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Does mitochondrial compartmentation of CO_2 exist in man?

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Summary. A comparison was made between the specific radioactivity of urea, and that of CO_2 in breath, in urine and in arterialized blood, during a 36-h continuous infusion of 0·5 mCi and 100 mmol of sodium bicarbonate ($NaH^{14}CO_3$) into six normal male volunteers. After a period of equilibration, the mean specific radioactivity of urea was found to be only 16% below that of end expiratory CO_2 and a similar amount below that of CO_2 both in arterialized blood and in urine. This difference may be explained by isotopic dilution of $^{14}CO_2$ by metabolic CO_2 produced in the splanchnic tissues. It is concluded that, in these normal subjects, there is little or no compartmentation between cytosolic CO_2 and the mitochondrial CO_2 used for urea synthesis.

Key words: CO_2 compartmentation, expiratory CO_2, mitochondrion, urea formation.

Introduction

Many concepts concerned with metabolic regulation have developed from a knowledge of the activity of enzymes and the concentration of metabolites within tissues (Newsholme & Leech, 1985). However, enzymes are not evenly distributed within the cell. For example, the enzymes involved in the citric acid cycle, and beta-oxidation are found in mitochondria, whereas glycolytic enzymes are found in the cytosol. Substrates and co-factors may also be unevenly distributed within the cell. These observations may have important implications both for metabolic regulation, and the estimation of the flux of metabolites through metabolic pathways, using tracers. For example, in certain tracer studies it is frequently assumed that the specific activity or enrichment of the substrate at its site of metabolism is close to that in the tissue as a whole or in the blood that perfuses the tissue. However, this may not be so, and consequently erroneous results of flux may be obtained (see below).

Incomplete equilibration of labelled substrates within a cell may occur because of permeability restrictions imposed by membranes and viscous matrix. In metabolic pathways in which enzymes and substrates are located in different parts of a cell, incomplete equilibration may occur if diffusion or transport of the labelled substrates to

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their site of metabolism is not fast enough to overcome the above restrictions. Compartmentation of substrates may even occur in different parts of the same organelle such as a mitochondrion (Von Glutz & Walter, 1975; Schoolwerth & LaNoue, 1980) or involve relatively diffusible substrates such as CO_2 (Hems & Saez, 1983; Hems, 1984). For example, using liver cell preparations and radiolabelled substrates it has been shown that substantial quantities of CO_2 produced intramitochondrially, do not equilibrate with cytosolic CO_2, but are preferentially incorporated into urea through the operation of initial intramitochondrial reactions (Hems & Saez, 1983; Hems, 1984). As a consequence there may be a several-fold difference between the specific activity of CO_2 and urea, which is formed from CO_2. This difference, which may vary with time, depends on the type of substrate used for oxidation (Hems & Saez, 1983). Thus, CO_2 produced as a result of pyruvate dehydrogenase activity has been found to be preferentially used for urea synthesis (Hems, 1984). These kinds of observations may be of major importance when the flux of metabolites are assessed using tracer techniques.

However, in contrast to the above observations, other studies involving perfused liver preparations have shown no difference in the specific radioactivities of CO_2 and urea, implying that compartmentation does not exist under the conditions of these studies (Marsolais *et al.*, 1987).

In view of these apparently conflicting results, no general conclusions can be made about compartmentation of CO_2 in hepatocytes *in vitro*, and certainly no conclusion can be made about the phenomenon *in vivo*. The purpose of this study was to investigate the extent of subcellular equilibration of CO_2 *in vivo*. This was achieved by comparing the specific radioactivity of urea with the estimated specific radioactivity of hepatic CO_2 during prolonged infusion of labelled sodium bicarbonate ($NaH^{14}CO_3$) into normal healthy subjects. A major discrepancy between the specific radioactivity of hepatic $^{14}CO_2$ and that of ^{14}C-urea (which obtains its carbon from intramitochondrial CO_2 in the urea cycle), would imply that hepatic compartmentation of CO_2 exists. A lack of such a difference would imply that no such compartmentation exists.

