Effect of protein restriction on sulfur amino acid catabolism in insulin-dependent diabetes mellitus

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Submitted 8 July 2002; accepted in final form 3 October 2002

Hamadeh, Mazen J., and L. John Hoffer. Effect of protein restriction on sulfur amino acid catabolism in insulin-dependent diabetes mellitus. Am J Physiol Endocrinol Metab 284: E382–E389, 2003. First published October 22, 2002; 10.1152/ajpendo.00295.2002.—Persons with conventionally treated insulin-dependent diabetes mellitus (IDDM) appear to be impaired in their ability to reduce fed-state urea production appropriately in response to dietary protein restriction (Hoffer LJ, Taveroff A, and Schiffrin A. Am J Physiol 272: E59-E67, 1997). To determine whether these conclusions apply to whole body sulfur amino acid (SAA) catabolism, we used samples from this protocol to measure daily urinary sulfate excretion and fed-state sulfate production after a high-protein test meal before and after dietary protein restriction. Eight normal subjects and six IDDM subjects treated with twice-daily intermediate- and short-acting insulin consumed a mixed test meal containing 0.50 g protein/kg after adaptation to 4 days of high protein intake (1.28 g protein/kg body wt) and again after 5 days of dietary protein restriction (0.044 g/kg). Adaptation to protein restriction decreased daily urinary sulfate and urea-N excretion by ~80%. Over the first 24 h of protein restriction, urinary sulfate excretion decreased more than urea-N excretion for both the normal and IDDM subjects. Under conditions of a high prior protein intake, fed-state sulfate production was normal for the IDDM subjects; protein restriction reduced fed-state sulfate production by 51% (normal subjects) and 59% (IDDM subjects; not significant). We conclude that whole body SAA metabolism is normal in conventionally treated IDDM before and after dietary protein restriction. SAA catabolism, as measured by fed-state sulfate production, may be a convenient and useful method to determine the extent of whole body protein dysregulation in IDDM.

THE PROTEIN WASTING associated with diabetes mellitus was known to ancient physicians (4), but it is only in recent years that the metabolic forces driving this phenomenon were identified. When insulin is withheld from persons with insulin-dependent diabetes mellitus (IDDM), their plasma branched-chain amino acid concentrations rise within several hours, and endogenous amino acid oxidation and urea production increase markedly (39, 40, 51). Proteolysis increases, whereas protein synthesis is unchanged in skeletal muscle. In the splanchnic tissues, both proteolysis and protein synthesis increase, the latter stimulated in part by the influx of amino acids from muscle (9, 38).

With the advent of the insulin era, protein wasting disappeared as a clinical problem in diabetes (4), and indeed, the protein economy of insulin-treated adults with IDDM appears to be normal (42, 45). Nevertheless, peripheral insulin injections do not fully normalize their insulin physiology, and concern about the increased plasma branched-chain amino acid concentrations of diabetic children was responsible in good part for the development of modern, intensive insulin therapies (47, 48). The conventional twice-daily insulin therapy (CIT) still used by most persons with IDDM is variably associated with mildly increased plasma branched-chain amino acid concentrations and metabolic rate (6, 8, 15, 19, 43, 47, 50); the physiological significance is uncertain.

We proposed that the lack of full physiological insulin replacement in persons with CIT-IDDM impairs their ability to adaptively limit amino acid catabolism when called upon to do so, but this is masked by the high level of protein consumption in conventional western diets (25, 26). Most people consume more than twice their protein requirement (18), and in such a situation there is no physiological need for efficient conservation of either endogenous amino acids or those entering the body from the diet. According to this hypothesis, persons with CIT-IDDM will be at nutritional risk only if their protein intake is reduced to near the requirement level.

The existing data support this hypothesis. Brodsky et al. (7) observed an important decrease in lean body mass and muscle strength in CIT-IDDM subjects when their protein intake was restricted to the average requirement for normal adults, 0.6 g/kg. We found that the rate of urinary N excretion on a protein-free diet (an indicator of the maximum capacity to conserve endogenous amino acids) was higher than normal in intensively treated IDDM (35). We subsequently reported that, although fed-state urea production was normal in CIT-IDDM subjects when adapted to a conventional high-protein (HP) diet, their fed-state urea production did not decrease appropriately when the...
test meal was consumed following a period of protein restriction (28).

