Assessment of daily and cumulative carbohydrate and fat balances in mice

J.P. Flatt

Department of Biochemistry, University of Massachusetts Medical School, Worcester, MA, USA

A method for measuring daily CO_2 production and oxygen consumption of mice is described, using 55-gallon plastic drums as sealed metabolic chambers, which are vented once a day. When gas exchange and food intake measurements are performed over many consecutive days (i.e., 15 days or more), an internal calibration can be performed to take into account individual variations in nutrient absorption and food spillage, as well as production of hydrogen by intestinal fermentation. This allows daily rates of carbohydrate and fat oxidation to be established with a high degree of accuracy. The method thus allows monitoring of daily and cumulative carbohydrate and fat balances over many consecutive days. This provides a totally new type of information, inaccessible by conventional studies limited to measurements of overall energy balance, but of great potential importance for the understanding of the corrective responses which bring about body weight stability.

Keywords: indirect calorimetry; carbohydrate metabolism; fat metabolism; metabolizable energy

Introduction

The regulation of energy balance has been studied extensively in view of its relevance to the problems of weight maintenance and of obesity.[1-4] The biological problem of weight maintenance is more complex than the mere adjustment of energy intake to expenditure since the body has to simultaneously maintain protein, carbohydrate, and fat balances.[5] The possibility to detect changes in the body's protein content by measuring nitrogen intake and excretion has allowed recognition long ago that the organism effectively strives to maintain nitrogen, and hence protein balance. Little is known, however, about the accuracy with which the organism tends to maintain carbohydrate and fat balances on a day-by-day basis. Given the rather small size of the body's glycogen reserves and the importance of maintaining adequate blood glucose levels, one would expect the biological significance of short-term deviations from the carbohydrate balance to be far greater than deviations from the fat balance, for which the body's reserve is far greater.[1,2,6] In order to gain insight into this problem, we developed a procedure for the determination of daily carbohydrate and fat balances in ad libitum fed laboratory animals, based on the measurements of nutrient intakes and of carbohydrate and fat oxidation rates by indirect calorimetry. We describe here a method for the determination of the 24 hr respiratory exchange of individual mice, which can be performed conveniently over periods of weeks or months. It proved important for the outcome of the indirect calorimetry calculations to include individual correction factors to take into account variations in food spillage and nutrient absorption from apparent food intake data, and to correct for the formation of small amounts of hydrogen by intestinal fermentation. The facts that cumulative carbohydrate balances are close to zero, whereas cumulative fat balances are proportional to body weight changes (slope = .61, R^2 = .99) support the validity of the method. Its application to monitor daily carbohydrate and fat balances in 10 female CD1 mice during 160 consecutive days is described in a companion paper.[7]

Materials and methods

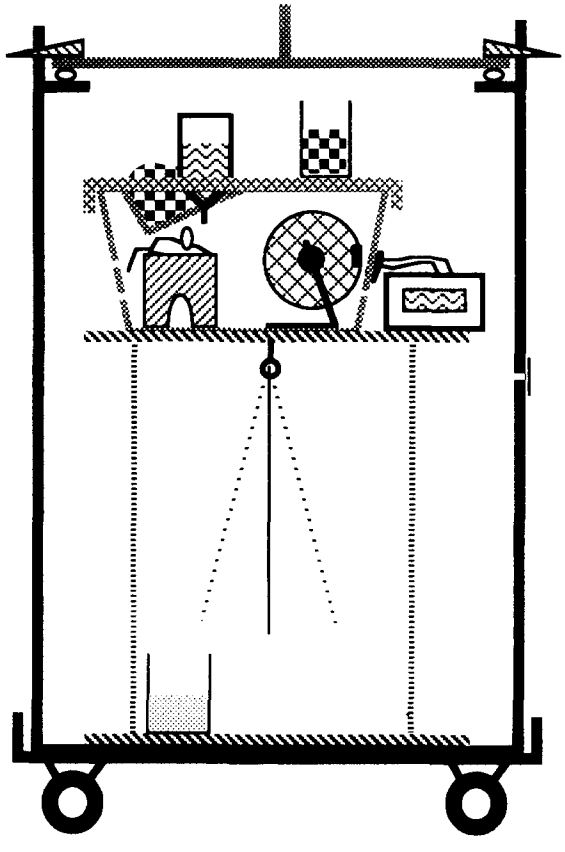
Mice were housed individually in conventional plastic cages with a wire mesh cover. A number of holes were drilled into the sides of the cages to enhance air circulation. Each cage contained a running wheel, magnetically linked to a counter which allowed recording each

Address reprint requests to Dr. J.P. Flatt, Department of Biochemistry, University of Massachusettes Medical School, Worcester, MA 01605, USA.
Received Februrary 16, 1990; accepted September 24, 1990.

Table 1 Experimental diets

Ingredients	*Lab chow*[a] (g/kg)	*High carbohydrate*[b] (g/kg)	*Mixed*[b] (g/kg)
[c]Casein [protein][a]	235	181	223
Sucrose		325	212
[c]Corn starch [carbohydrates][a]	(456)	344	223
Corn oil		16	20
Part. sat. vegetable oil [triglycrides][a]	65	33	199
[d]Vitamin mix [vitamins][a]	.2	10	12.3
[e]Mineral mix + $CaCO_3$ [Ash][a]	68	40	49
[f]Cellulose [fiber][a]	38	51	62
Nutrient Values			
[g]Gross energy (kcal/g)	4.19	4.27	5.18
Digestible energy (kcal/g)	3.50	3.80	4.66
% Energy as protein:CHO:Fat	24:61:15	18:69:13	18:37:45
mmol C/g diet	30.9	32.0	36.1
mmol O_2 for oxidation/g diet	34.1	34.7	43.6
FQ[h]	.906	.921	.827

