in eight healthy men. SS is optimally measured using serum $^{34}SO_4$ values between h 3 and 5. We compared this value of SS with the simultaneously measured CBS.

Serum concentrations that included h 2 were variably influenced by the tracer absorption phase, whereas those between h 3 and 5 consistently represented the elimination phase of serum $^{34}SO_4$ (Chapter 5, Figures 2 and 3). SS calculated using h 3-5 was associated with a better data fit for individual studies and a smaller between-subject

variance than SS calculated using h 2-4. We conclude that using data points before h 3 following oral tracer administration may result in greater variability and the overestimation of SS.

The SS was 79.7 ± 5.7 % of CBS, confirming results obtained by Lacroix et al (1965) in men and women (85 ± 10 %), Yu et al (1996) in men (83.9 %), and Barratt and Walser (1969) in rats (80 ± 0.4 %) when SS and CBS were compared directly. The reason for this constant difference between the SS and the CBS is unknown.

The oral administration of $^{34}SO_4$ provides a practical alternative for measuring SS, with the advantage of avoiding oral administration of a radioactive tracer. Serum samples are best obtained at h 3, 4, and 5 following tracer ingestion. Our results confirm that SS is 20% smaller than CBS in adults of normal body composition even after standard correction for Br penetration into erythrocytes.

SULFATE PRODUCTION AS A MEASURE OF SHORT-TERM SAA CATABOLISM

Studies were carried out to evaluate the use of sulfate production as an indicator of whole-body SAA catabolism so as to be able to examine nutritional factors regulating it in short-term, clinically practical study protocols. We determined sulfate Ra under basal steady state conditions using a primed continuous oral administration of sodium [^{34}S]sulfate and measured nontracer sulfate production after administering known amounts of intravenous sulfate, oral methionine, and an intravenous mixture of essential amino acids including methionine.

To test whether the tracer method accurately depicts sulfate production, we

compared the serum sulfate Ra indicated from a primed continuous oral administration of sodium [^{34}S]sulfate with sulfate production as measured by its urinary excretion. In a tracer approach modeled after the urea tracer method, sulfate Ra was significantly higher (20%) than the sulfate production rate indicated by the nontracer method. We found that organic sulfate became enriched in ^{34}S within a very short period of time, indicating tracer $^{34}SO_4$ incorporation into organic compounds (Chapter 6, Figure 2). This resulted in the overestimation of sulfate production by the tracer steady state methodology which we attribute to incomplete equilibration of the tracer with an intracellular sulfate pool because of the appearance of organic sulfate in the urine.

To determine whether the nontracer method predicts short-term acute changes in sulfate production, a known amount of sulfate corresponding to the amount of amino acid sulfur in a normal meal was administered intravenously following a 3-h basal period (Chapter 6, Figure 3). Cumulative sulfate recovery was 95% over 6 h and 98% over 9 h following magnesium sulfate infusion (Chapter 6, Table 2; both not significantly different from 100%). This is in agreement with the 90-100% recovery in urine obtained by Chakmakjian and Bethune (1966) 24 h following intravenous administration of an extremely large dose (362 mmol) of sodium sulfate.

We determined that sulfate production over a 3-h basal period accurately predicted sulfate production over the subsequent 9 h in 5 healthy men previously adapted to normal protein intake. Furthermore, hourly sulfate production was strongly correlated with hourly urinary sulfate excretion ($r = 0.96$, slope = 0.95, $P < 0.0001$), as expected when serum sulfate remains constant.

Theoretically, the sulfate space (SS) would most accurately depict sulfate distribution. This space is ~20% smaller than the CBS (Hamadeh et al, 1999). However, sulfate recovery following magnesium sulfate infusion was most accurate when the CBS was used to estimate the ECF volume. We conclude that the CBS (or even body weight x 0.2; Hamadeh et al, 1999) provides a suitable and convenient measure of ECF for determining changes in body sulfate content.

