Estimating energy expenditure from specific activity of urine urea during lengthy subcutaneous $NaH^{14}CO_3$ infusion

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Elia, M., M. G. Jones, G. Jennings, S. D. Poppitt, N. J. Fuller, P. R. Murgatroyd, and S. A. Jebb. Estimating energy expenditure from specific activity of urine urea during lengthy subcutaneous $NaH^{14}CO_3$ infusion. *Am. J. Physiol.* 269 (*Endocrinol. Metab.* 32): E172–E182, 1995.—Five healthy male subjects were continuously infused subcutaneously with [^{14}C]bicarbonate (12.3 μCi/day) using a mini pump for 5 days while in a whole body calorimeter. Energy expenditure was varied over a range of 1.35–1.75 times basal metabolic rate. Urine collections were obtained throughout the study and used to measure the specific activity of urea, from which CO_2 production was estimated. It was assumed that the recovery of label in gaseous CO_2 was 95% of that infused and that the specific activity of urea was 85% that of expired CO_2. Continuous daily collections of calorimeter air revealed that 95.6 ± 1.3% (SD) of infused label was recovered as gaseous CO_2, with little daily variation. Another 1.5 ± 0.4% was recovered as urinary urea. The estimated CO_2 production, calculated from the specific activity of urea in 24-h urine samples corrected for the small effects due to changes in the size and specific activity of the urea pool, was found to be 100 ± 5% of the calorimeter estimate for 1-day periods (20.80 ± 1.44 mol CO_2/day) and 100 ± 2% for 4-day periods. This study suggests that, in healthy subjects, the labeled [^{14}C]bicarbonate-urea method can provide reasonable estimates of net CO_2 production over the range examined.

carbon dioxide production; free living; energy expenditure; urea

THE LABELED BICARBONATE METHOD for estimating CO_2 turnover and energy expenditure has its origins in the late 1960s and early 1970s (5, 31, 32, 34–36). It is essentially an isotopic dilution technique, whereby an administered dose of labeled CO_2 (given as bicarbonate) is diluted by CO_2 produced endogenously by the body. The extent of this isotope dilution can be used to measure the rate of CO_2 production, particularly if the recovery of infused label in the gaseous CO_2 lost from the body is predictable and shows little variation between individuals. Although various short-term studies (2–12 h) have demonstrated considerable variation in the recovery of label in expired air (see Refs. 9, 14, 37), longer-term studies have shown considerably less variability, e.g., 94–97% in one of our studies undertaken in a whole body calorimeter (11). The specific activity or enrichment of multiple spot breath samples (9, 11, 13, 14) has been used to predict CO_2 production, but there may be important practical limitations because of the need to make measurements as frequently as once or twice each hour during waking hours and the lack of samples during sleep. Samples of blood and saliva have the same limitation as spot breath samples, but there are additional problems: the specific activity of acid-labile CO_2 in saliva is variable and generally lower than that in arterialized blood or CO_2 in expired breath, possibly because of isotopic dilution by locally produced CO_2 (11), and repeated blood samples may have to be taken in the restricted environment of the hospital.

Because urine is filtered continuously, a single urine collection (e.g., over 24 h including sleep) may provide a more convenient and perhaps a better estimate of the mean specific activity of CO_2 over the collection period than "spot" samples of other physiological fluids, especially spot [^{14}C]breath samples that have to be bubbled through a CO_2 trapping agent with an indicator (11). However, the rate of excretion of acid-labile CO_2 in urine can be very variable, depending on pH and urine volume. This means that samples of urine containing large amounts of acid-labile CO_2 will have a disproportionately large effect on the overall specific activity of urinary CO_2 and may produce a concomitant error in the estimated CO_2 production by the body. In addition, labeled CO_2 rapidly passes through the bladder wall (11) and almost certainly exchanges with CO_2 in blood and surrounding tissues. Therefore the specific activity of urinary acid-labile CO_2 may be modified after formation of urine (especially when there is a large gradient between the specific activity of CO_2 in blood and urine), and this could affect the estimation of CO_2 production by the body with this technique. A further problem concerns the presence of only small amounts of acid-labile $^{14}CO_2$ in acid urine, which causes practical difficulties in the making of accurate measurements, especially if there is further loss or isotopic exchange with atmospheric CO_2.

We have previously suggested a novel alternative approach in which CO_2 production is calculated from the specific activity of urinary urea, which is formed from CO_2 (11). Urea is abundant in urine, and its excretion is usually much more constant than that of bicarbonate. Furthermore, even if there were some transfer of urea across the bladder wall, this would be likely to have little effect on the results for at least two reasons. First, the slow turnover of the urea pool relative to that of the circulating pool of CO_2 means that the blood/urine gradient of urea specific activity is likely to be relatively small. Second, the concentration of urea in urine is usually so much higher than that in blood that any transfer of urea from blood to urine through the bladder is likely to be small compared with the amount already present in the bladder. Furthermore, urea is almost exclusively excreted in urine. By measurement of the

size and specific activity of the urea pool at the beginning and end of the experiment, appropriate correction factors can be generated. However, this approach is more indirect, because the specific activity of urea is used to predict that of expired CO_2, which is in turn used to predict net CO_2 production. Previous observations suggested that the specific activity of urea is likely to be ~15% lower than that of CO_2 in arterial blood or breath (15). This is due to isotopic dilution of CO_2 in splanchnic tissues and the extent of urea formation from unlabeled arginine derived from the diet and protein breakdown.

The purpose of the present study was to undertake the first prospective study of this new bicarbonate-urea method to assess its value for estimating CO_2 production and energy expenditure in subjects who are undertaking variable physical activity over several days in a whole body calorimeter. The study also aimed to obtain information about bicarbonate kinetics over extended periods of time by use of the subcutaneous route of infusion, which, as far as we are aware, has not been previously employed in humans. The use of a portable mini pump to infuse labeled bicarbonate subcutaneously could make this technique feasible in free-living situations.

METHODS

Subjects

The subjects were five healthy males with a mean age of 34 ± 10 yr, mean weight of 70.8 ± 5.2 kg, mean height of 1.765 ± 0.018 m, and a body mass index (BMI) between 20 and 25 kg/m^2. Body composition, assessed using dual-energy X-ray absorptiometry (DXA; Hologic, QDR-1000W enhanced body composition analysis), gave values of 14.4 ± 4.5% fat, whereas skinfold thicknesses (7) gave values of 17.0 ± 4.2% fat.

None of the subjects was taking medications, none had a relevant past medical history, and none was a smoker. They were all weight stable, and none had changed normal dietary habits in the few days before the study.

Protocol: Procedures Before Entering the Calorimeter

Obtaining physiological fluids. On the morning of the study the subjects had a sample of venous blood taken for the measurement of plasma urea concentration, which was used to calculate the priming dose of labeled urea (see *Calculating the bolus dose of [^{14}C]urea*). Plasma bicarbonate and blood hematocrit were also measured. The subjects emptied their bladders shortly before the start of the bicarbonate infusion and at the same time provided a urine sample for the measurement of basal specific activity of provided urinary urea. A spot breath sample was also collected (see below) for the estimation of background specific activity of expired CO_2.