[a] Purina Formula Chow #5008, Ralston Purina Co., St. Louis, MO; composition based on data from manufacturer, the digestible carbohydrate content being computed by difference.
[b] Prepared to these specifications by Teklad, Madison, WI.
[c] Casein contains 87% protein, 1% fat, 2% Ash; corn starch contains 90% starch, 0.35% protein, 0.3% fat.
[d] Vitamin Fortification Mix, Teklad #40080; contains 47% starch.
[e] "Bernhart-Tomarelli" Mineral Mix, Teklad #170750; contains 12% sucrose.
[f] Diets adjusted to contain approximately 10 g fiber/1000 kcal; the cellulose used contains about 25% moisture.
[g] By bomb calorimetry in our laboratory.[8]
[h] FQs were computed from the known macronutrient content of the diets by calculating the amounts of CO_2 produced and of O_2 consumed using the Loewy indirect calorimetry coefficients.[9,10]



day of the number of revolutions which the animals chose to perform. The mice had unrestricted access to one of two synthetic diets prepared by TEKLAD (Madison, WI) to contain 18% of their energy as casein, with porportions of fat and carbohydrate (half as starch, half as sucrose) either similar to lab chow (13% of total energy as fat = "High carbohydrate diet"), or to a mixed western diet (45% of total energy as fat = "Mixed diet") (*Table 1*). Both contained approximately 10 grams of cellulose per 1000 kcal. Each cage was placed on a platform in a 55-gallon plastic drum, in the top of which a large circular opening had been cut out (*Figure 1*). The drums were closed with 1/4 inch thick plexiglass covers, seated on one or two coils of latex tubing resting on the circular shelf left after

Figure 1 Use of a 55-gallon drum as a metabolic chamber for measuring the 24-hr respiratory exchange of a mouse. The presence of a block of wood allows the animals to gnaw (possibly reducing "shredding" of food) and provides a "tunnel" which is a much used shelter. The number of revolutions performed per day on the running wheel is recorded by a magnetic detector and counter. In addition to the water bottle and food in the cover, a similar amount of food is kept on top of the cage to allow for corrections of weight changes due to moisture. Some 10 g of anhydrous $CaCl_2$ are added every day to a beaker in the bottom of the barrel to limit the humidity in the chamber. Additional explanations are given in the text.

cutting out the openings in the drums. Using eight wooden wedges pushed into slots cut into the upper rims of the drums, the plexiglass covers were tightly pressed against the latex coil(s), providing a hermetic seal. Through the transparent plexiglass covers, the mice were exposed to a 12 hr light-dark cycle. This set-up allows measurement of the respiratory exchanges of individual mice during an entire day (during which the P_{CO_2} increases to 1.2–2%, while the P_{O_2} declines from 21% to 19–20%), except for an approximately 45-minute-long period every day in the early afternoon (when food intake is low), to permit venting (with a fan), cleaning, and measuring of body weights and food consumption by weighing the covers of the animals' cages (or the food containers) with the supply of food contained therein. Similar portions of feed were kept in separate containers in each drum and weighed daily, to allow appropriate corrections for changes in the weight of the feed caused by gain or loss of moisture, which proved important, particularly with high carbohydrate diets and lab chow. To limit the humidity in the barrels, small portions of anhydrous $CaCl_2$[9] (about 10 g/day)[11] were added daily to a container kept in the bottom of the barrel.

Gas measurements were performed with a Perkin-Elmer Medical Gas Analyzer (model MGA 1100B) just after sealing the drums (i.e., at about 3 PM) and again before they were opened (i.e., at about 2 PM on the following day). This instrument has separate detectors for the measurement of oxygen, carbon dioxide, nitrogen, and argon, and provides data in terms of partial pressures for these four gases, whose sum is set to be 100%. This allows calculation of the O_2/N_2 and CO_2/N_2 ratios at the beginning, and at the end of a period of observation. (If metabolic rates are to be established for different periods of the day, several gas measurements can be made during the day without opening the drums.) Gas exchange calculations are based on the use of these ratios, the assumption being made that the amount of N_2 present in the drums is unchanged by the animal's metabolism. In this manner, respiratory exchange of O_2 and CO_2 can be established without having to take into account changes in partial pressures due to temperature and atmospheric pressure fluctuations, to changes in water vapor content, and to differences in the volumes of CO_2 produced and of O_2 consumed. The volume of each drum was determined by measuring the weight of water which it could hold. This volume, minus the volume of the equipment used in them (calculated from weights and densities), gives value for V used in the following equations. Using C_i, O_i, N_i, C_f, O_f, N_f to designate % CO_2, % O_2, and % N_2 in the initial and final states, respectively, the equations used are:

$$CO_2 \text{ produced} = (C_f \times N_i/N_f - C_i) \times V \quad (1)$$

$$O_2 \text{ consumed} = (O_i - O_f \times N_i/N_f) \times V \quad (2)$$

The gas analyzer's channels were calibrated using gas mixtures prepared gravimetrically and verified by gas analysis (i.e., "primary gas standards" obtained from Matheson Co., East Rutherford, NJ). The measurements of the composition of air and of the calibrated gas mixtures were highly reproducible. In determinations made on 20 different days, the coefficient of variation affecting the P_{CO_2} P_{O_2}, and P_{N_2} values were 0.25%, 0.09%, and 0.02%, respectively. The average respiratory quotient (RQ) observed in 47 determinations on burning ethanol was 0.674 ± 0.012 (SD), an error of 1% relative to the theoretical RQ values of 0.667 for the oxidation of ethanol. Reference gas mixtures and ambient air were measured daily before, at the mid-point, and after the determinations of the drums' gas contents. Suitable corrections were incorporated into the gas exchange calculations to compensate for deviations from the reference values, including interpolations to correct for possible drift in the reference values while drum measurements were being performed. To test the seal of the drums, four drums were filled with a gas mixture containing about 10% CO_2, 10% O_2, and 80% N_2. The drums' gas contents were measured repeatedly over a period of 9 consecutive days. The average loss in their P_{CO_2} averaged 0.59% ± 0.08% per day (e.g., a drop in P_{CO_2} from 10.00 to 9.94% in 24 hr), whereas the P_{O_2} increased by 0.97% ± 0.18% (e.g., an increase in P_{O_2} from 10.00 to 10.1% in 24 hr). Such leakage is too small to affect the measurement of gas exchange in mice.

