Nitrogen balance of healthy Dutch women before and during pregnancy[1–3]

Mina Mojtahedi, Lisette CPGM de Groot, Harry A Boekholt, and Joop MA van Raaij

ABSTRACT

Background: Experimental studies including longitudinal nitrogen balance studies could provide insight into protein metabolism in pregnancy.

Objective: Our aim was to determine the development of nitrogen balance during pregnancy compared with nitrogen balance before pregnancy in women consuming imposed constant diets. We also tracked changes in muscle mass and lean body mass by measuring urinary 3-methylhistidine (3-MeH) and urinary creatinine.

Design: Nitrogen balance was determined over 8 d in 12 healthy Dutch women before pregnancy and at weeks 12, 23, and 34 of gestation. Complete daily diets were supplied during each balance period so that each subject's energy, protein, and macronutrient intakes were similar in amount and composition in all 4 balance periods.

Results: Throughout pregnancy there was no significant change in loss of nitrogen in feces and therefore no change in protein digestibility. The amount of nitrogen excreted in urine in late pregnancy (11.0 ± 1.4 g/d) was significantly ($P < 0.01$) less than in early pregnancy (12.6 ± 1.3 g/d). Nitrogen retention increased toward term, even though energy balance became progressively negative. The difference between the first (-0.4 ± 1.7 g N/d) and third (1.2 ± 1.6 g N/d) trimester was significant ($P < 0.05$). No differences were found in either 3-MeH or creatinine excretion between trimesters.

Conclusions: These urinary nitrogen excretion and nitrogen retention data show that when the dietary supply remains constant, nitrogen balance increases toward the end of pregnancy, suggesting a more efficient use of dietary protein later in pregnancy. Urinary 3-MeH and creatinine excretion indicated no change in protein metabolism. *Am J Clin Nutr* 2002;75:1078–83.

KEY WORDS Nitrogen balance, protein, pregnancy, women, metabolism, creatine, creatinine, 3-methylhistidine, Netherlands

INTRODUCTION

The FAO/WHO/UNU standards (1) recommend increasing dietary protein intakes throughout pregnancy. To reach the recommended amounts of protein would require an additional intake of 1.2, 6.1, and 10.7 g protein/d in terms of absorbed protein in the first, second, and third trimesters, respectively. These recommendations are based on theoretical calculations of protein deposition during pregnancy. Better insight into protein requirements may be gained from both nitrogen balance studies and protein turnover studies. Unfortunately, hardly any longitudinal nitrogen balance studies have been conducted in women whose diet is not ad libitum (2).

As yet, protein turnover studies have not been consistent in their findings or in identifying in which phase of pregnancy turnover is greatest. It was shown in rats that protein is stored in early pregnancy and mobilized in late pregnancy to meet the need for protein synthesis (3). No evidence of this has been found in nitrogen balance studies in humans. Nitrogen balance is calculated by deducting fecal and urinary losses of nitrogen from dietary nitrogen intake. Balance studies are thought to overestimate nitrogen balance because of the difficulty of measuring nitrogen losses from sources other than feces and urine. Indeed, a review of nitrogen balance studies in pregnancy showed the average nitrogen retention to be greater than what might be expected from theoretical calculations (4). However, an estimate of the amount of miscellaneous nitrogen losses can be made. The FAO/WHO/UNU recommendation (1) is that 8 mg $N \cdot kg$ body $wt^{-1} \cdot d^{-1}$ should be deducted from nitrogen retention values to account for these losses. Another discrepancy between nitrogen balance studies and the theoretical calculations is that nitrogen retention at the beginning of pregnancy is not as different from retention at the end of pregnancy as was previously thought (4). This discrepancy could be due to insufficient data from balance studies for different phases of pregnancy.

More insight into protein metabolism can be obtained by combining balance studies with measurements of 3-methylhistidine (3-MeH), which can be used as an indicator of muscle protein breakdown (5), and creatinine, which indicates the amount of lean body mass. When muscle protein is degraded, 3-MeH is released, and because it cannot be reused for synthesis, it is excreted in urine (6). Some investigators question the validity of using 3-MeH excretion as an indicator (7). However, rat studies

[1] From the Departments of Human Nutrition and Epidemiology (MM, LCPGMDG, and JMAVR) and Human and Animal Physiology (HAB), Wageningen University, Netherlands.

[2] Supported by a Socrates/Erasmus scholarship (to MM).