Subjects and methods

Radiolabelled sodium bicarbonate ($NaH^{14}CO_3$): 0·5 mCi and approximately 100 mmol in 500 ml was infused over 36 h at a rate of 14·0 ml h^{-1} into six normal male subjects (mean age 34 ± 5 years, weight 80 ± 7 kg and height 182 ± 6 cm). The studies were carried out in whole body indirect calorimeters which were maintained at 26°C. The subjects ate breakfast, lunch and an evening meal in the calorimeter (total intake 10·5 ± 0·4 MJ day^{-1}).

CO_2 production and O_2 consumption were monitored continuously during the study using infrared CO_2 and paramagnetic O_2 analysers. The analysers were calibrated every 2 h during the study using nitrogen (zero span) and a standard gas mixture containing 0·75% CO_2 (α-tested: BOC Ltd, Special Gases, London). The validity of the

standard CO_2 gas mixture was confirmed to within 1% by three other procedures: analysis by the Haldane apparatus; the increase in oxygen concentration after removal of CO_2 by soda lime; and by titration of a solution of hyamine/methanol before and after bubbling a known volume of gas (at measured temperature and pressure) through it. The oxygen analyser was calibrated using nitrogen (zero span) and atmospheric air (oxygen conc. 20·94%). The gas entering the analysers was dried with calcium chloride (mesh 8–16; BDH Chemicals Ltd, Dagenham, UK) which appears to be free of CO_2 adsorption (Elia *et al.*, 1986).

Basal metabolic rate (BMR) was measured during 1 h of bed rest after an overnight fast. Energy expenditure was calculated using the formula of Elia & Livesey (1988) which is based on measurements of gaseous exchange.

Spot breath samples of expired CO_2 were taken during the alveolar phase of expiration. They were collected into an accurately weighed amount of hyamine/methanol, with phenolphthalein as indicator, containing approximately 1 mmol of hyamine (BDH). The samples were generally obtained at 1 h intervals, but during two 1 h periods of exercise (cycling at 50 and 100 W) they were obtained at 15 min intervals. The strength of the hyamine: methanol (approximately 0·25 mol l^{-1}), was accurately determined by titration using phenolphthalein as indicator (repeat measurements on the same sample yielded results that were within 1% of each other). The subject exhaled end expiratory CO_2 into the hyamine: methanol mixture until the colour of the indicator just disappeared. Ten millilitres of scintillation fluid (Ready-Solv EP: Beckman-RHC Ltd, Bucks, UK) containing hyamine (3·3 mmol l^{-1}), was added, for scintillation counting. The hyamine was included in the scintillant to ensure that $^{14}CO_2$ remained in the scintillation fluid and did not escape into the air above the scintillation fluid. A quench curve reproducing these conditions had been constructed previously.

Urine from the bladder was passed 3 hourly into a graduated 250 ml cylinder containing 5 ml of 5 mol l^{-1} NaOH (CO_2 free). The cylinder was then sealed to prevent exchange of urinary CO_2 with atmospheric CO_2. Aliquots were stored at −20°C for future analysis. A small untreated sample of urine was taken for the measurement of pH.

Blood was taken from all subjects at the beginning and at the end of the study; in one subject, arterialized blood was taken regularly during the study from a cannulated, heated, dorsal vein of the hand, which was placed in a box containing air at a temperature of 68°C. The blood was stored at −20°C in sealed, almost fully filled tubes. It had been shown previously that such a procedure prevented both a loss of CO_2, and a decrease in the specific radioactivity of CO_2. Small losses of CO_2 occurred when these samples were thawed and refrozen in half-filled tubes, although the specific radioactivity remained unaltered.