The composition of the gas phase in the drums was determined by introducing a nine-inch-long metal canula connected to the gas analyzer's gas inlet through a small hole drilled in the side of the drums, which was sealed with plastic tape between measurements. No difference could be detected at the end of 24-hr periods when the canula was inserted through holes near the top or near the bottom of the drums (i.e., in 15 determinations, the ratio of the P_{CO_2} values so obtained averaged 1.0008 ± 0.0025). When animals are kept in the drums, small increases in temperature and of water vapor pressure (usually) cause a slight overpressure, preventing the contents of the drums from being diluted by ambient air when the holes are unsealed to allow introduction of the gas sampling probe. Since the output of the gas analyzer is in terms of percents of the sum of N_2 + O_2 + CO_2 + argon, the escape of a small volume of gas from the drum when the measuring port is opened does not affect the calculations of the gas exchange, as these are based on the ratio of CO_2/N_2 and O_2/N_2 and on the assumption that the amount of N_2 is constant. The amount of N_2 initially present is known from the volume of the gas phase in the drums and the initial % N_2 taking into account barometric pressure, ambient temperature, and relative humidity at the time of sealing the drums. Daily gas exchanges were calculated by extrapolating the values determined as just described to 24-hr periods, assuming CO_2 production and O_2 consumption to proceed during the interval in which the drums were opened at 2/3 of the rates at which they had occurred during the measured period of the day (since the drums were opened during a period of the day in which the mice are relatively quiet).

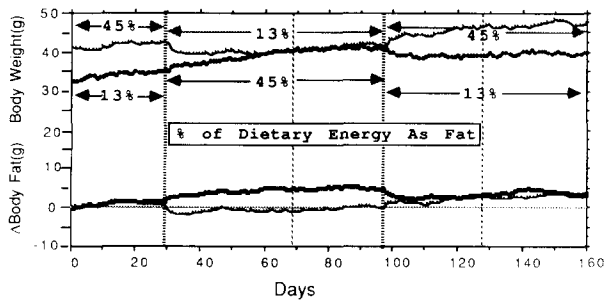


Figure 2 Average body weights and changes in body fat content in two groups of five female CD1 mice. The mice were preadapted to synthetic diets containing 18% of calories as protein, and either 13% or 45% as fat, and 69% or 37% as carbohydrate. The diets were switched on days 29 and 97. Changes in body fat content were calculated by cumulating the changes in fat content determined daily by indirect calorimetry and food intake measurements. For the purpose of the calculations presented here, the total experimental period of 160 days was subdivided into five segments of approximately one month's duration each.

Experimental animals

Most of the data presented were obtained in 10 female CD-1 mice (purchased from the Charles River Corp.) studied continuously over a period of 160 days, while 130 to 290 days old. Five mice initially received the high carbohydrate diet, the other five the mixed diet. Previously, they had been adapted to these diets so that their body weights would be relatively stable when the indirect calorimetry measurements were to begin. After one month, the diets were switched, and two months later the animals were returned to their initial diet, for the last months of the experiment. The time course of the experiment is shown in *Figure 2*. Some additional data to be considered were obtained with another group of 10 female mice, maintained alternatively on Purina lab chow or on the high carbohydrate diet, and in a group of 10 male CD1 mice receiving the mixed diet.

Calculation of carbohdyrate and fat oxidation and balances

It was assumed that the mice used in these studies maintain approximate nitrogen balance, and no attempt was made to measure their nitrogen losses. Protein oxidation was thus taken to be equal to the protein intake on a given day. The coefficients used in calculating dietary nutrient contents (*Table 1*) and changes in the body contents of protein, carbohydrate and fat due to the animal's metabolism are those proposed by Loewy,[9] as used by Lusk.[10] Livesey and Elia[12] have proposed recently to revise the values used for protein to 4.70 instead of 4.32 kcal/g, with an RQ of .835 instead of .801. When these values were used in a subsequent experiment with 10 male mice, a slight improvement in the outcome of the cumulative carbohydrate balances could be noticed (cf. *Figure 7*).

In order to calculate carbohydrate and fat balances with accuracy, it is obviously necessary to know the animals' nutrient intake as precisely as possible. As in previous experiments involving the synthetic diets,[6] we found that the cumulated amounts of CO_2 produced over many consecutive days accounted for a substantial amount of the carbon contained in the amount of food disappeared from the hopper, particularly in mice whose body weights had remained stable. This is shown by the intercept in *Figure 3* which has a value of 94% when no weight change occurs. In animals that gained or lost weight, carbon obviously was stored in (or lost from) their carcasses, mostly as adipose tissue triglycerides. When measurements are performed continuously over prolonged periods of time (e.g., 15 days or more), these amounts are relatively small in comparison to the cumulated CO_2 production (*Figure 3*). They were evaluated by considering that in the case of gradual weight changes in adult mice, 6 kcal are deposited (or lost) per gram of body weight change, which was assumed to comprise 0.6 g triglyceride, 0.06 g protein, 0.01 g glycogen per gram. Together, these substances contain 40.7 mmol C, which would require 57.2 mmol O_2 for their complete oxidation, with an RQ of 0.71. When these changes in carcass carbon content were taken into account, overall carbon recoveries averaged 93.7 ± 3.0% (i.e., 94.6 ± 2.4% and 92.8 ± 3.4% on the high carbohydrate and the mixed diets, respectively). Considering that food spillage and incomplete intestinal absorption factors obviously cause the amounts of nutrients effectively absorbed by the animals to be less than the nutrient content of the amounts of food removed from the food hoppers, the observed carbon recoveries are very high. We have, therefore, presumed that they give a fairly good estimate of the fraction of the nutrients effectively absorbed, relative to the nutrient content of the amount of food apparently consumed. In fact, the observed C-recoveries are consistent with the nutrient content of the diets calculated using the Atwater factors,[13] which yield energy values equal to 94% of

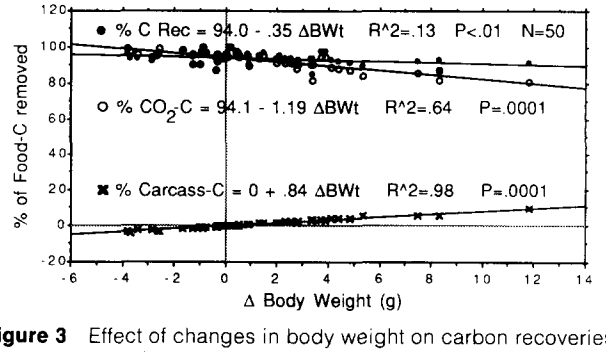


Figure 3 Effect of changes in body weight on carbon recoveries. The open dots show the proportions (in %) of food-carbon disappeared from the hopper which were recovered in the form of CO_2 in 10 ad libitum fed CD1 mice during 5 consecutive periods of approximately one month's duration each. The crosses show the amount of carbon gained by (or lost from) the carcass (assuming that 60% of body weight change is triglyceride, 6% protein, and 1% glycogen), expressed as percentage of apparent food-carbon consumption. The full circles show the overall carbon recoveries (in %), obtained by relating the sum of cumulative CO_2 production plus changes in carcass carbon content to the amount of food-carbon removed from the food hopper.