[3] Address reprint requests to LCPGM de Groot, Department of Human Nutrition and Epidemiology, Wageningen University, PO Box 8129, 6700 EV Wageningen, Netherlands. Email: lisette.degroot@staff.nutepi.wau.nl.

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TABLE 1
Characteristics of the study subjects[1]

Age at onset of pregnancy (y)	28.4 ± 2.5
Height (m)	1.70 ± 0.06
Weight (kg)	61.4 ± 9.1
BMI (kg/m^2)	21.3 ± 3.0
Body fat (%)	27.5 ± 6.1
Parity	0.4 ± 0.5
Duration of gestation (wk)	40 ± 1
Weight gain over pregnancy (kg)[2]	12.2 ± 2.9
Birth weight of baby (g)	3583 ± 257
Length of baby (cm)[3]	52.8 ± 1.5

[1] $\bar{x}$ ± SD; n = 12.
[2] Weight at 1–7 d before delivery, minus prepregnancy weight.
[3] Measured 13 ± 7 d after delivery.

show that 90% of 3-MeH is from skeletal muscle and that urinary 3-MeH can be used as a marker of muscle protein breakdown (8). Urinary 3-MeH excretion was found to increase in late pregnancy, suggesting mobilization of protein during late pregnancy (9, 10). Creatine in muscle gradually degrades to creatinine, which is then excreted in urine (6). The daily production of creatine and subsequently creatinine depends on muscle mass, and the excretion rate of creatinine is relatively constant when the diet remains constant (11, 12). Creatinine excretion is therefore considered a reasonable index of lean body mass, provided that 3 consecutive 24-h urine samples are collected (13, 14).

The present study was carried out in conjunction with an energy balance study in which experimental diets were kept constant (15). We examined nitrogen balance, 3-MeH excretion, and creatinine excretion before and during pregnancy with the aim of determining the development of protein metabolism during pregnancy.

SUBJECTS AND METHODS

Study design

Nitrogen balance was determined in 12 healthy Dutch women before pregnancy and at weeks 12, 23, and 34 of gestation as part of an energy balance study in which energy balance was measured in respiration chambers (15). A few months before the study began, 1 d was spent familiarizing the subjects with the procedures and the respiration chambers and estimating the energy requirement for energy expenditure (EE). Two open-circuit respiration chambers were used simultaneously. On the evening preceding the 24-h EE measurement, the subjects came to the laboratory and entered the respiration chamber no later than 2300. After awakening at 0815 they followed a set meal and activity schedule that simulated housekeeping or work at an office. Gas-exchange measurements were made during a 24-h period, beginning and ending at 0800. The 24-h EE was derived from measurements of oxygen consumption and carbon dioxide production (15).

Each experimental balance period of the study lasted 8 d: 6 d at home and 2 d in the respiration chambers. For the duration of these balance periods, complete daily diets were supplied and the subjects were instructed to perform their regular light activities. The first 4 d of each balance period were considered to be an adjustment phase to the diet. Feces and urine were collected during the last 4 d of each balance period. The subjects spent the last 2 d in respiration chambers, during which 24-h EE was measured consecutively and their physical activity was similar to that of the original 1-d EE measurement. Prepregnancy baseline measurements were made in the follicular phase of the menstrual cycle. Two women were in the luteal phase of their cycle during the prepregnancy measurement and one woman had conceived at ovulation in the week before the baseline measurement. Approximately 1 wk after each measurement of EE, body weight was measured on a balance-beam scale (Berkel ED60-T; Rotterdam, Netherlands), calibrated to 0.05 kg, and body volume was assessed by underwater weighing, with a simultaneous correction for residual lung volume by helium dilution. Siri's equation (16) was used to calculate the prepregnancy percentage body fat from body density. Equations for estimating body fat mass in pregnancy were used for the body density measurements during pregnancy (17), which took into account the (estimated) alterations and composition of the maternal fat-free mass throughout pregnancy.

Subjects

Twelve women were recruited through advertisments in local newspapers. All subjects were nonsmokers, white, from the middle-upper socioeconomic class, and residing in the town of Wageningen or surrounding areas. They had a body mass index (in kg/m^2) of 18.0–28.4 and percentage body fat of 18–36%. All were judged to be healthy through medical history screening and urinalysis. The protocol of the study was approved by the ethical committee of the Division of Human Nutrition of Wageningen University, and all the women gave written, informed consent before the study began. Eleven women delivered at term (gestation: 264–291 d) and one was classified as postterm (296 d). Characteristics of the women are given in **Table 1**.