We have now carried out measurements on serum and urine samples already obtained for that study (28) to determine the effect of dietary protein restriction on an independent indicator of amino acid catabolism, namely whole body sulfate production. Sulfate is the predominant end product of sulfur amino acid (SAA) catabolism; conversely, urinary inorganic sulfate excretion is almost entirely due to SAA catabolism (21). Urinary sulfate production decreases after dietary SAA (34) or total protein (23) restriction, and, when measured over 24-h periods, it closely mirrors SAA consumption (44) and N excretion (23, 33, 41). When measured over shorter time periods, urea production and sulfate production have been reported to diverge, with proportionately greater SAA retention postprandially and greater release in the postabsorptive state (11). This variation may be due to the replenishment of tissue stores of glutathione (GSH) during the fed state and their depletion during an overnight fast (12, 17). Such fluctuations in nonprotein SAA retention are detectable in humans, since sulfate production can be accurately determined over periods as short as 6 h as urinary sulfate excretion corrected for any change in extracellular fluid sulfate content (21).

We postulated that splanchnic amino acid turnover is increased by the mild insulin deficiency associated with CIT-IDDM (9, 38) and that this should yield abundant SAA for hepatic GSH synthesis, even in the postabsorptive state. Unlike in the fasting (30) or protein-deficient (29) rat, hepatic GSH stores are normal in the IDDM rat (36). The general catabolic state induced by endotoxin or tumor necrosis factor administration also increases hepatic GSH in the rat as long as SAA or protein is provided in its diet (31, 32). If the GSH stores of CIT-IDDM persons are more completely filled in the basal state, their sulfate production might be higher than normal following consumption of an HP test meal (3, 49). We also wondered whether CIT-IDDM persons reduce their daily sulfate excretion normally during protein restriction and whether, following a period of adaption to protein restriction, their ability to retain the SAA in a mixed test meal is normal.

**METHODS**

**Subjects and protocols.** The subjects were eight normal adults (4 men and 4 women: age 25 ± 2 yr, weight 64 ± 4 kg, body mass index 22 ± 1 kg/m²) and six adults with uncomplicated C-peptide-negative IDDM (4 men and 2 women: age 21 ± 1 yr, weight 72 ± 3 kg, body mass index 23 ± 0 kg/m²) receiving CIT. Data on the trial protocol, metabolic control of the IDDM subjects, and fed-state urea production have previously been described in detail (28).

The IDDM subjects had blood hemoglobin A₁c concentrations of 10.2 ± 1.2% (normal range 4.6–8.5%). Capillary blood glucose concentrations were measured seven times daily using glucose oxidase reagent test strips (Boehringer Mannheim Canada, Laval, QC, Canada). Fasting blood glucose was 9–13 mmol/l, typical of CIT-IDDM (13). Urine was collected in serial complete 24-h collections. All subjects consumed an HP diet for 4 days, after which the first test meal study was conducted on the morning of day 5. A 5-day period of protein restriction followed, and on the morning of day 11, the test meal procedure was repeated. All of the research participants gave their written consent to participate in the study, which was approved by the local Institutional Review Board (28); the additional sulfate and urea measurements reported here fall within the guidelines for approval of the original study.

**Adaptation diets.** Daily energy intake was 37 kcal/kg body wt (155 kJ/kg) on both the HP and low-protein (LP) diets, appropriate for their measured resting energy expenditure (28). The protein in the HP diet was whole milk protein Optifast-70 (provided as a gift by Sandoz Nutrition, Whitby, ON, Canada) supplemented with glucose polymer and protein-free diet powder (Product 80056; Mead-Johnson, Evanston, IL, provided as a gift) and provided 87 g protein/day, with fat, carbohydrate, and protein constituting 29, 58, and 13% of total energy intake. The LP diet was similar to the HP diet but without the milk protein and with the absent protein energy replaced by glucose polymer and corn oil. This diet provided 17 g protein/day with fat, carbohydrate, and protein constituting 37, 62, and 0.5% of total energy intake. The IDDM subjects consumed 300 kcal/day (1,250 kJ/day) additional energy to compensate for urinary glucose losses. On days 1–4, the diet provided a mean of 1.28 g protein/kg. On day 5, after completion of the test meal procedure, the subjects consumed two LP meals 3 h apart, for a total protein intake that day of 0.54 g/kg, while meeting the full energy requirement. On days 6–10, the diet provided a mean of 0.044 g protein/kg, and on day 11 (2nd test day), only the test meal was consumed.