those calculated with the Loewy coefficients. We have, therefore, considered that average carbon recoveries can be established by relating the sum of the carbon converted to CO_2 plus that incorporated into (or lost from) the carcass, to the cumulated amounts of food-carbon removed from the food containers. Furthermore, by establishing carbon recoveries for each animal individually, it becomes possible to take into account individual variations in food spillage and intestinal absorption. Since carbon recovery factors are quite high (84 to 100% on synthetic diets), with the lower presumably due primarily to increased food spillage, the same factor was applied in calculating protein, carbohydrate, and fat absorption from the food consumption measurements and the diets' known macronutrient contents. The fact that nutrient absorption can be established individually, without the need for collection and analysis of spilled food particles and excreta (which generally remains inaccurate in spite of painstaking efforts), represents a considerable practical advantage.

When performing the indirect calorimetry calculations after taking into account individual carbon recoveries, as described above, cumulated carbohydrate balances of -5 to -10 g were obtained. Such large negative carbohydrate balances are obviously not realistic. In trying to resolve this apparent discrepancy, we considered that intestinal fermentation could lead to the formation of hydrogen and/or of methane, and that this could explain the outcome of the indirect calorimetry calculations. As shown by the examples in equations 4 and 5, release of hydrogen or of methane causes the measured oxygen consumptions to be below the values expected for the complete oxidation of a given amount of nutrients (equation 3), leading to erroneously high RQ values, and a consequent overestimation of carbohydrate oxidation rates.

$$4\,C_6H_{12}O_6 + 1\,C_{16}H_{32}O_2 + 47\,O_2 \quad \text{(glucose) (palmitate)} \xrightarrow{RQ = 0.85} 40\,CO_2 + 40\,H_2O \tag{3}$$

$$4\,C_6H_{12}O_6 + 1\,C_{16}H_{32}O_2 + 45\,O_2 \xrightarrow{RQ = 0.89} 40\,CO_2 + 4\,H_2 + 36\,H_2O \tag{4}$$

$$4\,C_6H_{12}O_6 + 1\,C_{16}H_{32}O_2 + 45\,O_2 \xrightarrow{RQ = 0.89} 39\,CO_2 + CH_4 + 38\,H_2O \tag{5}$$

Using a breath analyzer available in the hospital (for the study of lactose malabsorption, revealed by increased appearance of H_2 in the exhaled air), we observed concentrations of hydrogen ranging from 6 to 12 parts per million in the gas of drums in which four mice had been kept for 24 hr. This corresponded to H_2:CO_2 ratios five to fifteen times higher than those observed in normal humans. In our mass spectrometric gas analyzer, CH_4 or other volatile carbon compounds would be detected in the CO_2 channel, if such compounds were to be produced instead of, or in addition to H_2 (equation 5), and the RQ would appear to be 0.89, as in equation 4. Tests to detect the presence of methane by mass gas spectrometry were performed thanks to the collaboration of Dr. Reed Hoyt at the U.S. Army Natick Laboratory, but the amounts detected (1 to 2 ppm) were not higher than in ambient air.

Therefore, we have considered that the O_2 consumptions observed were too low because of the production of hydrogen by intestinal bacteria. Since hydrogen production is not measured in the procedure described here, this phenomenon is taken into consideration in performing the indirect calorimetry calculations by the following corrective procedure: For each animal, the sum of the cumulated O_2 consumption observed, plus (or minus) the amount of oxygen needed for the oxidation of the carbon stored in (or lost from) the body (i.e., 57.2 mmol of O_2 per gram of body weight change) was calculated. This sum was divided by the amount of oxygen needed for the oxidation of the amount of nutrients effectively absorbed (i.e., amounts of nutrients mixtures disappeared from the hopper, multiplied by the C recovery observed for that mouse), calculated from the carbon content and the known FQ of the diet (*Table 1*). The amounts of oxygen accounted for came to 99.2 ± 1.2% and 98.6 ± 0.9% of the expected value for the two synthetic diets. The "missing" amount of oxygen (i.e., the O_2 presumed to be needed for the oxidation of all of the H_2 formed during a period of calculation) was divided by the total amount of food-C absorbed by a given mouse, to provide individual "O_2-correction factors." These were applied to calculate increments to be added to the observed daily oxygen consumptions, in proportion to the amounts of nutrients absorbed on given days. This presumes that the process of intestinal fermentation is proportional to the amount of food consumed on a given day. These correction factors ranged from $-.004$ to .039 (mean ± SD = .011 ± .011) mol of O_2 per mol of food carbon absorbed. (For comparison, the correction factor would be 0.05 in the situations described by equations 4 or 5). This corrective procedure does not apear to be biased at the onset toward influencing in a particular manner the calculation of the carbohydrate balances rather than the fat balances. Yet, when it was included in the indirect calorimetry calculations, the cumulative carbohydrate balances all became quite small (*Figure 4*).