Experimental diets

The experimental diets provided the prepregnancy energy intake because previous longitudinal studies failed to show significant changes in energy intake during pregnancy (18, 19). For each subject, an experimental diet was designed to supply the prepregnancy energy requirement estimated from the 24-h EE measurement taken before the study. Of the total metabolizable energy of the diets, 50% was supplied by carbohydrate, 35% by fat, and 15% by protein. These diets were provided throughout the 8 d of each nitrogen balance experiment, so that for each balance period the diets supplied similar amounts of energy, protein, and macronutrients and used similar foods. The experimental diets used 37 food products that are common in Dutch diets and were chosen for their homogeneous composition and ease of sampling and storage. All food products of the experimental diet were sampled, and daily portions were weighed to the nearest 0.1 g. The subjects were instructed to eat all the food provided. Before the balance periods, the metabolizable energy of the experimental diets was estimated by using Dutch food-composition tables (20).

Sample analysis

The actual metabolizable energy intake during the balance periods was determined by analyzing the energy content of all food items, feces, and urine, which was measured with an adiabatic bomb calorimeter (IKA-Calorimeter C4000; Janke and Kunkel, Heitersheim, Germany). Nitrogen in foods and excreta was determined by the Kjeldahl method, with mercury as a catalyst (21). For the analyses of 3-MeH, urine samples were collected in bottles with 2 mL of a 2-mol HCl/L solution. Samples were hydrolyzed with a 6-mol HCl/L solution. The hydrolysate was evaporated and then dissolved in a lithium buffer (pH 2).

TABLE 2
Dietary intake, digestibility, urinary excretion, and retention of nitrogen before and during pregnancy[1]

	Before pregnancy	During pregnancy		
		Week 12	Week 23	Week 34
Gross nitrogen intake (g/d)	13.8 ± 1.4	14.0 ± 1.5	14.0 ± 1.4	13.7 ± 1.5
Gross nitrogen ($g \cdot kg^{-1} \cdot d^{-1}$)	0.23 ± 0.03	—	—	—
Nitrogen digestibility (%)	87.9 ± 2.3	87.4 ± 2.7	89.2 ± 3.9	88.5 ± 3.6
Digestible nitrogen ($g \cdot kg^{-1} \cdot d^{-1}$)	0.20 ± 0.03	—	—	—
Nitrogen excreted in urine (g/d)[2]	12.0 ± 1.0^{a,b}	12.6 ± 1.3^{a}	11.9 ± 1.5^{a,b}	11.0 ± 1.4^{b}
Nitrogen retention (g/d)[3]	0.2 ± 0.9^{a,b}	−0.4 ± 1.7^{a}	0.5 ± 1.1^{a,b}	1.2 ± 1.6^{b}

[1] $\bar{x}$ ± SD; n = 12. Values in the same row with different superscript letters are significantly different, $P < 0.05$ (Tukey's studentized range test).
[2] $P < 0.01$.
[3] $P < 0.05$.

The hydrolysate was used as a normal sample for amino acid analysis in the physiologic mode of an automatic amino acid analyzer (Biotronic LC 6001; Biotronik, Wissenschaftliche Geräte, GMbH, Frankfurt, Germany). During chromatography, 3-MeH is eluted directly after histidine. The creatinine in urine produces an orange-yellow color after reaction with picric acid. The intensity of the color is a measure of the creatinine content. The color intensity was measured at a wavelength of 490 nm.

Statistics

Data were analyzed with the use of SAS (version 6.12; SAS Institute, Inc, Cary, NC). Results are presented as means ± SDs. Longitudinal data were analyzed by analysis of variance for repeated measures and Tukey's studentized range test for multiple comparisons ($\alpha = 0.05$).

RESULTS

The main results are shown in **Tables 2** and **3**. As expected from the study design, nitrogen intake and metabolizable energy intake did not differ significantly between the balance periods. Energy intakes were 8.8 ± 0.9 MJ (144 ± 18 kJ/kg)/d before pregnancy, 8.7 ± 1.0 MJ/d in week 12 of gestation, 8.9 ± 0.9 MJ/d in week 23, and 8.7 ± 1.1 MJ/d in week 34. Throughout pregnancy there was no significant change in loss of nitrogen in feces and therefore no change in protein digestibility. The mean amount of digestible nitrogen was 12.2 ± 1.1 g/d (0.20 ± 0.03 g/kg) before pregnancy and 12.2 ± 1.3, 12.4 ± 1.1, and 12.1 ± 1.2 g/d in weeks 12, 23, and 34, respectively. Nitrogen digestibility as a percentage of the gross nitrogen intake is shown in **Table 2**.