**Test meal.** The test meal was prepared by combining 4.21 g/kg Ensure High Protein with 5.79 g/kg Glucerna (a gift from Ross Laboratories, Columbus, OH) to which beet sugar (Rogers Sugar, Winnipeg, MB, Canada) was added to provide per kilogram of body weight 0.50 g protein and 10 kcal energy (19% protein, 37% fat, and 44% carbohydrate). The amino acid S content was 0.30 mg/ml for Ensure High Protein and 0.31 mg/ml for Glucerna, based on data supplied by Abbott Laboratories (Saint Laurent, QC, Canada).

**Test meal procedure.** On the test days (days 5 and 11), urine was collected over the 6 and 9 h following test meal consumption. Blood samples were drawn in the postabsorptive state and hourly after the meal from an arterialized arm vein kept patent with 77 mmol/l NaCl. The subjects drank water every hour to maintain proper hydration. To achieve postprandial blood glucose levels typical of conventionally treated IDDM, subjects with IDDM administered an optimal dose of the intermediate-acting and one-third of the estimated ideal short-acting insulin dose 30 min before consuming the test meal. The same short-acting insulin regimen was followed for both test meals.

**Analytical methods.** Arterialized venous blood was collected in sterile collection tubes containing sodium heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) kept on ice. The blood was centrifuged at 1,400 g for 10 min at 4°C, and the plasma was stored at −30°C until analysis. Plasma and urinary urea were measured on a Beckman CX3 discrete analyzer. Plasma and urine were analyzed for inorganic sulfate by ion exchange chromatography with conductivity detection ( Dionex 2110; Dionex, Sunnyvale, CA), as previously reported (5).

**Calculations.** Sulfate production was calculated as urinary inorganic sulfate excretion corrected for changes in extracellular fluid (ECF) sulfate content (21). The postmeal urea-N/S production molar ratio was calculated as the moles of urea-N
excreted over 6 or 9 h corrected for changes in total body water (TBW) urea-N content divided by the moles of inorganic sulfate excreted corrected for changes in ECF content over the same time period. TBW was estimated from age, height, and weight (1). The ECF volume was estimated as body weight × 0.2 (21, 22). SAA retention (%) was calculated as

\[
\text{SAA retention} = \frac{(\text{total amino acid S intake} - \text{sulfate produced})}{\text{total amino acid S intake}} \times 100 (8)
\]

Statistical analyses. Three-way ANOVA was used to determine significant differences in plasma sulfate and urea concentrations, the factors being diet (high vs. low protein), group (normal vs. IDDM), and time (SigmaStat version 2.03; SPSS, Chicago, IL). Two-way repeated-measures ANOVA was used to determine significant differences in 24-h urea and sulfate excretion and 24-h urinary urea-N/S molar ratio, the factors being time and group. Two-way repeated-measures ANOVA was used to determine significant differences in postmeal sulfur metabolism and postmeal urea-N/S production ratio, the two factors being diet and group. Within the same group, plasma urea and sulfate concentrations were analyzed by two-way repeated-measures ANOVA, the two factors being diet and time. Within the same group and diet, 24-h urea and sulfate excretion, 24-h urinary urea-N/S molar ratio, and plasma urea and sulfate concentrations over time were analyzed by one-way repeated-measures ANOVA. When significance occurred, the Newman-Keuls test was used post hoc to determine the source of difference. Student’s unpaired t-test was used to determine significant differences between the normal and IDDM groups. Differences were considered significant at \( P \leq 0.05 \). Results are presented as means ±SE unless otherwise indicated.