Results

In order to limit body weight changes during the initial phase of the experiment, the mice were allowed to become adapted to the diet which they were to receive. Body weights in the two groups of five mice had been comparable before they were transferred from lab chow to the synthetic diets, whereupon they increased noticeably in the animals receiving the mixed diet containing 45% of dietary energy as fat during the weeks preceding the experiment. Their body weights averaged 41.0 ± 1.4 g (SD) at that time,

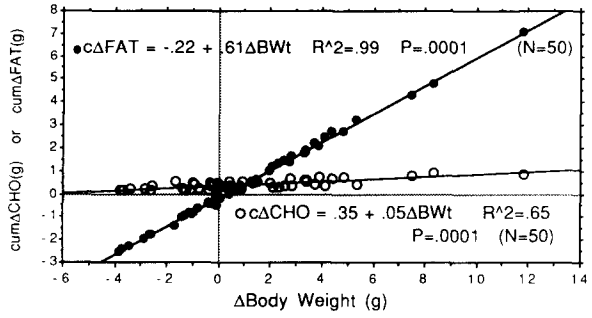


Figure 4 Relationship between cumulative carbohydrate and fat balances and changes in body weight. The open circles (○) show the cumulative carbohydrate balances and the closed circles (•) the cumulative fat balances observed in 10 mice during 5 consecutive experimental periods of approximately one month each.

as compared to 32.5 ± 1.4 g for the mice maintained on the high carbohydrate diet whose macronutrient content is comparable to that in laboratory chow (i.e., 13% of dietary energy as fat). The increase in body weight and in body fat known to be induced by diets with substantial fat contents[6] is also clearly evident in *Figure 2*, which illustrates the time course of the experiment. When the diets were switched, the mice transferred from the high carbohydrate to the mixed diet gained weight; this was associated with deposition of body fat, as shown by cumulating the daily fat balances determined by indirect calorimetry. On the other hand, the animals that were changed from the mixed diet to the high carbohydrate diet lost weight, mostly as fat. These changes were reversed when the mice subsequently were returned to their initial diets.

Since it is not feasible to validate the method by sequential measurements of body composition in a given animal, and since variations in body composition between mice are far greater than daily changes in a single animal, a direct verification of the results obtained for daily carbohydrate and fat balances is impossible. However, it is possible to assess whether the changes in body composition computed by cumulating daily carbohydrate and fat balances over extended periods (e.g., one month) are consistent with observed body weight changes, which reflect primarily changes in the fat mass and to a lesser extent the associated expansion (or shrinkage) of the supporting lean tissue mass. Considering that 10 animals were individually monitored and that the 160-day-long experiment was subdivided into five periods of approximately one month each (cf. *Figure 2*), 50 sets of data were available to evaluate the accuracy and validity of the procedure by relating cumulative carbohydrate and fat balances obtained by the proposed calculations to weight changes observed during the corresponding periods. As shown in *Figure 4*, the cumulative carbohydrate balances were small (0.35 g + .05 g glycogen per gram of body weight change) and the fat balances correlated very closely with the observed weight changes (R^2 = .99), with a slope indicating gains or losses of .61 g of fat per gram of body weight change. This is consistent with our experience from other studies, where the body composition of female CD1 mice was determined by chemical carcass analysis and showed the following relationship: Body fat (g) = −15.4 + .61 Body weight (g); R = .87, N = 205. The results obtained by cumulating data based on indirect calorimetry measurements over one-month periods are thus remarkably consistent with the "boundary conditions" which must logically apply; namely, that cumulative carbohydrate balances must be relatively small in view of the animals' limited storage capacity for glycogen, whereas changes in body weight in full-grown animals are primarily due to changes in their adipose tissue fat stores and the related expansion of the lean body mass.

To evaluate whether the proposed procedure allows to establish correction factors individually tailored to different mice, we compared the C recoveries calculated during two consecutive months on the same diet for the different animals. The correlation apparent in *Figure 5* (upper panel) demonstrates that these C recoveries are indeed reflecting individual characteristics (R^2 = .39 and P = .003; if one outlying value is disregarded, R^2 = .73 and P = .0001). Since the variables are affected equally by experimental error, the slope of the line was calculated according to Brace.[14] In this procedure, the slopes of the correlations are given by dividing the slope of the regression line calcu-

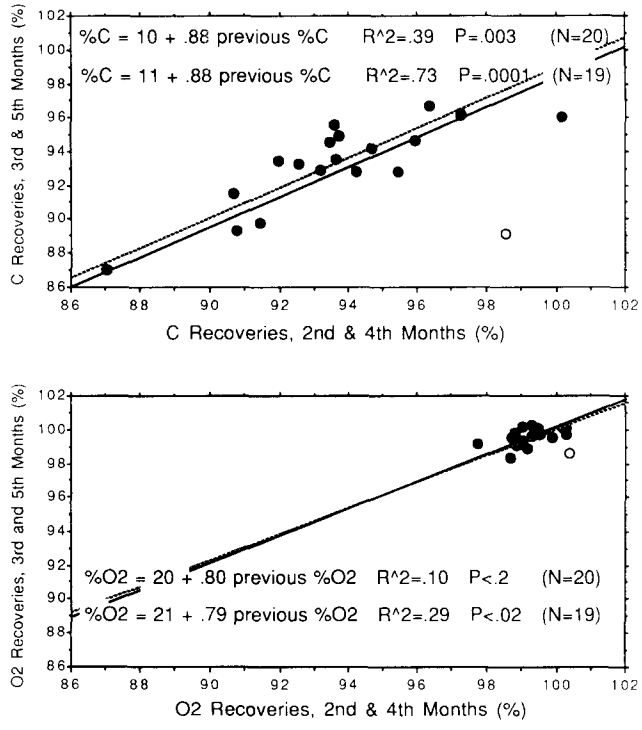


Figure 5 Reproducibility of carbon and oxygen recoveries. The points show the values obtained for 10 female CD1 mice during two consecutive periods while they were on the same diet (i.e., 3rd month versus 2nd month, and 5th month versus 4th month for each animal). Since the numerical values for the abscissa and the ordinate are equally affected by experimental errors, the slope of the correlation was calculated according to Brace.[14] Statistical parameters were calculated on the 20 data pairs (full line), as well as by disregarding one set of values (shown as open circle), which appears to be a manifest outlier in both regressions.

lated by conventional computation of the regression line of Y versus X (in which it is assumed that the X-values are not affected by experimental error) by the value of R. The values of .88 ± .26, or .88 ± .13 (SEM), respectively, are not significantly different from unity. Similarly, the rates of H_2 formation reflected in the ratios of observed to expected O_2 consumption for individual mice were compared during consecutive experimental periods (lower panel of *Figure 5*). In view of the close clustering of these values, individual characteristics were less obvious statistically (R^2 = .10, $P < .2$, or R^2 = .29, $P < .02$ if the outlying value, which came from the same aberrant set, was also disregarded), with slopes[14] of .80 ± .58 or .79 ± .30 (SEM), respectively.