The mean urinary excretion of nitrogen was 12.0 ± 1.0 g N/d before pregnancy and on average 11.8 ± 1.4 g N/d during pregnancy. Toward term urinary nitrogen excretion was significantly lower than in early pregnancy, with a tendency to be highest in the first trimester. Along with the reduction in excretion, nitrogen retention increased toward term. The difference between the first and third trimesters was significant.

As shown in Table 3, the mean urinary excretion of 3-MeH was 172 ± 51 μmol/d before pregnancy and on average 159 ± 32 μmol/d during pregnancy. Urinary excretion of 3-MeH showed a tendency to be lowest at week 12 of gestation and higher toward term. The mean urinary excretion of creatinine was 1.10 ± 0.24 g/d before pregnancy and on average 1.15 ± 0.20 g/d during pregnancy. The differences in both 3-MeH and creatinine excretion between trimesters were not significant. Similar patterns emerged when urinary excretions were expressed per kilogram fat-free mass.

Fat-free mass increased toward term. The mean value for fat-free mass was 44.2 ± 5.1 kg before pregnancy and on average 48.2 ± 6.0 kg during pregnancy. For fat-free mass, the difference between the amount before pregnancy and in the third trimester was significant, as was the difference between the first and third trimesters.

DISCUSSION

In this study we measured baseline nitrogen balance before pregnancy and 3 times from early to late pregnancy. The results show that nitrogen balance increases toward the end of pregnancy, suggesting a more efficient use of dietary protein later in pregnancy.

The results of the present study reflect the alterations in protein metabolism under strictly standardized conditions throughout 4 experimental balance periods: both energy intake and the physical activity pattern were kept constant. The former condition was

TABLE 3
Urinary excretion of 3-methylhistidine (3-MeH) and creatinine, body weight, and fat-free mass before and during pregnancy[1]

	Before pregnancy	During pregnancy		
		Week 12	Week 23	Week 34
3-MeH excreted in urine (μmol/d)	172 ± 51	147 ± 25	163 ± 33	166 ± 37
Creatinine excreted in urine (g/d)	1.10 ± 0.24	1.16 ± 0.21	1.15 ± 0.21	1.14 ± 0.17
Body weight (kg)[2]	61.4 ± 9.1^{a}	62.1 ± 10.3^{a}	67.2 ± 9.9^{a,b}	73.0 ± 10.3^{b}
Fat-free mass (kg)[3]	44.2 ± 5.1^{a}	44.7 ± 5.6^{a}	47.6 ± 6.4^{a,b}	52.4 ± 6.0^{b}

[1] $\bar{x}$ ± SD; n = 12. Values in the same row with different superscript letters are significantly different, $P < 0.05$ (Tukey's studentized range test).
[2] $P < 0.05$.
[3] $P < 0.005$.