The concentration and specific radioactivity of acid labile CO_2 in urine were measured as follows: a known quantity of urine of predetermined pH, was placed in the base of a 100 ml capacity flask. A buffer (4 ml) of potassium carbonate (0·1 mol l^{-1}—BDH) and potassium bicarbonate (0·2 mol l^{-1}—BDH) containing a phenolphthalein indicator (absorbance, approximately 0·6 at 554 nm) was added to a well

situated adjacent to the top of the flask. The well contained a central channel to allow free movement of gases from the base of the flask. After sealing the top of the flask, 5 ml of 150 g l^{-1} phosphoric acid was added to the urine via a thick rubber airtight seal, located on the side of the flask. The flask was then agitated gently for 8 h, to allow the CO_2 to be released and trapped by the buffer. The buffer was then withdrawn, its absorbance measured for the determination of acid labile CO_2, and a weighed amount taken for scintillation counting (as above). The quantity of radiolabelled CO_2 (d.p.m.) in the scintillation vials was calculated from quench curves established specifically using the same buffer and volume as used in the experiments. The concentration of acid labile CO_2 was determined from a standard curve and the specific radioactivity of CO_2 (d.p.m. μmol^{-1}) was then calculated, after making allowances for its recovery, which was about 95%. Standard solutions containing acid labile CO_2 (Sigma Chemical Co. Ltd, Poole, UK) and $^{14}CO_2$ (Amersham International plc, Amersham, UK) were run under the same conditions in the same assay.

The concentration and specific radioactivity of CO_2 in arterialized blood was determined in essentially the same manner as for the urine bicarbonate, except that modified Conway dishes were used to replace the flasks; 3 ml of buffer containing 0·036 mol l^{-1} potassium carbonate and 0·072 mol l^{-1} potassium bicarbonate were placed in the central reservoir, and 1–3 ml of 200 g l^{-1} sulphosalicylic acid (BDH) was introduced via a hypodermic needle into the blood through an easily sealable airtight opening in the lid. Solutions containing standard amounts of acid labile CO_2 and $^{14}CO_2$ were used in each assay. For radioactive counting a separate quench curve was constructed to accommodate the specific conditions of this assay.

The specific radioactivity of urinary urea was determined as follows. Firstly, the concentration of urinary urea in the untreated sample of urine (also used for pH measurement) was determined using a modification of a method suggested in a kit from Boehringer Mannheim (Kit 124770: BCL, Lewes, UK). The methods involved the colorimetric determination of ammonia before and after enzymatic hydrolysis of urea with urease (Fawcett & Scott, 1960). The urine was diluted 30-fold for ammonia determination and 900-fold for urea determination. Secondly, two weighed 1 ml samples of urine were put into scintillation vials. To sample 1 was added enough 150 g l^{-1} phosphoric acid to achieve a pH <3. The sample was then mixed for about 1 h, to ensure that all labile CO_2 had been allowed to escape into the atmosphere. This was confirmed by subjecting labelled bicarbonate standard to the same procedure. The urine was then neutralized, and 10 ml scintillation fluid added for radioactive counting. Sample 2 was mixed with 0·1 ml urease (Sigma, U1500) containing 3000 units ml^{-1} and incubated for 2 h at 50°C, a temperature frequently used for the accelerated rate of urea hydrolysis (see BCL Kit 124770). In each case a specifically constructed quench curve was used to calculate the radioactivity present. Preliminary studies had shown that the hydrolysis of urea was complete under these conditions and that all CO_2 formed during this hydrolysis was lost to the atmosphere. Sample 1 was then subjected to the same procedure as that for sample 2. The radioactivity associated with urea was

the difference between two samples. The specific radioactivity (d.p.m. μmol^{-1}) was then calculated.

The recovery of infused $^{14}CO_2$ in expired air was about 95% in all subjects. A further 2% was recovered in urine. If all the remaining radioactivity was located in bone, it is estimated that the effective whole body dose equivalent produced by this radiation is about 5 μS (cf. 5–6 μS produced by exposure to 1 day of natural radiation). However the value may be somewhat higher if some of the $^{14}CO_2$ expired in breath had cycled through bone.

The study was approved by the Local Ethical Committee, and all subjects gave their informed consent.