The proposed indirect calorimetry calculations involve the use of two correction factors (i.e., the % of food-Carbon effectively absorbed, and the factor used to add the O_2 needed for the oxidation of H_2), of which the latter is established only after the former has been set. The question thus arises as to whether the ultimate fit of the data may be merely due to the fact that the second factor compensates for any possible errors that may have been committed in establishing the first. To test this possibility, we performed calculations in which the values used for % Carbon effectively absorbed were deliberately taken to be greater or smaller than the value obtained experimentally. For each altered % C recovery value, the correction factor to make the O_2 uptake plus the O_2 needed for the oxidation of the tissue gained equal to the oxygen needed for the oxidation of the nutrients absorbed was recalculated. Daily carbohydrate and fat oxidation rates and balances were then recomputed using these "altered" pairs of correction factors in four mice. As shown in the upper panel of *Figure 6*, the cumulative carbohydrate balances calculated with these pairs of correction factors show gains or losses which exceed the changes in the animals' glycogen content that can reasonably be expected. This is the case even if the C-recovery factor is set to be only 1% above or below the experimentally established value. The cumulated fat balances (lower panel of *Figure 6*) also show that the values most consistent with the observed weight changes are obtained when the experimentally determined C-recovery factor is used. The validity of the corrective procedures used is thus further supported by the fact that their outcome is accurate only for one particular pair of values for the two correction factors, presumably the pair of values closest to reality.

In a subsequent experiment involving 10 male mice, the indirect calorimetry calculations were performed using either the classical (Loewy)[9,10] or the revised calorimetry factors for protein proposed by Livesey and Elia in 1988.[12] As can be seen in *Figure 7* (upper panel), cumulative carbohydrate balances are about 3 times smaller when the revised factors are used, indicating a slight, but definite improvement. The difference is very small in relation to total energy turnover (e.g., about .4% in the animal with the largest body weight change) and its impact on daily carbohydrate

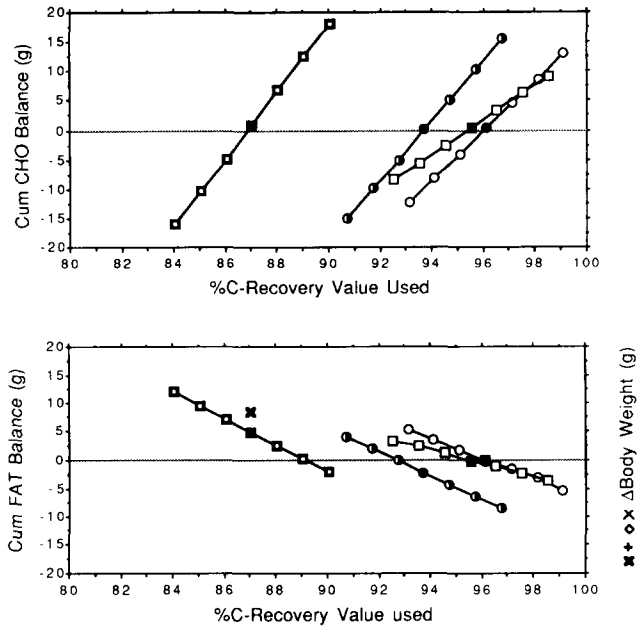


Figure 6 Effect of carbon recovery values on cumulative carbohydrate and fat balances. The full circles and full squares show the values obtained for the cumulative carbohydrate (upper) and fat balances (lower panel) according to the calculations described in the text, for two mice maintained on the high carbohydrate diet with 13% (circles) and two mice maintained on the mixed diet with 45% of dietary energy as fat (squares). After deliberately altering the carbon recovery values by increments or decrements of 1%, the calculations were repeated, yielding the values plotted as open circles and squares. The figure shows that errors in carbon recovery values cannot be compensated by adjustment of the oxygen correction factor in computing cumulative carbohydrate and fat balances. (The crosses in the lower graph show the weight changes observed in each of the four mice during this experimental period).

and fat balances, as well as on the cumulative fat balances (lower panel of *Figure 7*) is too small to be noticeable. The ability of the experiment set-up to detect a minor difference in the coefficients for a nutrient, which provides less than 20% of the diet's energy content, offers still further evidence about the method's accuracy. It also provides a set of biological data supporting the future adoption of the revised protein calorimetry factors proposed by Livesey and Elia.[12]

The experimental procedure was also used to assess nutrient absorption when mice were fed conventional laboratory chow. Ten female CD1 mice subdivided into 2 groups of five alternatively had free access to Purina Lab Chow #5008 or to the synthetic diet high carbohydrate formula which has a similar macronutrient content (*Table 1*). The amount of C effectively absorbed, corrected for changes in body weight, came to 81.9% of the digestible carbon content of the amount of lab chow removed from the food hoppers, whereas the carbon recoveries averaged 94.4% when the same mice were maintained on the synthetic diet (see intercepts in *Figure 8*). These data suggest that the "digestible energy" content of commonly fed lab chows may generally be less than predicted from their composition. In the case of the batch of lab chow

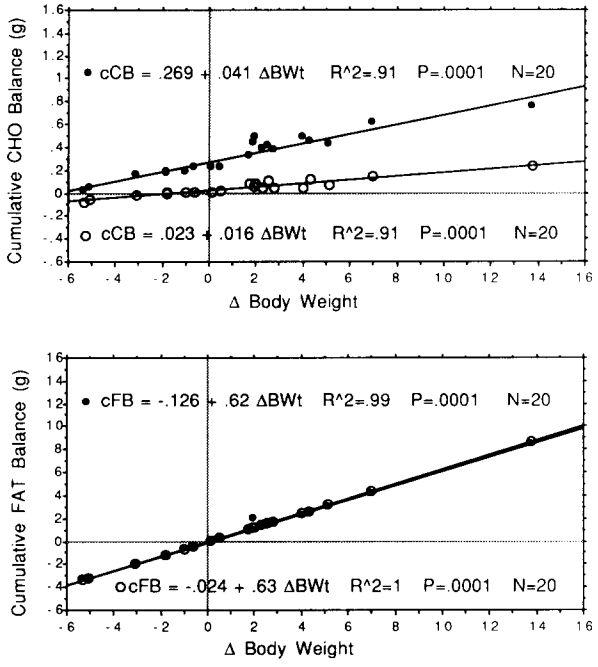


Figure 7 Impact of different calorimetry coefficients for protein on cumulative carbohydrate and fat balances. The data were obtained in 10 male mice during 2 periods of one month of observations on a diet providing 18% of energy as protein, 41% as fat, and 41% as carbohydrate. The full circles show the results of calculations using the conventional coefficients for protein oxidation (4.32 kcal/g; RQ = .80, as proposed by Loewy).[9,10] The open circles show the results obtained when the coefficients for protein oxidation proposed by Livesay and Elia[12] (4.70 kcal/g; RQ = .835) were used instead in the indirect calorimetry computations and in calculating the diets' macronutrient content.

tested here, believed to contain 3.5 kcal of digestible energy per gram, only 2.9 kcal/g of pellets removed from the food hoppers were absorbed effectively, whereas 3.6 of 3.8 kcal/g of synthetic diet removed from the hopper were absorbed.