Measuring the radioactivity in the bicarbonate solution, inserting the subcutaneous cannula, and starting the infusion. Three accurately weighed samples (~0.25 g) of [^{14}C]bicarbonate (CFA3, Amersham International, Little Chalfont, Bucks, UK) were taken for scintillation counting from the end of the administration set before it was connected to the cannula (see below for procedure). A further set of samples was taken at the end of the study in a similar way, as well as from the stock solution and syringe.

Under aseptic conditions, a cannula (model 22G, Wallace, Colchester, UK) was inserted subcutaneously under a moderate amount of subcutaneous adipose tissue (to minimize possible transcutaneous loss of label), and a transparent plastic adhesive dressing was used to secure the cannula and part of the extensible rigid spiral extension tube that delivered to the subject an alkaline solution of sterile pyrogen-free labeled bicarbonate-carbonate solution (pH 10.0–10.5; 18.95 × 106 dpm/g; 300 mosmol/l) from a volumetric mini pump (1.44 g solution 12.3 μCi/day in small pulses, one every 18 min). The total amount of solution infused during the study was calculated both volumetrically and gravimetrically. Preliminary studies had established that there was essentially no loss of label through the syringe and administration set (closed system) over 5 days.

A priming dose of urea [0.6–1.2 ml of saline (9 g/l) containing urea at a concentration of 5 mmol/l and 0.32 × 106 dpm/ml; 29952–6, Sigma Chemical, Poole, Dorset, UK] was administered through the cannula immediately before it was connected to the bicarbonate solution. The precise amount given was calculated as indicated in *Calculating the bolus dose of [^{14}C]urea.*

The total whole body equivalent radiation exposure, calculated using the bicarbonate kinetics observed in this study, was estimated to be ≤1 day's natural background radiation.

Protocol: Procedures After Entering the Calorimeter

Diet. Subjects received a diet providing a daily energy content of 1.5 × basal metabolic rate (BMR) as predicted from the Schofield equations (27), containing 47% of its energy from carbohydrate, 40% from fat, and 13% from protein (23). The food was consumed at breakfast (0930–1000), lunch (1300–1330), and dinner (1930–2000) in approximately isoenergetic amounts. Although any food not eaten was weighed and taken into account when the total energy intake was calculated, it was found to be negligible. The total mean energy intake was 10.9 ± 0.6 MJ/day, with essentially no variation from day to day.

BMR. Measurements of BMR were carried out between 0800 and 0900 on *days 1–4* after an overnight fast.

Exercise. No formal exercise was undertaken on *day 0*, but on *days 1–4* the subjects exercised on a bicycle ergometer for 40 min three times daily (1110–1150, 1510–1550, and 1710–1750). During *days 1* and *2* the subjects exercised at either 25 W (low exercise) or 75 W (high exercise), and during the subsequent 2 days (*days 3* and *4*) the level of exercise was reversed. The order of the two high and two low exercise days was randomized (three subjects undertook the high exercise first (*days 1* and *2*), and two subjects undertook the low exercise first).

Body weight. Body weight was measured daily (shortly after 0900) by use of an electronic balance (sensitive to 0.001 kg) kept within the calorimeter.

Urine collection. On *day 0* urine specimens were collected every 2 h between 0900 and 2100. Subjects were encouraged to drink 150 ml of water every hour to ensure an adequate urine flow during each 2-h period. A separate urine collection was obtained between 2100 (*day 0*) and 0700 (*day 1*). On subsequent days urine collections were obtained between 0900 and 2100, 2100 and 2300, 2300 and 0700, and 0700 and 0900.

Specific activity of breath samples. On *days 0, 2,* and *4,* measurements of the specific activity of end-expiratory CO_2 were made at hourly intervals between 0900 and 2300. However, they were taken 10 min before the hour (1150, 1550, and 1750) immediately after the exercise periods on *days 2* and *4*. Expired CO_2 was passed through a calcium chloride drying column (mesh 8–16 and fused granules 1–2 mm, Fisons Scientific Equipment, Loughborough, UK) and then trapped in an accurately weighed solution of hyamine hydroxide 10

times (600 3005, Canberra Packard, Pangbourne, Berks, UK) in methanol of known titratable molarity (typically 6.0- to 6.5-g solution, 0.5 mol/kg).

Blood samples. Samples of finger tip capillary blood were obtained using the Lancet autolet package (Owens Mumford, Medical Division, Brook Hill, Woodstock, Oxon, UK) and collected in microcapillary heparinized tubes (17458, Sarstedt, Leicester, UK) to measure urea concentration. A separate venous blood sample was taken at 0900 on *day 5*. This blood sample was subjected to the same analyses as the venous blood sample obtained on *day 0* (urea, bicarbonate, and hematocrit).

Trapping of labeled and unlabeled CO_2 leaving the calorimeter. CO_2 leaving the calorimeter was trapped as described previously (15) except that the two cylinders in series contained a higher concentration of hyamine in methanol (initially 0.5 mol/kg; ~200 g in the first cylinder and ~100 g in the second). The specific activity of calorimeter air was also measured at 0900 and 2100 by passing calorimeter air through a vial containing hyamine-methanol-phenalphthalein.

Measurement of CO_2 production and O_2 consumption. The calorimeter (capacity of 28 m^3), which was ventilated at a rate of 200 l/min, provided measurements of CO_2 and O_2 concentration of ingoing and outgoing air (22). Gaseous exchange was calculated by the method of Brown et al. (4), which yields a fast measurement response time. Details of the procedures for the standardization and calibration of gases are given elsewhere (11). The linearity of the analyzers with increasing concentration of gases (O_2 and CO_2) was also assessed and taken into account when gaseous exchange was calculated.

Biochemical procedures. Urine nitrogen was measured by the Kjeldahl technique and creatinine by the Jaffé method. Urinary urea concentration was assayed by an end-point colorimetric method that measured the increment ammonia concentration associated with the enzymatic hydrolysis of urea (124770, Boehringer Mannheim, Lewes, East Sussex, UK). Plasma urea was measured by a kinetic method (071098.9, Unikit III, Urea UV; Roche Diagnostics, Welwyn Garden City, Herts, UK) that is more rapid than the colorimetric method. Circulating bicarbonate was measured by both a colorimetric (11) and an enzymatic method (CO_2 130-UV, Sigma Chemical). Urine pH was measured with a standard pH meter.

Titration of hyamine-methanol solution. Details of the procedure of titration [with 0.2 N HCl (Analar Grade, measured density 1.001 g/l); 19070, British Drug House (Suppliers) Merck, Lutterworth, UK] of an accurately weighed aliquot of the hyamine-methanol solution (~4 g of ~1 M solution), to which ~20 ml cold methanol were added, are given elsewhere (11).