When subjects were adapted to a low (control study) or high (standard diet control study) protein diet the day before they were studied, their serum sulfate concentrations were relatively constant over a 9-12 h observation period, so there was no need to account for changes in ECF sulfate (Chapter 6, Figures 1 and 4). This suggests that simple urinary sulfate excretion could be used in many settings as a convenient measure of basal sulfate production. Furthermore, results obtained at h 6 corresponded well with those obtained at h 9. Thus, acceptably accurate results can be obtained following acute short-term changes in sulfate production in study protocols that last only 6 h, an important practical advantage when carrying out metabolic studies in the clinical setting.

Since oral methionine did not stimulate insulin secretion, and hence protein synthesis, it is likely that the sulfur retained in the body after oral methionine was stored in nonprotein sulfur-containing compounds, such as glutathione, an important cysteine reservoir (Cho et al, 1981; Fukagawa et al, 1996; Tateishi et al, 1981). Similarly, the majority of the sulfur retained in the body following the amino acid infusion must have been retained in a nonprotein form, as after oral methionine alone.

The conclusion that a significant percent of the S in an oral methionine dose or an intravenous mixture of methionine and essential amino acids is retained in nonprotein bound form is supported by comparing urea-N and S balances. These results suggest a selective retention of sulfur compared to nitrogen in the fed state. This temporal offset between sulfate and urea production has previously been described after whole meal ingestion by Cheema-Dhadli and Halperin (1993).

We conclude that a ^{34}S-sulfate tracer approach to measuring whole-body sulfate production is inaccurate, likely owing to incomplete exchange of the tracer with intracellular organic compounds. However, the simple nontracer approach of measuring urinary sulfate excretion, corrected for changes in ECF sulfate, appears to accurately and conveniently depict sulfate production over observation periods as short as 6 h. Endogenous sulfate production measured over a 3-h basal period can be used to measure endogenous production over subsequent several hours when subjects are studied in the normal postabsorptive state. Our use of this method suggests that following ingestion of methionine, either alone or together with other essential amino acids, a significant fraction is stored within a nonprotein bound form, which could represent glutathione.

SULFATE PRODUCTION DEPICTS FED-STATE ADAPTATION TO PROTEIN RESTRICTION

We tested the reproducibility of postprandial urea and sulfate production upon repeat testing. We also investigated the use of sulfate production as an alternative to urea to measure whole-body amino acid oxidation over 6 and 9 h following the nonsteady

state consumption of a protein-restricted test meal in persons previously adapted to high and low protein diets. We also measured N and S metabolic balance, NPU and SAA retention. We found postprandial urea and sulfate production were reproducibly decreased following adaptation to protein restriction. Changes in sulfate production paralleled those of urea-N and changes were evident within 6 h following test meal consumption. We showed that the low protein (0.25 g/kg) test meal is effective for detecting these changes.

We also tested whether the abnormal protein turnover present in conventionally treated IDDM affects body protein economy. Urea-N and S metabolism in 6 subjects with IDDM on conventional insulin therapy and adapted to a conventional high protein diet was similar to that of control subjects.

Over 3 days of protein restriction, 24-h urinary urea and sulfate excretion reproducibly decreased by 74% and 70%, respectively (Chapter 7, Figure 1). This indicates successful adaptation to protein restriction by decreasing whole-body amino acid oxidation. A significant decrease (80-87%) in 24-h sulfate excretion following consumption of SAA-free diets has been observed previously (Lakshmanan et al, 1976).

It is noteworthy that 24-h sulfate excretion on days 3 and 4 were closely similar (Chapter 7, Figure 1), in agreement with previous data showing a leveling off in sulfate excretion after 48 h on a SAA-deficient diet (Lakshmanan et al, 1976). This indicates fast adaptation in sulfate excretion to dietary SAA intake. Though 3-4 days of low-protein intake may not be enough for complete adaptation, the adaptation that occurs is large and reproducible.