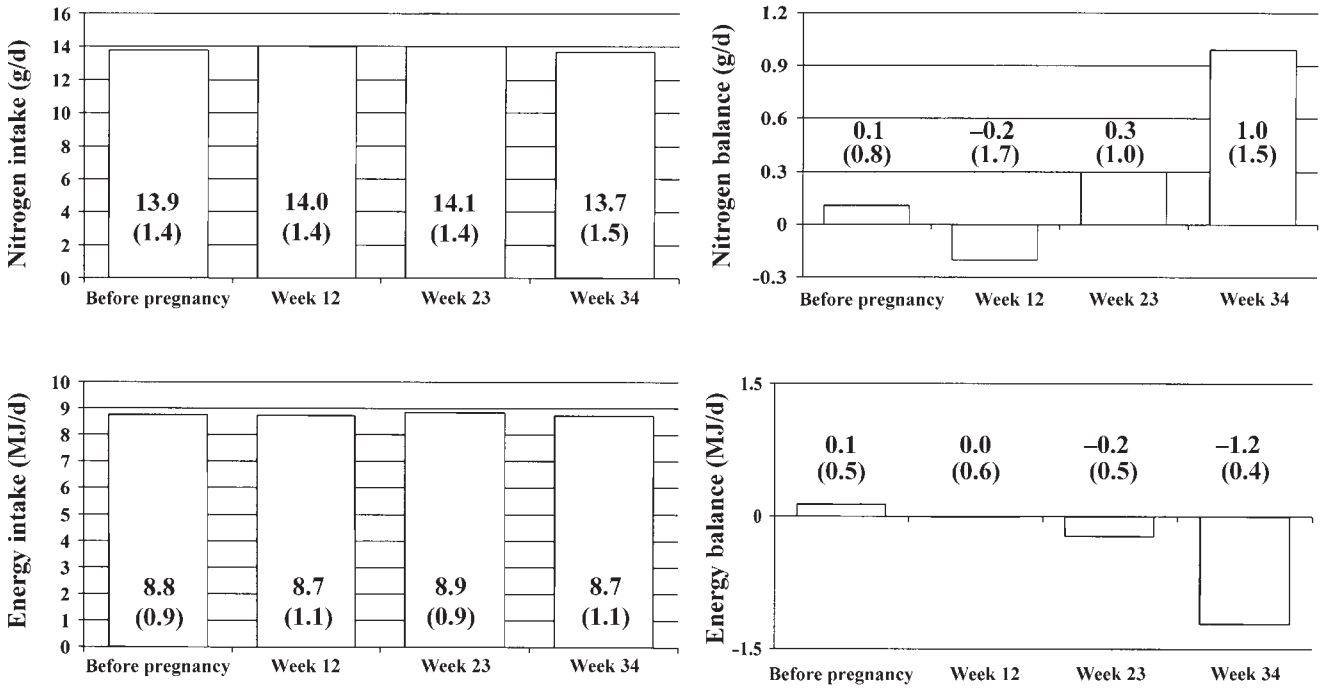

FIGURE 1. Mean (±SD) nitrogen and energy intakes and balances before pregnancy and during weeks 12, 23, and 34 of pregnancy for women with complete data (n = 10).

meant to resemble the assumed constancy of energy intake throughout pregnancy according to food consumption studies (18, 19). Yet, we previously concluded that energy balance becomes progressively negative during pregnancy (15). In 10 of the 12 women we studied, we fully assessed energy balance 4 times and observed that the gap between the imposed energy intake and 24-h EE increased over pregnancy (**Figure 1**). Our present findings show that even when dietary intakes do not meet energy requirements, nitrogen is conserved and that the association between nitrogen and energy balance is weak (r = –0.07 before pregnancy to r = 0.26 in week 34).

These findings are based on strictly standardized balance measurements. Despite being well controlled, nitrogen balance studies have some methodologic constraints, such as overestimation of nitrogen retention and limited duration. Also note that measurements carried out over three 8-d balance periods would not necessarily capture changes occurring over an entire pregnancy. Despite the view that nitrogen balance studies overestimate nitrogen retention because of the difficulty of determining miscellaneous nitrogen losses (22, 23), nitrogen retention does not appear to have been overestimated in the present study. Nitrogen retention in the present study was 3.4 $mg \cdot kg^{-1} \cdot d^{-1}$ before pregnancy and -6.4, 7.4, and 16.4 $mg \cdot kg^{-1} \cdot d^{-1}$ in weeks 12, 23, and 34 of pregnancy, respectively. These values do not seem to leave a margin for miscellaneous losses of 8 $mg \cdot kg^{-1} \cdot d^{-1}$ as is suggested in FAO/WHO/UNU recommendations (1). We therefore assume that there was no significant overestimation of retention in this study. As for the duration of the adjustment period and collection period of the present study, 4 d for each is generally considered acceptable. UNU/World Hunger Programme (24) recommends an adjustment period of ≥5 d. Urinary excretion of nitrogen becomes stable 3–8 d after a change in diet, and no significant differences have been found in excretion at 10–14 d (25). Most researchers accept values that have been measured starting 3–6 d after a change in diet (26). UNU/World Hunger Programme (24) also recommends a minimum period of 5 d for collection of urine samples. Day-to-day variation in urinary nitrogen excretion can result in a CV of ≤50% (27). In a review of long-term balance studies, Rand et al (28) nevertheless came to the conclusion that short-term balance studies are fairly reliable in estimating the nitrogen requirements of healthy adults fed a diet with protein intakes close to recommended levels.