Results

The intra-assay coefficient of variation for the measurement of specific activity of CO_2 in physiological fluids generally ranged from about 2 to 4%. The interassay coefficient of variation was similar. A low coefficient of variation was observed when the change in absorbance produced by CO_2 was high and associated with high radioactive counts. Thus, the specific activities of CO_2 in samples that were serially diluted before analysis were found to be essentially the same as each other but the coefficient of variation increased with progressive dilution. As a result of these observations, the volume of blood or urine used in the assays was selected in order to produce a large change in absorbance.

The radiolabelled bicarbonate ($NaH^{14}CO_3$) in the infusion was gradually incorporated into the CO_2 and urea pools of the subjects over the first 12 h of the study. This was demonstrated by a rapid increase in the specific activity of CO_2 and a relatively slower increase in the specific radioactivity of urea until a plateau was reached. During the final 12 h infusion period the specific activity of urea was more stable than that of CO_2, which varied inversely with total CO_2 production (Table 1).

Comparison of the mean specific radioactivities of the breath CO_2 and urea, for each of the six subjects over the final 12 h period, demonstrated a correlation coefficient (r) of 0·79 ($P<0·02$) with a slope of 0·9.

Table 1. Comparison of specific activities (d.p.m. μmol^{-1}) of breath CO_2, urine CO_2 and urinary urea for the final 12 h of continuous infusion of $NaH^{14}CO_3$

	H since commencement of infusion				
	24–27*	27–30	30–33**	33–36	Mean
CO_2 production (mol)	4·12 ± 0·28	3·29 ± 0·27	4·99 ± 0·45	3·46 ± 0·30	3·97
Specific activity of breath CO_2	27·3 ± 2·2	31·9 ± 2·6	22·8 ± 2·0	29·5 ± 2·2	27·9
Specific activity of urine CO_2	27·8 ± 2·0	30·3 ± 2·0	23·2 ± 2·0	28·6 ± 1·1	27·5
Specific activity of urinary urea	23·4 ± 2·0	23·0 ± 4·6	23·7 ± 3·0	23·1 ± 4·3	23·3
% Specific activity urea/breath CO_2	85·7	72·1	103·9	78·3	83·5

Includes 1 h of exercise at *50 W and **100 W respectively.

The mean specific radioactivity of the arterialized blood CO_2 was 98·6% that of breath CO_2; the correlation coefficient (r) was 0·99 ($P<0·001$) and there was no significant difference between these values when subjected to a paired t-test.

The mean concentration of CO_2 in whole blood was found to be $20·0 \pm 1·7$ mmol l^{-1} at the beginning of the study and $23·3 \pm 3·6$ mmol l^{-1} at the end of the study. The basal metabolic rate, which was calculated to be 323 ± 28 kJ h^{-1} was associated with the production of $0·60 \pm 0·06$ mol CO_2 h^{-1}. The recovery of label in expired CO_2 (12–36 h) was $95·6 \pm 1·1$%. A further $1·9 \pm 0·4$% was recovered in urine, mainly as urea and acid labile CO_2.

Discussion

A number of conclusions concerned with the compartmentation of CO_2 between mitochondria and the cytosol have been based on tracer studies. Liver cell preparations supplied with labelled substrates (Hems & Saez, 1983; Hems, 1984) have shown differences in the specific activity of CO_2 and urea (which is synthesized from CO_2 in a series of reactions that begin in the mitochondrion). In this *in vivo* study in humans we have measured the specific radioactivity of urea, which is produced almost entirely within the liver; and have compared it with the estimated specific radioactivity of hepatic CO_2 (see below). The comparison was made during the last 12 h of the labelled bicarbonate infusion (24–36 h) when the values of specific radioactivity were relatively stable. The earlier periods were considered unsuitable for such comparisons because the specific radioactivity of CO_2 and that of urea increased rapidly as the label equilibrated within the body pools. The variation in the specific radioactivity of CO_2 during the last 12 h of the infusion was largely due to variations in CO_2 production, which followed meal ingestion and exercise. The relative constancy of the specific radioactivity of urea during this period can be explained by the slow turnover rate of the urea pool (about 10–12 h) compared with that of the freely exchangeable CO_2 pool which is 1–2 h (Elia *et al.*, 1988).