Discussion

Since carcass analysis obviously could not be used to sequentially establish changes in the body composition of individual mice, the accuracy of the method had to be judged on the basis of the cumulated carbohydrate and cumulated fat balances which it yields. The results of such tests (*Figures 4 to 7*) contribute different lines of evidence to validate the proposed experimental method and the rationale in the calculations serving to compute carbohydrate and fat oxidations. (A large set of data obtained by applying these procedures is presented in a companion paper.)[7] The corrective procedures explained above may be regarded as effecting an "internal calibration," since they serve to make the cumulated gas exchange data consistent with the amounts of nutrients oxidized and stored. This can best be justified when the contribution attributed to changes in body composition is small compared to the overall metabolic exchange (*Figure 3*). This is the case for extended, continuous periods of observation, because some inaccuracy in the evaluation of changes in carcass energy content then will not introduce much error in establishing the correction factors. It should be noted that the food intake and gas exchange data reflect changes in the animals' carbohydrate and fat content, and that the daily carbohydrate and fat balances thus include changes in the intestinal nutrients content. Since the daily measurements are concluded (and initiated) in mid-afternoon, at a time of low feeding activity, intestinal contents are then usually rather small.

In principle, the determination of energy expenditure by indirect calorimetry is based on the measurements of CO_2 production, oxygen consumption, and nitrogen excretion, which together allow calculation of the changes in the body's protein, carbohydrate, and fat content due to metabolism.[10] However, since the rate of protein oxidation is a quantitatively minor and relatively constant process, it may be (and in fact quite often is) assumed to occur at a fixed fraction of total energy expenditure (e.g., 15%). Errors due to such an assumption, as well as errors committed for other reasons in assessing rates of protein oxidation have only a very small impact on the determination of overall rates of energy expenditure.[12] However, errors in assessing protein oxidation have a greater impact in assessing rates of carbohydrate and fat oxidation. Thus, an underestimate of protein oxidation by 1 kcal, leads to an overestimate of carbohydrate and fat oxidation of 1/3 and 2/3 kcal, respectively. It is therefore important to consider the possible consequences of the assumption inherent to our procedure, according to which daily protein oxidation is set to be equal to the amount of protein contained in the food disappeared from the hopper, multiplied by the C recovery factor. To this effect, one may consider the average daily protein, carbohydrate, and fat intakes (*Table 2*) and assume that daily differences between protein oxidation and protein intake vary, for example with a coefficient of variation (CV) of ± 15% relative to aver-

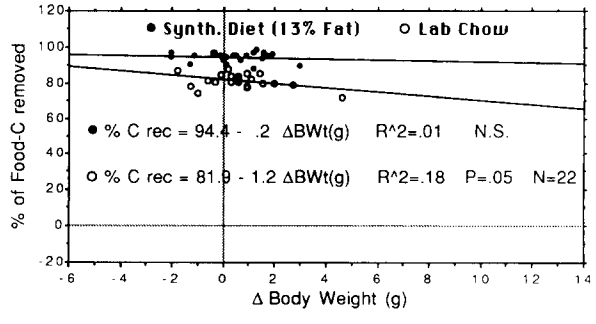


Figure 8 Carbon recoveries on lab chow and synthetic diet. Ratio (as %) of the sum of carbon released as CO_2 plus gained or lost from the carcass, divided by total amount of food-C removed from hoppers. Ten ad libitum fed female CD1 mice were studied during 84 consecutive days; 5 mice were provided initially with Purina Lab Chow #5008, while the other five received a synthetic high carbohydrate formula with a similar macronutrient distribution (cf. *Table 1*). After 50 days, the diets were exchanged for the remainder of the period of observation.

Table 2 Average daily substrate turnover and balances

	High carbohydrate diet kcal/day (CV)	*Mixed diet* kcal/day (CV)
Energy intake	15.3 ± 3.4 (± 22%)	16.8 ± 3.2 (± 19%)
Energy expenditure	15.3 ± 2.0 (± 13%)	16.4 ± 2.0 (± 12%)
Energy expend./g b.wt.	0.40 ± 0.06 (± 16%)	0.39 ± 0.06 (14%)
Energy balance	0.01 ± 3.1 [± 20%][a]	0.4 ± 2.9 [± 18%][a]
Protein intake	2.8 ± 0.6	3.0 ± 0.6
Protein balance[b]	0 ± 0.4 [± 15%][a]	0 ± 0.45 [± 15%][a]
Carbohydrate intake	10.6 ± 2.3	6.2 ± 1.2
Carbohydrate oxidation	10.4 ± 2.1 (± 21%)	6.1 ± 1.2 (± 20%)
Carbohydrate balance	0.03 ± 1.3 [± 12%][a]	0.07 ± 0.9 [± 14%][a]
Error[c]	0 ± 0.14 [± 1.3%][a]	0 ± 0.15 [± 2.5%][a]
Fat intake	2.0 ± 0.4	7.6 ± 1.4
Fat oxidation	2.0 ± 2.5 (± 124%)	7.1 ± 2.0 (28%)
Fat balance	−0.02 ± 2.8 [± 139%][a]	0.36 ± 2.8 [36%][a]
Error[c]	0 ± 0.27 [± 14%][a]	0 ± 0.30 [± 4%][a]

[a] Coefficient of variation (CV) in %, relative to intake. $N = 800$.
[b] Assuming that long-term nitrogen balance is maintained and that differences between protein oxidation and protein intake occur with a CV of ± 15%.
[c] Errors on carbohydrate and fat oxidation due to protein imbalances described above in footnote[b].

age daily protein intake, a CV often observed in studying nutrient requirements. This would introduce a kind of "wobble," but little cumulative error, since the protein content in adult animals is relatively stable. The difference between protein oxidation and protein intake would thus average zero, with a standard deviation of ± .45 kcal/day. The errors introduced thereby into the calculated rates of oxidation of carbohydrates and fats, and in the presumed carbohydrate and fat balances, would thus vary with an SD of about ± .15 and ± .30 kcal/day, respectively. This is only about one-tenth of the standard deviation affecting these balances (*Table 2*). Thus, it can be concluded that the consequences that protein oxidation is equal to protein intake appears to introduce only a small error into the assessment of carbohydrate and fat oxidation rates and balances, compared to the variability introduced by other biological variations and other measurement errors.