Measuring the specific activity of urinary urea. The volume of urine containing ~12.5 mmol urea was added to a 500-ml conical flask containing 100 ml of 1 M citrate buffer, pH 5.6. Variable amounts of distilled water were added to achieve a constant volume of 300 ml. An airtight screw top was then firmly fixed while the side arm of the flask was connected to a vacuum pump for 60–90 min to remove acid-labile CO_2 (preliminary studies showed that essentially all [^{14}C]bicarbonate added to urine was removed by this procedure). The pump was then disconnected.

A scintillation vial containing an accurately weighed amount of previously titrated solution of hyamine hydroxide (~5 ml 1 M solution)-methanol-phenolphthalein was suspended into the flask on a specially constructed wire platform that was attached to the cap of the flask.

A suspension (2 ml) of urease in water (1,000 μm/ml; urease powder from Jackbeans, U1500, obtained from Sigma Chemical) was added to the flask, which was then tightly sealed and placed on a horizontal orbital mixer. When the hyamine-methanol-phenolphthalein solution decolorized, after ~70–90 min, the scintillation vial was removed. Hyamine hydroxide and scintillant were then added, as described below.

Procedure for scintillation counting. Hyamine hydroxide 10× was added to the scintillation vials that had already trapped CO_2 to ensure that they contained 2.5 mmol of titratable hyamine hydroxide. When 7.0 ml of scintillant (Hionic Fluor; 601339, Canberra Packard) were added, the final pH was 10. There was undetectable loss of label from the vial over 1–2 days and only 1.0–1.5% over 4 mo. The background radioactivity, which was subtracted from the radioactivity of all test samples, was low [≤ 100 disintegrations $\cdot$ min^{-1} (dpm) $\cdot$ $vial^{-1}$] because of the use of high-quality scintillant and hyamine hydroxide.

Calculating the bolus dose of [^{14}C]urea. The bolus dose of urea was calculated using the following equation: priming dose of urea (dpm) = urea pool size (mmol) × specific activity of urea at near equilibrium, where the pool size was calculated from the product of the urea distribution volume [in liters, with the assumption that it is the same as that for water (3); see Ref. 29 for estimating body water from weight, height, and age] and the plasma urea concentration (mmol/l)/0.93. The factor 0.93 is the proportion of water in plasma (found to be 0.93 ± 0.005 after freeze-drying the plasma of our subjects). The specific activity of urea at near equilibrium was thus calculated as [^{14}C]bicarbonate infused/day × 0.95 × 0.85/CO_2 produced, where CO_2 produced (mol/day) was assumed to be 1.35 BMR (MJ)/0.535 (MJ/mol). In this last calculation it was assumed that energy expenditure on *day 0*, which was a sedentary day, was 1.35 × BMR as predicted by the Schofield equations (27) (actual value was found later to be 1.26 ± 0.04 measured BMR). The equation also assumes that the recovery of label in gaseous CO_2 was 0.95 of that infused (11) and that the specific activity of urea was ~0.85 that of CO_2 in arterialized blood or breath [close to the values of 0.84 (15) and 0.83 (16) found previously]. It was also assumed that the energy equivalent of CO_2 was 0.535 MJ/mol, which applies to subjects close to nutrient balance while ingesting a Western type diet with a food quotient of ~0.85 (8, 10, 12). It is appreciated that some of these assumptions, especially the estimated CO_2 production rate, are very approximate, but the priming dose only aimed to achieve a value for urea specific activity that was not very different from the near steady-state value obtained during that day and to attain a near-steady state value earlier than if a priming dose had not been given.

Calculation of energy expenditure (EE) from measurement of O_2 consumption and CO_2 production. Energy expenditure was calculated using the equation of Elia and Livesey (12): EE (kJ) = 15.818 O_2 + 5.176 CO_2, where O_2 and CO_2 are in liters at standard temperature and pressure (STP).

Recovery of labeled bicarbonate as gaseous $^{14}CO_2$. The amount of label recovered as gaseous CO_2 over a 12-h period was estimated as the product of the label trapped by the hyamine in the cylinder (continuous collection) and the flow ratio [flow through the calorimeter (STP) to flow through the cylinder hyamine (STP)]. Any accumulation or loss of radioactivity in the air of the calorimeter chamber (product of the specific activity and quantity of CO_2 in the calorimeter air) was taken into account when recovery of infused label between 12- or 24-h measurement periods was calculated.

The hourly recovery of gaseous CO_2 on *day 0* was calculated from the product of the hourly CO_2 production rate and the specific activity of breath CO_2, which was sampled hourly.

Calculation of CO_2 production and energy expenditure from the specific activity (SA) of urinary urea. CO_2 production was calculated according to the following equations

Method 1)

$$CO_2 \text{ production (mol/day)} = \frac{0.95 \times 0.85 \times \text{infused dpm/day}}{\text{SA of urea (dpm/mol)}}$$

In this equation the specific activity of urea was calculated in two ways: *A*) specific activity of a single 24-h urine sample (equivalent to pooling all the urine samples), i.e., total 24-h urinary urea divided into the total 24-h label in urea; *B*) mean of the specific activities of two 12-h urine samples (total label in urea/total urea excreted)

Method 2) CO_2 production (mol/day) = 0.95

$$\times 0.85 \left[\frac{\text{infused dpm (}\textit{period 1}\text{)}}{SA_1(\text{dpm/mol})} + \frac{\text{infused dpm (}\textit{period 2}\text{)}}{SA_2(\text{dpm/mol})}\right]$$

where SA_1 and SA_2 are the values for specific activity obtained during periods 1, 2, and so on. This equation employed data from *A*) two 12-h periods (total urea excreted over 12 h/total amount of label in this urea), and *B*) all individual urine collections obtained during the day, which were not of equal duration.

These different methods have individual advantages and disadvantages (see Ref. 11), but because, as expected, the rate of urinary urea excretion and its specific activity showed no major change during the 24-h period, the results obtained by these methods were very similar.

A small correction to take into account the changes in the size and specific activity of the urea pool from one day to the next (0900–0900) was also made on the basis of plasma urea concentration and estimates of the distribution volume of urea (see *Calculating the bolus dose [^{14}C]urea*) and its specific activity, which was assumed to be similar to that obtained in the 2-h urine samples (0700–0900). Thus changes in the amount of urea in the pool were added to (when there was accumulation) or subtracted from (when there was loss) the amount of urea excreted in urine. Similarly, the change in the amount of label in the urea pool was added to (when there was accumulation) or subtracted from (when there was loss) the amount of label in the urea that was excreted in urine. In practice this correction was small and, in general, made $\leq 1\%$ difference in the estimated net CO_2 production by individual subjects.