Twenty-four hour sulfate excretion closely correlated with 24-h urea-N excretion ($r = 0.95$, $P < 0.0001$; Chapter 7, Figure 2), in agreement with previous observations showing close correlations between urinary sulfate excretion and urinary urea-N (Jourdan et al, 1980) and total N excretion (Jourdan et al, 1980; Lakshmanan et al, 1976).

Baseline and postprandial urea and sulfate concentrations were reproducible upon repeat testing ([^{15}N]ALA versus [^{15}N]SPI) (Chapter 7, Figures 3 and 4).

Serum sulfate concentrations did not change significantly over the 9 h following the test meal (Chapter 7, Figure 4). This obviated the need to correct sulfate production for any changes in ECF, so that sulfate production was equivalent to its urinary excretion. This ascertains the use of urinary sulfate excretion as a measure of sulfate production postprandially, an advantage when carrying out metabolic studies in the clinical setting.

Cumulative urea-N and S production 9 h following the test meal reproducibly decreased with the LP diet (Chapter 7, Table 3). These changes were similar in magnitude to the decrease in cumulative N and S production 6 h following the test meal. The observed increase in conservation of dietary protein was closely reproducible upon repeat testing.

Results obtained over collection periods of 6 h corresponded well with those obtained over 9 h. Therefore, the use of collection periods as short as 6 h can provide accurate information on changes in whole-body protein conservation under normal fed-state conditions and following adaptation to protein restriction.

Urea-N production was reduced by a similar magnitude (29%) as that following a test meal high in protein (31%; 0.50 g/kg of protein) (Hoffer et al, 1997). Metabolic N

balance and NPU significantly improved from negative to positive values with the LP diet. In the present study, the 0.25 g protein/kg test meal was as sensitive and may prove more useful for detecting mild catabolic states since it requires more efficient immediate conservation of dietary amino acids.

Contrary to our expectations, the persons with IDDM previously adapted to a high protein intake demonstrated a normal response in postprandial sulfur and urea-N metabolism. We had anticipated that a low protein meal would be able to detect differences in the efficiency to retain dietary amino acids even when the prior habitual diet was high in protein. Several reasons could explain the outcome. First, because subjects were adapted to high protein intake, amino acid catabolism was unrestricted since the mechanisms governing adaptation were at zero efficiency and would require more than one meal to adapt to protein restriction. Second, though the subjects with IDDM were hyperglycemic, they were not extremely so, and hyperglycemia is not a very accurate indicator of insulin deficiency in diabetes. A poorer glycemic control could provide the extreme conditions necessary for a meal low in protein to detect inefficiency in metabolic protein economy (see page 288 for further explanation). Future studies are needed to further elucidate the mechanisms governing successful adaptation in subjects with IDDM.

We conclude that normal adaptation to protein restriction is associated with a reduction in postprandial urea and sulfate production that is highly reproducible on repeat testing. A protein level of 0.25 g/kg in the test meal detects adaptation and could prove to be more sensitive for detecting metabolic dysregulation than a test meal with a higher

protein level. Sulfate production effectively depicts fed-state adaptation to protein restriction. The sulfate production measurement is less prone to error than urea since sulfate distributes in a smaller volume than urea, and under different dietary intakes serum sulfate levels do not change significantly 9 h following the test meal, obviating the need, in some cases, for any correction to account for change in its ECF pool size. Postprandial adaptive changes in urea and sulfate production can be accurately depicted over observation periods as short as 6 h. Whole-body protein economy on standard high protein intake is not different between healthy controls and persons with IDDM on conventional insulin therapy.