RESULTS

The diet and study protocol were well tolerated by all of the subjects without significant weight loss. Daily urinary urea and sulfate excretion levels were constant during the HP phase of the study, with a urinary urea-N/S molar ratio of ~38 (Figs. 1 and 2). Urea and sulfate excretion decreased by >80% within 4 days of protein restriction (Fig. 1). In the first 24 h of protein restriction, urea-N excretion decreased by 21% for the normal subjects, whereas sulfate excretion decreased by 41%. This corresponds to a preferential sparing of 71 μmol/kg of SAA, or ~20% of daily SAA consumption on the HP diet. The urea-N/S excretion molar ratio increased and then returned to the level observed during the HP diet (Fig. 2). Similar results were obtained for the IDDM subjects.

Daily urinary urea-N and sulfate excretion levels were closely related in both the normal and the IDDM subjects (Fig. 3). The \( y \)-intercepts and slopes of the two regression lines were similar (\( P > 0.4 \)), the slopes indicating a urea-N/S molar ratio of 38–39. Individual regression lines for daily excretion urea-N/S molar ratio for both groups provide similar results (normal: 39 ± 1; IDDM: 38 ± 2; \( P = 0.7 \)).

Plasma sulfate and urea concentrations were measured on each of the two test days (Fig. 4). Postabsorptive plasma urea concentrations were ~70% lower after protein restriction than before it for both the normal and the IDDM subjects, whereas plasma sulfate concentrations were 9–16% lower. After the test meal, plasma urea concentrations increased only slightly when the previous diet was high in protein but more than doubled when the same test meal was consumed following protein restriction, with no differences between the normal and IDDM subjects. Plasma sulfate concentrations increased only very slightly after the test meal under both HP and LP conditions.

Fig. 1. Daily urinary sulfate and urea-N excretion of normal subjects (A, □) and subjects with conventionally treated insulin-dependent diabetes mellitus (IDDM; B, ■) before (days 1–4) and during (days 6–10) protein restriction. Mixed test meals containing 0.5 g protein/kg were consumed on days 5 and 11. Different letters indicate significant differences within the same group (\( P < 0.05 \)). There were no significant differences between the normal and IDDM subjects.
The effects of HP and LP diets on fed-state SAA metabolism are shown in Table 1. The conclusions are similar whether based on urinary collection periods of 6 or 9 h. Fed-state sulfate production on the HP diet was higher than normal in the IDDM group, but the increase was only borderline statistically significant ($P = 0.081$ over 9 h; $P = 0.058$ over 6 h), with no change in the urea-N/S production ratio. The fed-state urea-N/S production ratio was 49 (at 6 h) and 44 (at 9 h) for both normal and IDDM subjects; this is significantly higher than the 24-h urinary excretion ratio of 38 ($P < 0.001$).

When the test meal was consumed after 5 days of protein restriction, fed-state sulfate production was 50% lower than when the prior diet had been high in protein; this occurred similarly for the normal and IDDM subjects (Table 1). Consequently, whole body SAA balance, which was neutral (at 6 h) or negative (at 9 h) when the prior diet was HP, was now strongly positive. After protein restriction, the postmeal urea-N/S production ratio increased by ~60% (at 6 h) and 40% (at 9 h) for the normal subjects, indicating a net body gain of nonprotein SAA. The increase in the urea-N/S production ratio was even greater for the IDDM subjects: 100% (at 6 h) and 90% (at 9 h) ($P < 0.05$). This was not due to greater SAA retention by the protein-restricted IDDM subjects but to their failure to decrease fed-state urea N production normally (28).

**DISCUSSION**

In this study, we investigated the effect of mixed test meals high in protein on whole body sulfate production by normal and CIT-IDDM subjects, first when adapted to a habitual HP diet, and again after 5 days of adaptation to protein restriction with maintenance energy provision. Urea appearance and tracer kinetics related to this study have previously been published (28). They indicated that when normal subjects and otherwise healthy persons with IDDM consumed an HP test meal, their postmeal N balance was similar and close to zero, and transfer of the $^{15}$N in a tracer dose of [H]$^{15}$N alanine included in the test meal into urea was...
also similar. However, when the same test meal was consumed after a period of protein restriction, the adaptive reduction in postmeal urea production (and corresponding increase in postmeal N balance) and the reduction in transfer of $^{15}$N from $[^{15}$N]alanine into urea were significantly less for CIT-IDDM subjects. IDDM subjects receiving intensive insulin therapy had a fully normal adaptive response. We regarded this as evidence that CIT-IDDM may be incompatible with a fully successful metabolic adaptation to dietary protein restriction (28).