After bolus injections of labeled urea into the blood, there is a "urinary delay time" of 0–1.5 h in normal subjects (28) before the urea specific activity in urine matches that in blood. This is at least partly due to the time taken for filtered urine to enter and leave the bladder, especially when there is residual urine in the bladder after voiding. Some compartmentation of [^{14}C]urea within the kidney cannot be excluded, but in the present experiment, it is the changes in the near steady state of urea specific activity (2-h urine samples) that were being sought, so that small corrections in energy expenditure could be made over 24-h periods (see RESULTS).

To calculate energy expenditure from net CO_2 production, it was assumed that the energy equivalent of CO_2 was 535 kJ/mol CO_2 (see above).

Assessing transcutaneous loss of $^{14}CO_2$ close to the infusion site. A continuous 6-h subcutaneous infusion of [^{14}C]bicarbonate ($\sim 18.95 \times 10^6$ dpm/g; 1.3 mg/min) was administered into the abdomen of five healthy subjects and subcutaneously into the upper chest of another five subjects, according to methods described above. A thick plastic nonadhesive sheet (150 cm^2) was taped to the skin to cover the cannulated area, which was ventilated with air at a rate of 1 l/min. Labeled CO_2 leaving this area was then passed through sintered glass and trapped in hyamine.

CO_2 production was measured using a ventilated hood system. Measurements were made for 30 min at a time and repeated throughout the study, with 15-min intervals between measurements. The subjects were not prevented from moving during any part of the study. The movements were minor and were similar during and between the hood measurements. Spot breath tests for measuring the specific activity of end-expiratory CO_2 were undertaken before and after each hood measurement. The recovery of $^{14}CO_2$ in breath was calculated as the product of specific activity of end-expiratory CO_2 and CO_2 production rate and was expressed as a percentage of the amount infused.

Statistics

Results are expressed as means ± SD. Comparison between methods was made using the bias (mean difference) and standard deviation of the difference (see Ref. 2). Some of the paired data were also analyzed using Student's *t*-test.

Ethical Approval

Ethical approval was obtained from the local ethics committee, and subjects gave their informed written consent.

RESULTS

Body Weight and Energy Balance

During the period of the study, early morning body weight remained remarkably stable, with mean values at the beginning of *days 0, 1, 2, 3, 4, 5*, and *6* of 70.8, 70.9, 71.0, 71.1, 71.1, 71.0, and 71.1 kg.

Energy balance on *days 1–4* was close to zero [−0.36 ± 0.7 MJ/day; energy intake, 10.9 ± 0.6 MJ/day; energy expenditure, 11.3 ± 0.7 MJ/day; with an associated respiratory quotient (RQ) of 0.831 ± 0.010]. The subjects were in a small positive energy balance during the two low-exercise days (0.44 ± 0.38 MJ/day) and in negative energy balance on the two high-exercise days (−1.15 ± 0.49 MJ/day). The energy balance on *day 0* was +2.0 ± 0.9 MJ. The physical activity level, or ratio of total energy expenditure to BMR (PAL), was 1.26 ± 0.04 on *day 0*, 1.67 ± 0.06 on the high-exercise days, and 1.46 ± 0.08 on the low-exercise days.

Nitrogen balance (assuming 1.5 g of N loss per day via feces and skin) was close to zero (0.9 ± 1.5 g N/day between *days 1* and *4*). Urea, ammonia, and creatinine were present in urine in the nitrogenous ratio of 89:5:6, respectively, and together they accounted for 97% of total urinary N.

CO_2 Measurements

The amount of CO_2 leaving the calorimeter, as measured by the infrared analyzer, agreed closely with that measured by titration (infrared-titration method = 0.15 ± 0.15 mol for 12-h samples; titration value × 100/infrared value = 99.0 ± 1.2%).

Dose of Label Administered

The dose of radioactivity administered daily was 12.32 ± 0.11 μCi/day (27.354 ± 0.240 × 10^6 dpm/day).

The measured radioactive content of the solution was 18.938 ± 0.081 × 10^6 dpm/g at the beginning of the study and 18.968 ± 0.098 × 106 dpm/g at the end of the study (end value, 100.16 ± 0.16% of initial value). Preliminary studies had established that solutions of a lower pH (pH ~7–8) were associated with some loss of label through the tubing/syringe. The infusion of the more alkaline solution in this study (~60 μl/h) did not produce visible signs of a local inflammatory reaction.

Recovery of Label

The mean daily recovery of label in gaseous CO_2 (continuous collection method) between *days 1* and *4* was 94.8 ± 1.6, 95.8 ± 0.9, 95.6 ± 0.7, and 96.2 ± 1.3%, respectively, (mean 95.6 ± 1.3%). The 24-h recovery of labeled CO_2 was essentially identical during days of high activity and low activity (95.7 ± 1.3 vs. 95.5 ± 1.3%, respectively). The additional recovery in urinary urea increased the total recovery by 1.5 + 0.4% to produce mean values of 96.4, 97.2, 97.1, and 97.8 on *days 1–4*, respectively. The lowest individual recovery of label in gaseous CO_2 over the 4 days was 93.3, and the highest was 97.9%. The calculation took into account any accumulation or loss of label from the calorimeter chamber between the beginning and end of each daily period (0900–0900). However, the corrections were small and made <0.5% difference to the result in 14 of the 20 individual 24-h periods (*days 1–4*). Urinary acid-labile CO_2 was not measured, but the amount present in urine with a pH 5.8–7.3 is estimated to make <0.5% difference to the recovery of label (11).

On *day 0*, the hourly recoveries of CO_2 (subcutaneous infusion) were found to be similar to those obtained during an intravenous infusion under similar conditions in an earlier study (see Ref. 11 and Fig. 3). The total amount recovered between 0 and 12 h was found to be 83 ± 3% by the continuous collection method after correction for the quantity of label that accumulated in the calorimeter air.

The amount of radioactivity recovered (13.67 ± 0.12 × 10^6 dpm infused) between 0900 and 2100 on *days 2* and *4* was 13.9 ± 0.5 × 10^6 dpm by the continuous collection method, again after allowances had been made for changes in the quantity of label in the calorimeter air between 0900 and 2100. The corresponding values by the multiple spot breath sample method were 14.6 ± 1.1 × 10^6 dpm (use of mean specific activity between hourly measurements) and 13.4 ± 0.8 × 10^6 dpm (use of specific activity obtained at the end of each hour).

Recovery of label during the entire exercise period is difficult to assess, because the only spot values of specific activity of breath CO_2 were obtained before and immediately after the exercise. Nevertheless, the lowest limit for the recovery (11) can be calculated by multiplying the CO_2 production rate with the lowest specific activity that was obtained at the end of exercise (~140% for the hour that included the high-intensity exercise). In the hour after the high-intensity exercise there was a compensatory low recovery (~70%). The fluctuations produced by the low-intensity exercise followed the same pattern but were less marked.