^{15}N TRANSFER FROM DIETARY [^{15}N]ALANINE AND [^{15}N]*SPIRULINA PLATENSIS* INTO UREA FOLLOWING PROTEIN RESTRICTION

We previously observed an adaptive increase in first pass dietary protein conservation in normal subjects adapted to a low protein intake as depicted by a marked reduction in the transfer of ^{15}N added to a mixed meal as [^{15}N]ALA into plasma and urinary urea. To determine the validity of using the fate of [^{15}N]ALA as a marker of dietary amino acid N, we tested whether the reduction in the transfer of ^{15}N added to a meal as [^{15}N]ALA into urea was comparable to that for [^{15}N]SPI and observed the distribution of ^{15}N labels in serum amino acids and the time course of serum amino acid concentration over 9 h following a mixed test meal for the two ^{15}N tracer sources ([^{15}N]ALA versus [^{15}N]SPI). As was anticipated, far less of the ^{15}N in the whole protein source made it into urea over the time course of the study. The important finding was

that the reduction in the transfer of ^{15}N added to the test meal as [^{15}N]ALA following protein restriction was comparable to that for [^{15}N]SPI. This occurred despite quite different patterns of postprandial serum amino acid enrichments with the two tracers.

We also tested whether subjects with IDDM on conventional insulin therapy exhibit first pass dietary protein conservation similar to that of controls when tested with a low-protein meal and [^{15}N]ALA. ^{15}N recoveries in serum and urinary urea and serum ^{15}N amino acids over the 9 h following consumption of the test meal were similar for the IDDM and control groups. This agrees with our previous conclusion, based on a study using a high-protein test meal, that conventionally treated IDDM is associated with normal postprandial amino acid handling as long as the prior diet is high in protein.

The difference in the time profile distribution of the ^{15}N amino acid enrichments between the [^{15}N]ALA and [^{15}N]SPI treatments is due to the different ^{15}N tracer sources, and was not evident between the HP and LP diets for the same ^{15}N tracer source. The rapid appearance of ^{15}N in serum glutamine, leucine and isoleucine within 2-4 h following consumption of the test meal with [^{15}N]ALA indicates rapid interchange of the amino group between these amino acids. However, the typical increase in serum ^{15}N amino acid enrichments following consumption of the test meal with [^{15}N]SPI is mainly due to tracer dietary amino acids. The splanchnic processes underlying the different patterns of amino acid enrichments are, nevertheless, reproducible upon repeat testing within the same ^{15}N tracer source. Thus, the similar decrease in ^{15}N recovery observed following protein restriction for both [^{15}N]ALA and [^{15}N]SPI indicates true reduction in first pass amino acid oxidation.

In a previous study, ^{15}N recovery decreased by 43% following consumption of a high protein test meal (0.50 g protein/kg) in normal subjects adapted to protein restriction (0.05 g protein/kg.d) (Hoffer et al, 1997). In the present study, the decrease in ^{15}N recovery following protein restriction was similar in magnitude (38%; NS). The low protein (0.25 g protein/kg) test meal was equally effective in showing a reduction in the transfer of dietary ^{15}N from the meal into urea but may be more useful in depicting subtle increases in the efficiency to conserve dietary amino acids.

Amino acid concentrations followed similar patterns and amino acid enrichments were similar for the IDDM and control groups (Chapter 8, Figure 9). Postprandial ^{15}N recovery in serum and urinary urea and the time profile of serum amino acid enrichments were similar for both groups (Chapter 8, Table 1), in agreement with our previous observation (Hoffer et al, 1997).

Serum leucine and isoleucine concentrations were lower and serum alanine, methionine, aspartate and urea concentrations similar for the subjects with IDDM compared to the control group (Chapter 8, Figures 3, 4, 5 and 9). This is important to recognize, because the mild catabolic state that occurs in untreated or poorly treated IDDM is associated with an increased serum concentration of the branched-chain amino acids, leucine, valine and isoleucine (Carlson and Campbell, 1993; Nair et al, 1987; Trevisan et al, 1989; Vannini et al, 1982). The subjects in the present study, despite their hyperglycemia, may have had sufficient amounts of circulating insulin to suppress the catabolic effects of insulin deficiency on protein metabolism. It will be important in future field studies exploring the protein metabolic state of persons with IDDM to

include both glucose control and a convenient marker of protein catabolism, such as leucine, in the analysis.

