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Underestimation of metabolic rates owing to reincorporation of $^{14}CO_2$ in the perfused rat liver

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$^{14}CO_2$ production by perfused rat livers was simulated by infusing $NaH^{14}CO_3$ into the perfusate. Recovery of label as $^{14}CO_2$ gas + perfusate bicarbonate was 45–85%. Rates of $^{14}CO_2$ exchange in the liver are 3–70 times greater than net rates of CO_2 production. Therefore $^{14}CO_2$ reincorporation can lead to significant underestimations of rates of oxidation of ^{14}C-labelled substrates in liver.

Metabolic rates measured in a variety of experimental models are frequently expressed as rates of oxidation of ^{14}C-labelled substrates to $^{14}CO_2$. Although it is well known that $^{14}CO_2$ can be reincorporated by exchange reactions, it is generally assumed that these processes are quantitatively negligible. As far as we know, the extent to which $^{14}CO_2$ exchange in the liver might underestimate rates of substrate oxidation has not been evaluated. We have investigated this question by using rat liver perfusions. Production of $^{14}CO_2$ was simulated by a constant infusion of a tracer of [^{14}C]bicarbonate. We report that a substantial fraction of $^{14}CO_2$ is converted in the liver into compounds non-volatile in acid. This suggests that many reported metabolic rates calculated from the production of $^{14}CO_2$ are, in fact, underestimated.

Experimental

Liver perfusions

Livers from schedule-fed Sprague–Dawley rats were perfused with recirculating buffer (Krebs & Henseleit, 1932) containing 4% dialysed bovine serum albumin and glucose (15mM or 4mM in perfusions of livers from fed or starved rats respectively). The surgical technique and the perfusion apparatus have been described previously (Brunengraber *et al.*, 1973). Where indicated, a 1mM concentration of oleate was maintained by a primed-constant infusion (Brunengraber *et al.*, 1978).

After an equilibrium period of 30min, $NaH^{14}CO_3$ was infused into the effluent line of the perfusion reservoir at a rate of 2.5×10^5 d.p.m./min for 90min. The effluent CO_2 from the oxygenator and the CO_2 present in the perfusate bicarbonate pool at 120min were trapped as described previously (Endemann *et al.*, 1982). The flow rate of the gas mixture (O_2/CO_2, 19:1) passing through the oxygenator was kept at 150ml/min in all experiments to avoid variations in the rate of CO_2 exchange. During the experiment, duplicate samples of perfusate (0.25ml) were taken at 5min or 10min intervals for counting of radioactivity. One set of samples was counted without processing. The second set of samples was incubated with one drop of acetic acid for 30min to eliminate $^{14}CO_2$ before adding the counting fluid. The rate of $NaH^{14}CO_3$ infusion was measured at the end of each experiment. In control experiments, livers were perfused for 30min and were then removed from the apparatus before starting the infusion of $NaH^{14}CO_3$.

Liver glycogen was isolated after alkaline digestion (Good *et al.*, 1933) and counted for radioactivity. Labelling of perfusate glucose was measured as described by Mallette *et al.* (1969). Counting efficiencies for all samples were determined by recounting the radioactivity of each vial after addition of an internal standard of [^{14}C]toluene.

Tracer kinetics

In control experiments without livers, the rate of $^{14}CO_2$ exchange in the oxygenator was calculated from the profile of the specific radioactivity of bicarbonate in the perfusate, by using the following reasoning. It is assumed that, in the perfusate, bicarbonate and CO_2 behave as a single pool. As a tracer of $H^{14}CO_3^-$ is continuously infused at a rate of Id.p.m./min into the perfusate, the radioactivity

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present in the bicarbonate pool, $A(t)$, increases with time in relation to its specific radioactivity, $S(t)$:

$$\frac{dA(t)}{dt} = I - kS(t) = I - \frac{kA(t)}{M} \quad (1)$$

where k is the rate of CO_2 exchange in the oxygenator (μmol/min) and M is the constant total amount of $CO_2 + HCO_3^-$ present in the perfusate (μmol). Integration of this expression and division by M yield the specific radioactivity of the bicarbonate pool:

$$S(t) = \frac{I}{k}(1 - e^{-kt/M}) \quad (2)$$

Experimental values of $S(t)$ were fitted to a generalization of eqn. (2) which permitted a non-zero intercept, by using the non-linear regression procedure NLIN of SAS Computer System (Hellwig & Council, 1979) running on an IBM 370/168 computer.