Continuous collection of label also suggested significantly higher recoveries ($P < 0.001$) of label during the day (0900–2300) than during the night (2300–0900) (4.4 ± 1.1% above and below the mean value for all the 24-h periods). This difference was associated with a change in the specific activity of expired CO_2 (which reflects the specific activity of the rapidly turning over pool of CO_2 at 0900 and 2300 (1,870 ± 145 dpm/mmol at 0900 and 1,100 ± 103 dpm/mmol at 2300). The amount of label retained in the body between 2300 and 0900 was estimated to be 1.2 ± 0.3 × 10^6 dpm (mean of all days between *days 1* and *4*). The recovery of $^{14}CO_2$ during the day (0900–2100), estimated from the product of hourly CO_2 production, and the specific activity of CO_2, obtained at the end of each hour, was 101 ± 6% (and as much as ~10% higher when the mean specific activity between hourly periods was used).

Diurnal Changes in the Specific Activity of CO_2 and Urea

The specific activity of expired CO_2 varied inversely with CO_2 production. Figure 1 shows such changes for the high-exercise day. During exercise, when CO_2 production was high, the specific activity of CO_2 rapidly decreased but rose again between exercise periods. The highest recorded values for the specific activity was at 0900 after a period of sleep and BMR measurement. The diurnal changes during the low-exercise day followed a similar pattern, but, as expected, the changes produced by the exercise were less marked (data not shown).

The specific activity of urinary urea fluctuated much less than that of CO_2. The values in urine samples obtained between 0700 and 0900 were consistently higher than those obtained between 1900 and 2100, by a mean of 20 ± 7% (by 17 ± 6% on the low-exercise days, and by 23 ± 6% on the high-exercise days). The circulating urea concentration remained stable during the period of study, with the following mean values on consecutive days: 4.5, 4.4, 4.4, 4.5, and 4.6 mmol/l (SD 0.35–0.70). The plasma bicarbonate concentrations at the beginning and end of the study were similar, 23.6 ± 2.0 vs. 23.4 ± 3.1 mmol/l, respectively (equivalent values for whole blood, 19.4 ± 1.7 mmol/l vs. 19.4 ± 1.9 mmol/l). The hydration of plasma and whole blood was found to be 93.0 ± 0.5 and 80.8 ± 0.8%, respectively, with no change between the beginning and the end of the study. The hematocrit also remained unchanged (46 ± 1%).

There was little change in the specific activity of urea in urinary samples obtained between 0700 and 0900 at the end of *days 1–4* of the study. Furthermore, the values obtained at the end of the high-exercise days (1,162 ± 94 dpm/mmol) were similar to those obtained at the end of the low-exercise days (1,207 ± 105 dpm/mmol). The specific activities of expired CO_2 obtained at 0900 on different days were also similar: 1,903 ± 87, 1,883 ± 94, 1,856 ± 141, 1,858 ± 141, 1,836 ± 197, and 1,912 ± 142 dpm/mmol on *days 1, 2, 3, 4, 5,* and *6*, respectively. The intraindividual within-day coefficient of variation in the specific activity of urinary urea (all samples) was 7.5 ± 2% (6.6 ± 2.0% on

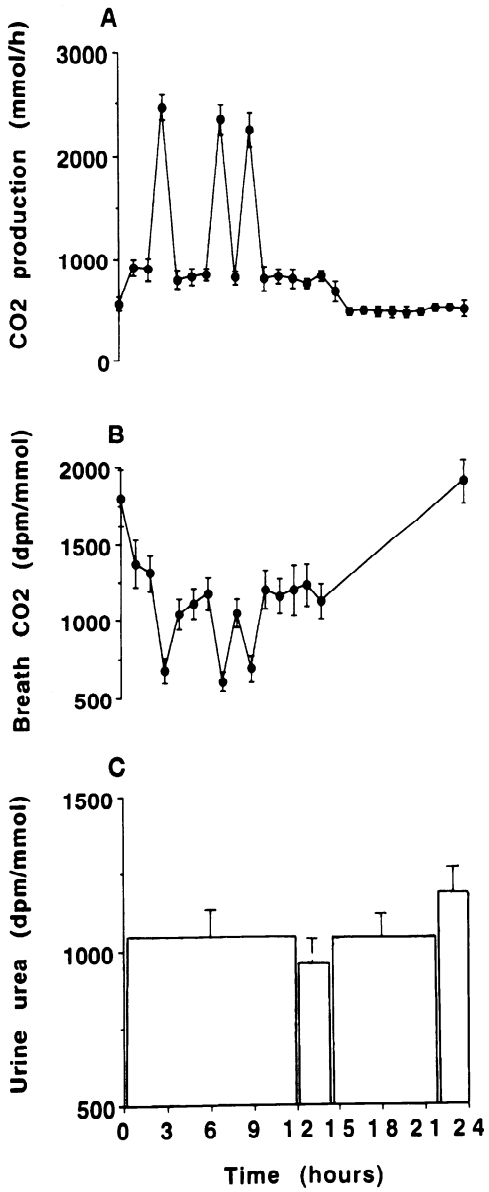

Fig. 1. Relationship between CO_2 production (*A*) and the specific activities (SAs) of $^{14}CO_2$ (*B*) and [^{14}C]urea (*C*) during a 24-h period (day of high-intensity exercise). The three peaks of CO_2 production (troughs in SA) are the result of exercise on a bicycle. The highest SAs of CO_2 (0900) occur during periods of inactivity [sleeping and resting, measurement of basal metabolic rate (BMR)]. *Time 0* on graph represents 0900.

low-activity days and 8.5 ± 1.6% on high-activity days). The intraindividual within-day coefficient of variation was 4 ± 2%.

On *day 0*, the specific activity of urea in two hourly urine samples showed little variation after the priming dose (Fig. 2); given its half-life (~10 h) (21), substantial changes should have occurred if the dose of label administered had been grossly inappropriate. The increase observed after an overnight period of sleep (measured between 0700 and 0900 the next morning) was typical of the pattern observed on other days. The recoveries of gaseous $^{14}CO_2$ during the same period were similar to those found previously (11): 56 ± 20% between 0 and 3 h, 81 ± 8% between 3 and 6 h, 88 ± 5% between 6 and 9 h, and 91 ± 4% between 9 and 12 h. Although in this study we measured the urea specific activity following a [^{14}C]urea prime, we have obtained information without a [^{14}C]urea prime in a previous study (15): the specific activity of urinary urea approached its near steady-state value (observed between 24 and 36 h) rather more slowly (~32% by 6 h, 70% by 12 h, and ~95% by 21 h) than that of breath CO_2 (see above).

In this study changes in the specific activity of urinary urea after *day 0* (*days 1–4*) again occurred more slowly than in that of CO_2 (Fig. 1). The specific activities of both breath CO_2 and urinary urea were higher on the low-exercise days and fluctuated less than on the low-exercise days.