The sulfate and plasma and urinary urea measurements reported in this paper are new. They show that the first 1–2 days of protein restriction were associated with a greater reduction in sulfate than urea excretion, followed by a return of the urinary urea-N/S ratio to values during the initial HP diet. Fed-state sulfate production was normal in the CIT-IDDM subjects following consumption of an HP test meal; their daily sulfate production decreased normally over a 5-day period of protein restriction, and, unlike with fed-state urea production, their fed-state sulfate production decreased normally following protein restriction.

**Effect of protein restriction on daily urinary urea and sulfate excretion.** The patterns of daily urea and sulfate excretion preceding and following protein restriction are illustrated in Figs. 1 and 2. There was a prompt reduction in both urea and sulfate excretion after diet change. Table 1 shows that this was associated with increased urinary sulfate excretion and a reduced sulfur amino acid retention.

**Table 1. Postmeal sulfate metabolism in normal subjects and subjects with IDDM**

<table>
<thead>
<tr>
<th>Time of Measurement</th>
<th>9 h Postmeal</th>
<th>6 h Postmeal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary protein</td>
<td>HP</td>
<td>LP</td>
</tr>
<tr>
<td>S metabolism, μmol/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake</td>
<td>95.2 ± 0.0</td>
<td>95.8 ± 0.3</td>
</tr>
<tr>
<td>Production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>110.4 ± 5.2</td>
<td>54.2 ± 3.4*</td>
</tr>
<tr>
<td>IDDM</td>
<td>126.2 ± 7.0†</td>
<td>52.1 ± 4.4*</td>
</tr>
<tr>
<td>Balance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>−15.3 ± 5.2</td>
<td>41.4 ± 3.3*</td>
</tr>
<tr>
<td>IDDM</td>
<td>−31.0 ± 7.0‡</td>
<td>43.9 ± 4.4*</td>
</tr>
<tr>
<td>SAA retention, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>−16.0 ± 5.4</td>
<td>43.3 ± 3.5*</td>
</tr>
<tr>
<td>IDDM</td>
<td>−32.6 ± 7.3‡</td>
<td>45.8 ± 4.6*</td>
</tr>
<tr>
<td>Post-meal production N/S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>45.6 ± 1.0</td>
<td>65.0 ± 3.9*</td>
</tr>
<tr>
<td>IDDM</td>
<td>43.3 ± 0.5</td>
<td>82.1 ± 8.2†</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. HP, high-protein diet before study day; LP, low-protein diet before study day; IDDM, insulin-dependent diabetes mellitus; SAA, sulfur amino acids; N/S, urea-N/S molar ratio.*Significantly different from HP, two-way repeated-measures ANOVA, $P < 0.05$; †significantly different from Normal, two-way repeated-measures ANOVA, $P < 0.05$. ‡$P = 0.058$ for difference between normal and IDDM on the HP diet. §$P = 0.058$ for difference between normal and IDDM on the HP diet.
etary protein was restricted, and a close relation between them (Fig. 3). Dietary N and S intakes were similar for the normal and IDDM subjects, and their patterns of urea-N and sulfate excretion were the same, indicating that, over 5 days of protein restriction, CIT-IDDM subjects reduce urea and sulfate excretion normally. This does not prove that people with IDDM adapt entirely normally to protein restriction, since full adaptation to a marked reduction in protein intake requires at least 7 days (16). We previously showed (35) that urinary obligatory urinary N excretion of intensively treated IDDM subjects is higher than normal when assessed using the formal 10-day adaptation protocol. We may nevertheless conclude that no substantial defect in endogenous amino acid recycling was apparent for the CIT-IDDM subjects in this study.

The increase in the urea-N/S excretion ratio on the first or second day of protein restriction indicates preferential SAA sparing on those days. We speculate that some of the SAA released by the dissolution of labile protein stores at the onset of protein restriction (27) were recaptured temporarily for GSH synthesis.