The use of eqn. (2) to analyse data from experiments conducted in the presence of a liver assumes that constant k combines all processes of CO_2 exchange (in the oxygenator and in the liver). The model fitted the data well in general [the standard error of fit was within experimental errors of $S(t)$; there was no pattern in the residuals], allowing the use of the extrapolated plateau [$S(t)$ for $t = \infty$] in later calculations. However, parameter identification with eqn. (2) where two independent estimates of k are possible suggests that this model is too simple and the data are too few to describe precisely the rise of $S(t)$ to a plateau. As discussed below, the rate of $^{14}CO_2$ fixation by the liver increases during the course of the perfusion.

Results

In model perfusions without liver, the total radioactivity recovered in the effluent gas of the oxygenator and in the perfusate bicarbonate at 120 min was 95% of the label infused (Table 1). The rate of CO_2 exchange in the oxygenator, calculated from the extrapolated specific radioactivity of bicarbonate in the perfusate [$S(t)$ for $t = \infty$] by using eqn. (2), was 40 ± 8 μmol/min (mean ± S.E.M.; $n = 5$). In the presence of a liver, recovery of $^{14}CO_2$ varied from 45 to 85% depending on the metabolic status of the liver and the substrate present in the perfusate. In other words, 10–50% of the label infused was incorporated into compounds non-volatile in acid. (Note that all calculations take into account the recovery of label in control experiments.) Incorporation of $^{14}CO_2$ was significantly increased by starvation and decreased by diabetes. Ethanol and oleate significantly decreased the incorporation of $^{14}CO_2$ in livers from starved, but not from fed, rats. The specific radioactivity of bicarbonate in the perfusate (Fig. 1) varied roughly in parallel with the percentage recovery of $^{14}CO_2$.

Apparent total rates of CO_2 fixation were calculated by two methods. First, the rate of label infusion was divided by the extrapolated specific radioactivity of perfusate bicarbonate [$S(t)$ for $t = \infty$] to yield turnover rates that comprise $^{14}CO_2$ exchange in both the oxygenator and in the liver. The rate of $^{14}CO_2$ exchange in the oxygenator was deducted from the latter turnover rates, yielding rates of $^{14}CO_2$ fixation at equilibrium in the liver (Method A, Table 1). Secondly, the total $H^{14}CO_3^-$ incorporated (d.p.m.) was divided by the integrated specific radioactivity of bicarbonate in the perfusate, yielding an average rate of $^{14}CO_2$ fixation over the

Table 1. *$^{14}CO_2$ fixation in the isolated perfused rat liver*

The data are presented as means ± S.E.M. for five observations in each group. 'Recovery of $^{14}CO_2$' is the percentage of label infused recovered as the sum of CO_2 evolved during the experiment and perfusate bicarbonate plus CO_2 at 12 min. Method A is based on the turnover rate of perfusate $H^{14}CO_3^-$ and Method B on the total radioactivity (d.p.m.) incorporated during 90 min (see the Experimental and Results sections).

Group	Recovery of $^{14}CO_2$ (%)	Apparent total CO_2 fixation (μmol/min per g dry wt.) Method A	Method B	Urea synthesis (μmol/min per g dry wt.)
Model without liver	95.0 ± 1.1	—	—	—
Fed control	63.1 ± 3.9	53.5 ± 12	31.3 ± 6.0	0.30 ± 0.10
Fed + ethanol	67.2 ± 4.4	86.4 ± 13	36.4 ± 7.2	0.56 ± 0.31
Fed + oleate	68.0 ± 6.9	21.3 ± 1.8*	21.2 ± 5.6	0.51 ± 0.14
Starved control	45.2 ± 4.7*	63.9 ± 9.3	29.9 ± 5.2	1.2 ± 0.19
Starved + ethanol	72.5 ± 3.9†	42.4 ± 4.0	22.3 ± 3.5	0.36 ± 0.06†
Starved + oleate	66.9 ± 4.2†	25.4 ± 8.2†	12.2 ± 4.3†	0.33 ± 0.14†
Fed diabetic‡	84.5 ± 5.5*	29.5 ± 1.5	8.5 ± 3.9	1.0 ± 0.37

* Differs significantly from fed control ($P < 0.05$, by two-sided t test).

† Differs significantly from starved control ($P < 0.05$, by two-sided t test).

‡ Diabetes was induced by streptozotocin (65 mg/kg) 2 weeks before the experiment.