Predicting CO_2 Production and Energy Expenditure With the Isotope Method

Table 1 shows that the tracer method predicted CO_2 production over 4-day periods with an accuracy of 100 ± 2% (SD) (about ±0.4 mol/day). There was virtually no difference when the results were analyzed by the four

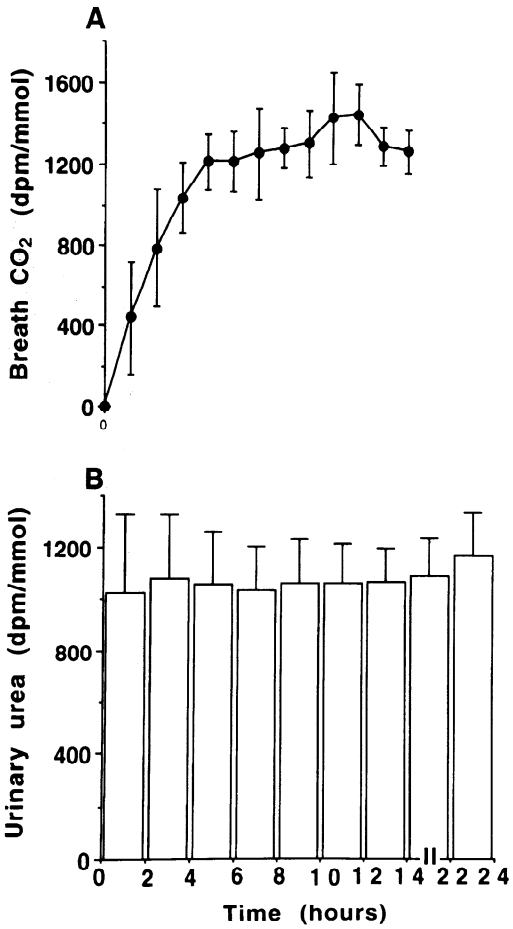

Fig. 2. Change in SA of urinary urea over a 24-h period after a bolus injection of urea at *time 0* (0900) on *day 0*, a sedentary day. Data points (*A*) are indicated at end of each period of urine collection. Labeled bicarbonate was begun at *time 0* (0900) and continued during entire period of study.

Table 1. *Comparison of CO_2 production between calorimeter (infrared analysis) and isotope* method*

	CO_2 Production (Calorimeter), mol/day	Calorimeter-Isotope, mol/day	Isotope × 100/ Calorimeter
1-Day periods			
Day 1	21.36 ± 2.45	−0.70 ± 0.84	103.8 ± 4.1
Day 2	20.92 ± 2.10	0.45 ± 1.04	98.3 ± 5.1
Day 3	21.03 ± 1.65	0.47 ± 1.12	98.0 ± 5.1
Day 4	20.50 ± 1.67	0.07 ± 1.06	98.0 ± 5.0
High-exercise days	22.26 ± 1.67	0.82 ± 1.01	96.4 ± 4.5
Low-exercise days	19.33 ± 1.37	0.67 ± 0.63	103.6 ± 3.4
All days	20.80±2.09	0.07±1.27	100.0±5.3
2-Day periods			
Days 1 and *2*	21.14±2.25	0.12±0.92	100.9±4.6
Days 3 and *4*	20.45±1.78	0.27±1.07	98.7±4.9
Days 2 and *3*	20.66±1.35	0.46±0.43	97.9±1.9
High-exercise days	22.26±1.57	0.82±0.87	96.3±3.9
Low-exercise days	19.33±1.32	−0.67±0.46	103.6±2.6
2 High- and 2 low-exercise days†	20.80±2.05	0.74±1.02	99.9±4.9
1 High- and 1 low-exercise days	20.66±1.35	0.46±0.43	97.9±1.9
All 2-day combinations	20.75±1.86	0.20±0.89	99.2±4.3
3-Day periods			
Days 1–3	20.89±1.56	0.07±0.42	99.8±2.0
Days 2–4	20.56±1.43	0.29±0.57	98.7±2.5
Days 1–3 and *2–4*	20.73±1.51	0.18±0.51	99.2±2.3
2 High- and 1 low-exercise days	21.18±1.54	0.50±0.60	98.3±2.6
1 High- and 2 low-exercise days	20.28±1.32	−0.02±0.31	100.2±1.6
4-Day periods	20.80±1.44	0.07±0.41	99.7±1.9

Values are means ± SD. *Based on specific activity of urea in a single 24-h urine sample (total urea/total label in that urea. See *Method 1* in text. †Mean results were obtained by combining values obtained on 2 consecutive high-exercise days with those obtained on two consecutive low-exercise days.

separate calculation procedures indicated in METHODS. Values for CO_2 production assessed by calorimetry (mol/day) over 4 days were found to differ from those obtained isotopically over the same period of time by 0.07 ± 0.41, 0.13 ± 0.38, 0.06 ± 0.39, and 0.23 ± 0.32 mol, as calculated by *methods 1A, 1B, 2A,* and *2B,* respectively. The values obtained by the isotopic methods were 99.7 ± 1.9, 99.7 ± 1.8, 99.8 ± 1.8, and 100.2 ± 1.7% of those obtained by calorimetry. All subsequent results in this paper are presented using *method 1A,* which is the simplest.

Table 1 shows the discrepancies (SD) between isotope and calorimeter methods: ±5% (±1.27 mol/day) for 1-day periods, ±4% (±0.9 mol/day) for 2-day periods, ±2.5% (about ±0.5 mol/day) for 3-day periods, and ±2% (±0.4 mol/day) for 4-day periods. There was a tendency for underestimation of CO_2 production on the high-exercise days (irrespective of whether the high-intensity exercise was carried out on the first 2 days or the last 2 days) and overestimation on the low-exercise days. However, the mean errors were not large (~3%). Furthermore, neither the %error or absolute error in CO_2 production on the low-exercise days was significantly different from either error obtained on high-exercise days (paired *t*-test). On the other hand, the ratio of total energy expenditure to BMR, which is often used as an indication of physical activity (PAL ratio), was found to be related to the %error ($r^2 = 0.26$; $P < 0.05$) and absolute error ($r^2 = 0.24$; $P < 0.05$). The PAL ratios ranged from 1.35 to 1.75, with mean values of 1.57 ± 0.12 (1.67 ± 0.06 on the high-exercise days and 1.46 ± 0.08 on the low-exercise days).

Isotopic estimates of total energy expenditure calculated by *method 1A* were 98.2 ± 1.8% of the calorimetric estimates over the 4-day periods (44.34 ± 2.49 vs. 45.20 ± 3.01 MJ) and 98.4 ± 5.5% over 1-day periods.