The specific radioactivity of CO_2 in the blood perfusing the splanchnic tissues (i.e. arterialized blood) was found to be essentially the same as the specific radioactivity of end expiratory CO_2. This is not surprising because the only tissue between the lungs and arterial blood is the heart, which contributes less than 1% of the CO_2 in the blood that is pumped around the body. The specific radioactivity of urinary CO_2 (derived largely from filtration of arterialized CO_2) was also found to be similar to that in arterialized blood and expired CO_2. Therefore, the mean specific radioactivity of urea is only about 16% below that of CO_2 in arterialized blood. This is in marked contrast to the several-fold differences between the specific radioactivities of urea and bicarbonate, which has been reported for liver cell preparations (Hems & Saez, 1983; Hems, 1984). However, the CO_2 in blood undergoes isotopic dilution by metabolically derived CO_2 as it passes through the splanchnic tissues. As a consequence, the specific radioactivity of hepatic CO_2 is likely to be lower than (although close to) that of urea,

as suggested by the following approximate calculations. Firstly, since the concentration of acid labile CO_2 in whole blood was about 20 mmol l^{-1}, and blood flow to the splanchnic area is about 1 l min^{-1} (it tends to be below this value as a result of exercise or above this value as a result of ingestion of food [Wahren *et al.*, 1971, 1976]), then it may be concluded that about 20 mmol of CO_2 perfuse the liver every minute. Secondly, although there is little information about CO_2 production by the splanchnic tissues of man, it is known that they contribute up to 30% of the basal metabolic rate of the whole body (see Grande [1980] for review). In our subjects the BMR was associated with the production of about 10 mmol CO_2 min^{-1} (0·6 mol h^{-1}). Both the production of CO_2 and respiratory quotient of the whole body and the splanchnic region may increase after administration of nutrients (Gil *et al.*, 1985). Therefore, despite lack of direct measurements, it is not unreasonable to assume that CO_2 production by the splanchnic tissues of our subjects occurred at a rate of 2–3 mmol CO_2 min^{-1}. Since this is equal to 10–15% of the CO_2 that perfuses the splanchnic tissues (see above) the specific radioactivity of CO_2 will be reduced by about 10–15%; which approximates to the difference of 16% obtained between the specific radioactivities of urea and CO_2 in arterialized blood and end expiratory CO_2. In these calculations it has been assumed that there is negligible net fixation of CO_2 by splanchnic tissues other than in urea. This is in accordance with the almost complete recovery (97·5%) of infused label in breath plus urine, and the presence in the liver of a small pool of CO_2 which has a rapid turnover.

Therefore, the data obtained from this study in man imply that there is little or no hepatic mitochondrial compartmentation of CO_2 *in vivo*. This is consistent with studies involving perfused livers (Marsolais *et al.*, 1987), but at variance with studies involving liver cell preparations (Hems & Saez, 1983; Hems, 1984). The apparently large compartmentation which has been observed in liver cell preparations (Hems & Saez, 1983; Hems, 1984) probably results from major manipulations of the metabolic environment of the liver cells. This is probably also true of the CO_2 compartmentation observed in perfused livers exposed to carbonate-dehydrase inhibitors. Under these non-physiological conditions, there is inhibition of urea synthesis from extracellular HCO_3^- but not CO_2 derived from glutamine oxidation (Haussinger, 1986). Thus, although there is a large potential for mitochondrial compartmentation of CO_2, there was little evidence for this under the physiological conditions of this study. Therefore, the present results emphasize differences between *in vivo* and *in vitro* studies and are of relevance to a number of studies involved with the assessment of the oxidation and fate of C-labelled substrates.

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