In conclusion, it appears that the method described here allows to establish how much carbohydrate and how much fat are oxidized in individual mice during 24 hr periods, with rather high precision and reliability. This is due in part to the fact that gas flow measurements, which are always difficult to achieve with high accuracy, are not required, since the volume of a 55-gallon (220-liter) drum is sufficient to allow the gas exchange of a single mouse to be measured over a whole day without interruption. The accumulation of CO_2 whose partial pressure can reach 2% in the largest and most active animals, appears to be well tolerated. Indeed, we have studied mice for periods of up to 345 consecutive days, during which they kept spontaneously using the running wheel, though with an intensity declining with age. Inherent to this set-up is the fact that the gas exchange can be established without interruption over ≥ 95% of each 24 hr period, so that it can be reliably extrapolated to a full day. Relating changes in P_{O_2} and P_{CO_2} to P_{N_2} provides another substantial advantage, as this avoids the need to make a number of corrections to take into consideration changes in partial pressures caused by variations in atmospheric pressure, temperature, and humidity, and due to the fact that CO_2 generally is not produced in amounts equal to the amounts of oxygen consumed. The procedure in effect provides for an "internal calibration" which permits assessment for each particular mouse as to how much of the nutrients removed from the hopper are effectively absorbed, and taking into account the production of hydrogen by intestinal fermentation. These features show that nutrient absorption is substantially less in animals fed conventional lab chow (82% of predicted digestible energy content) as compared to synthetic diet formulas (94%), and that the revised indirect calorimetry factors for protein proposed by Livesey and Elia[12] are probably more appropriate than those of Loewy[9] which are commonly used.[10] Furthermore, the measurements can be pursued conveniently using several drums in parallel over many consecutive days.

The procedure allows monitoring of daily carbohydrate and fat balances in small laboratory animals, in which spontaneous adjustments of food intake and energy expenditure can be studied. To our knowledge, data on daily carbohydrate and fat balances have been reported only in a few instances for human subjects studied in respiratory chambers for single days[15] or periods of up to ten days.[16] The experimental set-up thus offers special advantages in studying some of the metabolic phenomena by which weight maintenance may be brought about. The importance of being able to consider separately carbohydrate and fat metabolism is highlighted by the finding that carbohydrate oxidation is correlated positively with daily variations

in food intake, whereas fat oxidation is negatively correlated with variations in food intake.

Acknowledgments

The collaboration of K.E.G. Sargent and B.T. Krauss is gratefully acknowledged. Support for these studies was provided by NIH Grant DK 33214.

References

1 Mayer, J. and Thomas, D.W. (1967). Regulation of food intake and obesity. *Science* **156,** 328–337.
2 Bray, G.A. (1987). Obesity: A disease of nutrient or energy balance? *Nutrition Reviews* **45,** 33–43.
3 Sims, E.A.H. and Danforth, E. Jr. (1987). Expenditure and storage of energy in man. *J. Clin. Invest.* **79,** 1019–1025.
4 Stunkard, A.J. (1988). Some perspectives on human obesity: Its causes and treatment. *Bull. NY Acad. Sci.* **64,** 902–940.
5 Flatt, J.P. (1988). Importance of nutrient balance in body weight regulation. *Diabetes/ Met. Rev.* **4,** 571–581.
6 Flatt, J.P. (1987). Dietary fat, carbohydrate balance, and weight maintenance; effects of exercise. *Am. J. Clin. Nutr.* **45,** 296–306.
7 Flatt, J.P. (1991). Opposite effects of variations in food intake on carbohydrate and fat oxidation in ad libitum fed mice. *J. Nutr. Biochem.* **2,** 186–192.
8 Manual No. 147 (1978). Parr Instrument Co., Moline, IL.
9 Loewy, A. (1911). In *Oppenheimer's Handbook der Biochemie*, Jena **4,** 1.
10 Lusk, G. (1976). *Science of Nutrition*, p. 68, Johnson Reprint Corporation, New York.
11 Elia, M., McDonald, T., and Crisp, A. (1986). Errors in the measurement of CO_2 with the use of drying agents. *Clin. Chim. Acta*, **158,** 237–244.
12 Livesey, G. and Elia, M. (1988). The estimation of energy expenditure, net carbohydrate utilization and net fat oxidation and synthesis by indirect calorimetry: Evaluation of some errors with special reference to the detailed composition of fuels. *Am. J. Clin. Nutr.* **47,** 608–628.
13 Atwater, W.O. and Bryand, A.P. (1980). *Connecticut (Storrs) Agricultural Experiment Station*, pp. 73–123, 12th Annual Report, Storrs, CT.
14 Brace, R.A. (1977). Fitting straight lines to experimental data. *Am. J. Physiol.* **233,** R94–R99.
15 Abbott, W.G.H., Howard, B.V., Christin, L., Freymond, D., Lillioja, S., Boyce, V.L., Anderson, T.E., Bogardus, C., and Ravussin, E. (1988). Short-term energy balance: relationship with protein, carbohydrate, and fat balances. *Am. J. Physiol.* **255,** E332–E337.
16 Acheson, K.J., Schütz, Y., Bessard, T., Anantharaman, K., Flatt, J.P., and Jéquier, E. (1988). Glycogen storage capacity and de novo lipogenesis during massive carbohydrate overfeeding in man. *Am. J. Clin. Nutr.* **48,** 240–247.