Predictions of CO_2 production from the 12-h urinary urea measurements are less secure for at least two reasons: no measurements of the circulating urea concentrations were made that could be used to correct for changes in size of the urea pool (see calculations), and the assumption that the recovery of infused label as gaseous $^{14}CO_2$ is 95% both during the day (0900–2100) and night (2100–0900) is invalid (see *Recovery of Label*). Nevertheless, if it is assumed that recovery is 95% during both day and night, and that the circulating urea concentration at 2100 is midway between the preceding and subsequent values, then the isotopic estimate of CO_2 production (all 12-h periods) differed from the calorimetric estimate (10.4 ± 3.6 mol) by 0 ± 1.9 mol. The daytime isotopic estimates (0900–2100) were lower (by 1.4 ± 1.5 mol) than the calorimetric measurements (13.7 ± 1.8 mol), whereas the nighttime isotopic estimates (2100–0900) were higher (by 1.50 ± 0.7 mol) than the calorimetric measurements (7.1 ± 0.6 mol). The estimation of CO_2 production (0900–2100) by use of hourly measurements of specific activity of CO_2 during the day (0900–2100) was also found to underestimate CO_2 production during the same period by 0–20%, depending on method of analysis (see Ref. 11), level of physical activity, and whether the values of the specific activity of CO_2 were those obtained at the end of each hour or the mean values between consecutive hourly measurements.

Transcutaneous Loss of Label Close to the Infusion Site

The amount of label lost transcutaneously was $\leq 0.5\%$ of the amount infused subcutaneously in both the chest and abdomen. This suggests that virtually all the label entered the body to mix with the pools of bicarbonate. Indeed, the recovery of label in breath was not only similar between the two studies (Fig. 3) but also similar to the results of the calorimeter studies [this study, which involved a subcutaneous infusion, and a previous study (11), which involved an intravenous infusion of [^{14}C]bicarbonate; Fig. 3]. The large variation in recovery shortly after the start of the subcutaneous infusion is considered to be due largely to the difficulties in adequately accounting for the dead space of the cannula and gravitation effects that affected the exact position of the solution within the tubing before connection to the catheter.

DISCUSSION

This study has demonstrated the accuracy and feasibility of a novel method for estimating CO_2 production and energy expenditure (the bicarbonate-urea method) by use of the novel subcutaneous route for infusion and the longest infusion of labeled bicarbonate ever undertaken in humans. The study has also provided information about bicarbonate kinetics over prolonged periods of time, which is important to the interpretation of results obtained by the bicarbonate-urea method.

Bicarbonate Kinetics and Recovery of Label

Over 97% of the infused label could be accounted for as gaseous $^{14}CO_2$ (95.6%) and urinary urea (1.5%), with additional label being lost as acid-labile CO_2 (mainly as bicarbonate) in urine (estimated to be $<0.5\%$; see RESULTS) and to a lesser extent in feces. The small remaining amount ($\sim 2\%$) is considered to be due to fixation of CO_2 into "storage" nutrients (fat, carbohydrate, and protein) (9) and possibly entry into pools of CO_2 that turn over very slowly. Over the time frame of this study, these may be regarded as CO_2 sinks that are nonexchangeable with respect to $^{14}CO_2$. Although there was a tendency for the recovery of CO_2 to increase on consecutive days, which might suggest that some of these slowly turning over pools or storage nutrients may begin to recycle $^{14}CO_2$, the change was small (~ 1.0–1.5%) and statistically not significant.

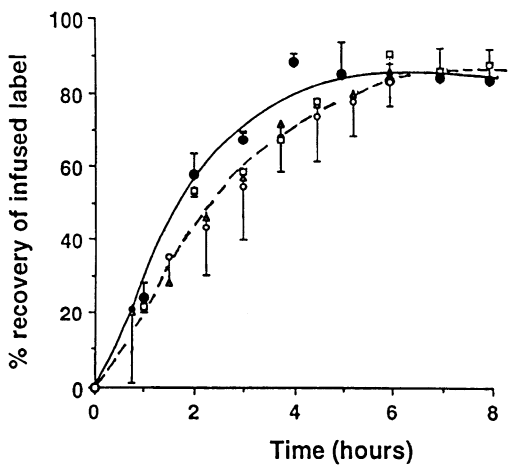

Fig. 3. Recovery of labeled bicarbonate as CO_2 after a constant unprimed subcutaneous infusion into the chest (△; $n = 5$), abdomen outside calorimeter (○; $n = 5$), and abdomen inside calorimeter (□; $n = 5$). For comparison, results from an intravenous infusion study carried out within a whole body calorimeter (11) are included (●; $n = 6$). For clarity, SDs of only the intravenous study (●) and one of the subcutaneous studies (○) are included.

It is possible to make an approximate estimate of the size of the "exchangeable" bicarbonate pool from the 24-h recovery of label during the first 24 h of infusion (83%), which was lower than the recovery on subsequent days by $\sim 12\%$ ($0.12 \times 27.35 \times 10^6$, or 3.3×10^6 dpm). If it is assumed that, 24 h after the start of the infusion, the specific activities of bicarbonate pools within the body are similar to each other and similar to the specific activity of expired CO_2, the size of the exchangeable pool of CO_2 can be readily calculated [size of exchangeable pool (mmol) = label in pool (dpm)/SA of CO_2 (dpm/mmol) = $3.3 \times 10^6/1{,}903$, or $\sim 1{,}700$ mmol]. This value agrees with an independent estimate obtained by assuming that the central circulating pool of CO_2 is in near equilibrium with the other pools of the body both at 0900 and 2300 (*days 1–4*). The amount of label retained in the body between 2300 and 0900 (1.2×10^6 dpm) divided by the change in SA of expired CO_2 [$\Delta 770$ dpm/mol, a change that is virtually identical to that in arterial blood (11)] gives another estimate of the size of the CO_2 pool (1,560 mmol). However, these estimates of the size of the exchangeable bicarbonate pool are greater than those suggested previously by Irving et al. (18) (~ 900 mmol) and those used by others, including Allsop et al. (1) (10–12 meq/kg; 700–840 meq for a 70-kg man), for calculating the priming doses of labeled bicarbonate in short-term metabolic studies lasting only a few hours. This can be explained by the much smaller recovery of label in breath in shorter studies. Label that enters pools of CO_2 that turn over relatively slowly or CO_2 that is fixed in intermediary metabolites or nutrients may not have sufficient time to recycle during periods of only a few hours, and therefore such pools may be described as nonexchangeable with respect to CO_2. During a longer period of time, the label may have sufficient time to recycle through the same pools, which can now be classified as exchangeable with respect to CO_2; therefore the calculated size of the exchangeable pool becomes larger. However, it is difficult to assess the proportion of CO_2 that cycles through true bicarbonate/carbonate pools and that which enters metabolic pools that fix CO_2 and later release it.

This study in healthy subjects demonstrates that, after an initial period of equilibration (*day 0*), there is a remarkable consistency in the 24-h recovery of infused [^{14}C]bicarbonate as $^{14}CO_2$ (*days 1–4*), irrespective of the level of physical activity. This consistency is an important prerequisite for the bicarbonate-urea method. However, it should be noted that the 24-h periods for measuring the recovery of labeled $^{14}CO_2$ began and finished at 0900 after a period of sleep and rest, when the size and amount of label in the bicarbonate pool are

likely to show little variation from day to day. This is because the amount of label in the pool is affected by CO_2 production (see below), which varies little during sleep and periods of rest after sleep.