Results from nitrogen balance studies show that nitrogen retention during pregnancy is greater than that predicted by the theoretical calculations of Hytten (29). These studies show that on average, nitrogen deposition is 1.3 g N/d for the first half of pregnancy and 1.8 g N/d for the second half (4). Although retention significantly increased toward term, the values for nitrogen retention determined in the present study were lower than those in most nitrogen balance studies and were also slightly lower than Hytten's theoretical calculations of nitrogen deposition. The negative energy balance in late pregnancy that we reported (15) could partly explain the low nitrogen retention. A more adequate diet would probably result in a higher nitrogen retention. However, in another balance study (30), in women in weeks 30–34 of gestation, the average nitrogen retention of 1.22 ± 0.83 g N/d was similar to that measured late in pregnancy in the women in late pregnancy in our study. Assuming that the subjects in our study were in equilibrium before pregnancy, the true nitrogen retention can be calculated by subtracting the mean value before pregnancy from the values at each trimester. The corrected retention would then be –0.6, 0.3, and 1.0 g N/d for weeks 12, 23, and 34 of pregnancy, respectively. These values are even lower than in the balance study by Johnstone et al (30), but they correspond

to the theoretical calculations of nitrogen deposition in the last trimester of pregnancy (29).

Our results showing increased nitrogen retention seem to be similar to those of Marino (1983) cited by King (2). Marino found that women in late pregnancy excreted less urinary nitrogen than did nonpregnant women and women in early pregnancy. However, in a nitrogen balance study in pregnant women by Naismith and Emery (10), no improvement in nitrogen balance was found, despite the increased needs of the growing fetus. In rats, the protein stored in early pregnancy was shown to be mobilized in late pregnancy to meet the need for protein synthesis in other tissue (3), but no evidence of this has been found from nitrogen balance studies in humans.

Kalhan (31) reviewed results from isotope studies that showed that changes in protein metabolism early in pregnancy not only meet current maternal needs for protein but also prepare the body for increasing fetal needs in late pregnancy. These findings included a decreased urea synthesis toward term and an increased nitrogen salvage (32, 33). In a cross-sectional study by de Benoist et al (34), protein synthesis and breakdown in pregnant Jamaican women were highest at week 12, after which they decreased throughout pregnancy. In other turnover studies, no significant differences in protein synthesis were found at any stage of pregnancy (9, 35). The latter study showed that both protein synthesis and catabolism increased toward term when expressed in relation to fat-free mass. In yet other turnover studies, protein synthesis was found to be increased between mid- and late pregnancy (36, 37), but no difference was found when synthesis was expressed in relation to body weight and fat-free mass (37). If it may be assumed that when turnover increases, the degradation of free amino acids increases, then it may also be assumed that urinary nitrogen excretion and thus the net loss of protein also increase. In this case our results agree with those of de Benoist et al (34). However, results from isotope studies are not quantitative, and it is therefore questionable to compare them with nitrogen retention. In addition, methods used in protein turnover studies vary so widely that it is difficult to relate their results to those from nitrogen balance studies.

Previous studies of urinary 3-MeH excretion showed that it increases toward term, suggesting an increased rate of protein degradation and possibly mobilization of maternal protein stores (9, 10). In the present study, excretion of 3-MeH did not change significantly toward term, which suggests that there was no breakdown of maternal muscle to meet the demands of growing maternal and fetal tissue. Urinary excretion of creatinine did not change either, which indicates that there was no significant change in muscle mass. Although nitrogen retention in our study was quite low, weight gain during pregnancy was average for Western women (38). The increase in fat-free mass during pregnancy was also as expected. The results for 3-MeH and creatinine indicate that no mobilization of maternal muscle mass was needed to achieve this. As noted, average changes in these indicators are subject to some speculation about whether they portray changes occurring throughout pregnancy or only during the highly standardized balance periods.

In the present study with an imposed, constant diet, dietary intake of nitrogen was the same during each balance period, and urinary nitrogen excretion decreased toward term, resulting in improved nitrogen balance toward the end of pregnancy. The urinary excretion of 3-MeH and creatinine indicated no mobilization of protein stores. We therefore conclude that dietary protein is used more efficiently in late pregnancy and that nitrogen may be retained not only in maternal muscle mass but also in other components of fat-free mass, such as the placenta and uterus.

Although shifts in protein metabolism are complex and change gradually throughout gestation, our findings contribute to understanding of the adjustments in nitrogen balance that occur when the dietary energy supply does not meet requirements and little behavioral flexibility exists.

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