Effect of test meal on sulfate production following adaptation to a conventional protein intake. As measured over either 6 or 9 h after the HP (0.5 g protein/kg body wt) test meal, sulfate production by the IDDM subjects was higher than normal, but the increase was small and only borderline statistically significant (Table 1). We recently measured sulfate production of CIT-IDDM subjects after they had consumed a test meal containing much less protein (0.25 g/kg), and sulfate production was normal under those conditions as well (23). Taking these results together, we conclude that CIT-IDDM is associated with normal or near-normal fed-state sulfate production. This fails to support our hypothesis that CIT-IDDM persons have increased visceral protein turnover or that such an increase, if it exists, is associated with more completely filled basa

Effect of test meal on sulfate production following adaptation to protein restriction. After partial adaptation to the LP diet, fed-state sulfate production by the IDDM subjects was normal. Unlike when the prior diet was high in protein, SAA in the test meal were now preferentially retained over other amino acids, as shown by the marked increase in the urea-N/S production ratio (Table 1). This presumably indicates replenishment of body GSH stores depleted by the LP diet. The greater urea-N/S production ratio for the IDDM subjects \( P < 0.05 \) was not due to greater SAA retention but rather to a failure to reduce their fed-state urea N production normally \( 28 \).

Physiological implications. These results indicate that CIT-IDDM subjects catabolize SAA normally in the 6 or 9 h after consuming a meal providing 0.5 g protein/kg, equivalent to approximately one-third of the habitual daily protein intake of North American adults (18), and they replenish their nonprotein SAA store normally when the test meal is consumed after a 5-day period of protein restriction. This occurs despite an impaired ability to reduce total amino acid catabolism as effectively as nondiabetic persons after protein restriction \( 28 \). We infer that postabsorptive tissue GSH stores are probably not importantly increased in CIT-IDDM and perhaps, therefore, that such persons are not in a hypercatabolic state despite postabsorptive \( \sim 10 \text{ mmol/l} \) and fed-state \( \sim 15 \text{ mmol/l} \) hyperglycemia consistent with the definition of CIT used in the Diabetes Control and Complications Trial \( 13 \).

This conclusion should be regarded as tentative, however, because the IDDM subjects in this study are not typical of all CIT-IDDM. In contrast to many \( 8, 15, 19, 43, 47, 50 \) but not all \( 6 \) studies of CIT, the plasma leucine levels and resting energy expenditure of our IDDM subjects were normal \( 28 \). This suggests that our treatment regimen resulted in peripheral insulinization that was adequate for purposes of body protein economy even in the presence of hyperglycemia. A similar situation exists in non-insulin-dependent diabetes, in which whole body protein kinetics are normal despite significant hyperglycemia \( 14, 46 \). It would be useful in future research to determine the effect of a protein meal (or even more simply, an SAA load) on sulfate production in IDDM subjects more insulin deficient than the ones in this study. The simple nonisotopic determination of SAA catabolism could be used to determine the level of insulin deficiency (as indicated by plasma branched-chain amino acid concentrations) at which whole body proteolysis and amino acid catabolism increase, as indicated by increased sulfate production.

Sulfate production has practical advantages over urea production for this purpose. Whole body sulfate production can be accurately calculated from its urinary excretion in a study as short as 6 h, but urea production cannot \( 20, 21, 23 \). Unlike urea, sulfate is not degraded significantly in the intestine. Moreover, its effective volume of distribution, the ECF space, is relatively small \( 22 \) and, as illustrated by the present results (Fig. 4), the pool size varies little after changes in diet or fed state \( 37 \). In contrast, plasma urea concentrations vary a good deal over time, and because urea distributes throughout TBW, considerable error can arise from calculations of the change in body urea content associated with changes in plasma urea concentration \( 20 \).

Perhaps the greatest problem with the approach described in this study is the lack of proof that changes in body nonprotein SAA stores truly correspond to changes in whole GSH content. Plasma GSH concentrations are measurably reduced in some diseases associated with whole body GSH depletion \( 2, 10 \), but no direct comparisons of plasma GSH concentrations or kinetics with whole body or tissue-specific GSH content are available. A confident application of the method described in this study will require its validation in a suitable animal model in which the noninvasive approach can be compared directly with plasma and tissue GSH measurements.

AJP-Endocrinol Metab • VOL 284 • FEBRUARY 2003 • www.ajpendo.org
We thank Mead-Johnson, Ross Laboratories, and Sandoz Nutrition for providing the products used in the test meals. This study was supported by a grant from the Canadian Institutes of Health Research.

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