Although the 24-h recovery of infused label as gaseous $^{14}CO_2$ shows little variation from day to day, there are important diurnal fluctuations. Infusion of [^{13}C]bicarbonate in inactive nonexercising individuals has also been found to be associated with an inverse relationship between CO_2 production and $^{13}CO_2$ enrichment over a 24-h period (17, 18), but the overall $^{13}CO_2$ recoveries were lower. In our study, values transiently exceeding 100% occurred during exercise (see also Refs. 11 and 33) and were compensated for by lower values in the postexercise period. Furthermore, the 12 hourly values during the day (0900–2100, a time of higher CO_2 production) were higher than those observed during the night (2100–0900), that is, 4–5% above and below the mean 24-h recovery on the low-exercise days and above and below the mean 24-h recovery on the high-exercise days. In all these respects it is important to demonstrate the extent to which [^{14}C]bicarbonate and [^{13}C]bicarbonate inputs are bioequivalent.

This study is the first to use the subcutaneous route of infusion to study bicarbonate kinetics and energy expenditure in humans. Only dissolved CO_2 can rapidly cross the vascular epithelium (20), and therefore carbonate/bicarbonate in the interstitial fluid must be protonated and converted to free CO_2 to enter the blood. It is interesting that some studies suggest that plasma bicarbonate has direct access to the carbonic anhydrase of the lung capillary (20), but whether the same applies to interstitial bicarbonate surrounding the capillaries of subcutaneous adipose tissue is uncertain.

The overall kinetics of CO_2 after subcutaneous infusion, as judged by the hourly recovery rates in breath, were found to be similar to those obtained during intravenous administration under similar circumstances (Fig. 3). Furthermore, the transcutaneous loss of label close to the infusion site was found to be very small, suggesting that virtually all the infused label entered the systemic circulation. Furthermore, if there were a large transcutaneous loss of label, the amount entering the systemic pool would be reduced, and the amount recovered in breath (0900–2100 by the multiple spot breath method) during the same period would be substantially lower than that trapped continuously from calorimeter air. Because this was not observed, the data again suggest that there is little transcutaneous loss of label. Fixation of CO_2 into fatty acids during lipogenesis from acetyl CoA is not considered important for biochemical reasons (9).

The Bicarbonate-Urea Method for Measuring Net CO_2 Production and Energy Expenditure

In addition to the multiple advantages of using urinary urea instead of urinary CO_2 to predict the net CO_2 production by the body (see introductory comments), the abundance of urinary urea means that the daily dose of administered radioactivity can be reduced by one to two orders of magnitude. However, the novel use of urinary urea for estimating CO_2 production is indirect, because it depends on the assumption that urea specific activity reflects that of the CO_2 in the expired air or arterial blood. Theoretically this relationship may vary in different circumstances because of isotopic dilution with unlabeled CO_2 produced in the splanchnic bed (liver and portal drained viscera) and urea formation from unlabeled arginine derived from the diet or protein turnover. A document addressing the quantitative aspects of the ratio can be obtained on request from the authors. It discusses the trivial effects of urea cycling and CO_2 production from colonic fermentation, the more important effects of compartmentation of urea cycle enzymes between periportal and perivenous hepatocytes, compartmentation of arginine metabolism (which partly derives from a slow hepatic transport system), and the effects of changes in hepatic perfusion and circulating CO_2 concentration.

Although the subcutaneous infusion of bicarbonate was well tolerated by our subjects (and by freely living subjects, unpublished data) and appeared to cause minimal inconvenience, the procedure is more invasive than the doubly labeled water method, which only requires the subject to drink the labeled isotope and produce intermittent urine samples (6). However, the cost of labeled [^{14}C]bicarbonate for a study of several days appears to be less than ~5% of that required for doubly labeled water. The cost of $H_2{}^{18}O$ makes the doubly labeled water method essentially prohibitive to many workers. Furthermore, mass spectrometry for measuring the enrichment of ^2H_2O and $H_2{}^{18}O$ requires expertise, is time consuming, and may involve considerable delays between sample collection and analysis. With the [^{14}C]bicarbonate-urea method, the analysis is simple and can be undertaken on the same day as the experiment by any laboratory with facilities for scintillation counting. It may also be possible to modify the method so that [^{13}C]bicarbonate is used instead of [^{14}C]bicarbonate, although this will inevitably increase both cost and the volume and osmolarity of the solution infused and require a mass spectrometer for analysis.

The isotopic prediction of CO_2 production using the bicarbonate-urea method over 12-h periods was found to be considerably less accurate than the 24-h measurements, irrespective of whether the estimates were made by analysis of breath $^{14}CO_2$ (integrated 12-h values during the day) or urinary [^{14}C]urea. This is partly because of the problems of isotope exchange and reentry, which are associated with higher recoveries of $^{14}CO_2$ during the day than during the night (see discussion on bicarbonate kinetics and recovery of label). It is also related to the change in the size and specific activity of the bicarbonate/urea pools. For example, with the bicarbonate urea method, the circulating urea concentration was not measured at 2100, making correction for possible changes in the urea pool size uncertain.

The use of a "steady-state" isotope dilution methodology to analyze changing specific activity of end products is a potential theoretical problem, although oscillations in the specific activity of the end product about a mean value over prolonged periods of time may be associated

with little error. Although fluctuations in specific activity of breath CO_2 can be subjected to theoretical mathematical models that take into account exchanges of label between bicarbonate pools, there is less scope for doing this with [^{14}C]urea, which has a relatively longer biological half-life and shows much smaller fluctuations in specific activity.

The bicarbonate-urea method may be of particular value in situations in which energy expenditure varies substantially from day to day, as in some clinical situations. For shorter periods of time of only a few hours, there may be more potential for estimating CO_2 production from repeated measurements of CO_2 specific activity (see above). In contrast, the doubly labeled water method can only provide accurate estimates of energy expenditure over much longer periods of time (6). All these methods measure CO_2 production, which means that it is necessary to choose an appropriate value for the energy equivalent of CO_2 to calculate energy expenditure (for detailed discussion see Refs. 8, 10, and 12).

Finally, it should be remembered that this is the first attempt to validate this new tracer method for estimating CO_2 production and energy expenditure. Although the results of this study suggest a reasonably good accuracy [±5% (SD) over 1 day and as little as ±2% over 4 days], which is as good, if not better, than many of those reported for doubly labeled water in adults (6, 17, 19, 24–26, 30), it is necessary to confirm the present results by independent studies, to bear some of the limitations of the method in mind when undertaking further validation studies, especially under different pathophysiological conditions, and to assess the practicalities of the method in the field.

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