We conclude that [^{15}N]ALA in a test meal provides information about adaptive changes in dietary amino acid catabolism equivalent to provision of a ^{15}N-labeled intact protein tracer. Postprandial serum amino acid concentrations following normal adaptation to protein restriction are highly reproducible on repeat testing irrespective of the ^{15}N tracer source. Postprandial amino acid enrichments are reproducible for the same ^{15}N tracer source irrespective of prior adaptation. In conditions of abundant dietary protein, persons with IDDM on conventional insulin therapy exhibit first pass dietary protein retention similar to that found with nondiabetic controls.

SUMMARY AND IMPLICATIONS

We determined that the primed continuous tracer urea infusion method can provide a valid measure of urea Ra only under basal conditions. However, changes in urea production can not be accurately detected when either non-steady-state kinetics or nonisotopically measured urea production is used to measure it.

The ESI-MS/MS method can be used to measure both sulfate concentrations and isotopic enrichments in serum and urine. The ^{34}S isotope of sulfur is stable and can be used in metabolic studies to measure ECF in humans, eliminating the requirement to use the radioactive isotope ^{35}S.

SS is best estimated by measuring serum $^{34}SO_4$ enrichment at h 3, 4, and 5 following tracer ingestion. SS is 80% that of CBS in adults of normal body composition

even after standard correction for Br penetration into erythrocytes. We also determined that the CBS, or even total body weight x 0.2, provides a suitable, non-invasive and convenient measure of ECF in subjects with normal body weights for the purpose of measuring sulfate production.

Sulfate production, as measured by its urinary sulfate excretion corrected for changes in ECF sulfate, is a valid measure of short-term SAA oxidation. Sulfate Ra, as measured from a primed continuous oral administration of sodium [^{34}S]sulfate, overestimates sulfate production by 20% owing to incomplete equilibration of the tracer with an intracellular sulfate pool.

Normal adaptation to protein restriction is associated with a reduction in postprandial urea and sulfate production that is highly reproducible on repeat testing. A protein level of 0.25 g/kg in the test meal detects adaptation and could prove to be more sensitive for detecting mild catabolic states than a test meal with a higher protein level.

Sulfate production effectively depicts fed-state adaptation to protein restriction. Postprandial adaptive changes in urea and sulfate production can be accurately depicted over observation periods as short as 6 h.

[^{15}N]ALA in a test meal provides information about adaptive changes in dietary amino acid catabolism equivalent to provision of a ^{15}N-labeled intact protein tracer.

Subjects with IDDM on conventional insulin therapy previously adapted to a high protein intake demonstrate whole-body protein economy comparable to that for healthy controls. Though a test meal low in protein did not disclose abnormalities in whole body postprandial protein economy in IDDM under the present conditions, it is possible that

the same test meal could unmask impairment in protein conservation in subjects with IDDM in poor glycemic control, hence under conditions of insulin-deficiency. In addition, we still need to determine the response of subjects with IDDM previously adapted to protein restriction to a low protein test meal. If the response is abnormal, it would add to the already existing evidence that protein requirement is above normal in IDDM. Under such conditions, caution should be exercised when recommending protein restriction in IDDM, and that insulin therapy should be optimal lest protein deficiency ensues. When monitoring intensive insulin therapy, it would be advisable to monitor both glucose control and serum BCAA concentrations as appropriate markers.

When carrying out metabolic studies in the clinical setting, accurate, noninvasive and convenient measures provide an important practical advantage. Nontracer sulfate production could be used in such settings: simple urinary sulfate excretion could be used as a convenient measure of basal sulfate production; acceptably accurate results can be obtained following acute short-term changes in sulfate production in study protocols that last only 6 h; the CBS, or even body weight x 0.2, provides a suitable and convenient measure of ECF for determining changes in body sulfate content; urinary sulfate excretion is an accurate measure of sulfate production following consumption of a mixed test meal, because serum sulfate concentrations remain virtually constant obviating the need, in some cases, to correct for changes in ECF sulfate; sulfate excretion rapidly adapts to dietary SAA intake; and sulfate production effectively depicts fed-state adaptation to protein restriction.

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