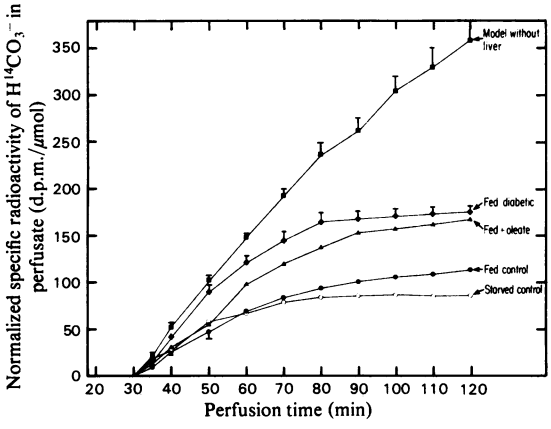

Fig. 1. *Profile of the specific radioactivity of bicarbonate in the perfusate*

The data are normalized for 1 μCi of $H^{14}CO_3^-$ infused over 90 min and are presented as means ± S.E.M. for five observations in each group. For the sake of clarity, four out of seven groups of experiments with liver are shown.

90 min period of infusion of label (Method B). In nearly all cases, Method A gave rates of $^{14}CO_2$ exchange higher than with Method B.

Net incorporation of $^{14}CO_2$ into urea contributed no more than 4 or 12% to total $^{14}CO_2$ fixation in livers from normal or diabetic rats respectively. Incorporation of label into compounds non-volatile in acid present in the final perfusate accounted for the following percentages of the total radioactivity incorporated: livers from normal fed rats, 9–13%; livers from normal starved rats, 3–5%; livers from fed diabetic rats, 32%. Radioactivity non-volatile in acid present in the final perfusate was distributed among glucose (44–68%), urea (7–36%), proteins (2–26%) and carboxylic + amino acids (10–26%). Labelling of liver glycogen accounted for less than 0.4% of the total radioactivity incorporated in each group.

Discussion

$^{14}CO_2$ generated in the liver cell can be reincorporated either transiently (acetyl-CoA carboxylase), or permanently (β-methylcrotonyl-CoA carboxylase, urea and pyrimidine synthesis). $^{14}CO_2$ fixation via exchange processes occurs in the cycle pyruvate–oxaloacetate–phosphoenolpyruvate–pyruvate. After carboxylation of pyruvate to [4-^{14}C]oxaloacetate and randomization of label between C-1 and C-4 of oxaloacetate (Hoberman & d'Adamo, 1960), a substantial amount of label can be incorporated by exchange reactions into glucose and glycogen. In addition, label can be exchanged with compounds in isotopic equilibrium with intermediates of the tricarboxylic acid cycle. Lastly, $^{14}CO_2$ can be incorporated by exchange via the reversal of reactions catalysed by phosphoenolpyruvate carboxykinase and malic enzyme (Chang *et al.*, 1966; Utter & Wood, 1951; Hsu, 1970). Müllhofer *et al.* (1977) have shown that, in rat livers perfused in open circuit with [^{14}C]bicarbonate, isotopic equilibrium is reached within minutes between perfusate bicarbonate, tricarboxylic acid-cycle intermediates and glucose.

Our data show that processes by which $^{14}CO_2$, or its equivalent $H^{14}CO_3^-$, is incorporated by the liver into compounds non-volatile in acid are significant and vary with the metabolic status of the organ. Comparison between the rate of $^{14}CO_2$ fixation at equilibrium calculated from the extrapolated specific radioactivity of perfusate bicarbonate (Table 1, Method A) and the integrated rate of $^{14}CO_2$ fixation (Method B) reveals that $^{14}CO_2$ fixation increases with time under our experimental conditions. This may be accounted for in part by the increasing accumulation of pyruvate and malate, the substrates of pyruvate carboxylase and malic enzyme. The non-steady-state character of $^{14}CO_2$ fixation probably explains the lack of agreement between the two estimates of k that come from the fitting of eqn. (2) (in which k would include a term of $^{14}CO_2$ fixation by the liver).

The rates of $^{14}CO_2$ fixation are much larger than the net rates of CO_2 production by the liver. The range of oxygen uptake by perfused livers from normal rats is 6–10 μmol/min per g dry wt. (Williamson *et al.*, 1969; Brunengraber *et al.*, 1973). Assuming a respiratory quotient (RQ) of 0.7 for livers perfused without ethanol (Forsander, 1968), this amounts to a net production of 4.2–7.0 μmol of CO_2/min per g dry wt. Comparing these values with the apparent total rates of $^{14}CO_2$ exchange (Table 1, Method A), it appears that the rate of $^{14}CO_2$ exchange in the perfused liver is 3–15 times the net rate of CO_2 production. In the presence of ethanol, the rate of oxygen uptake by the perfused liver is not altered, but the RQ is markedly decreased to almost zero in livers from starved rats (Forsander, 1968). If the RQ is 0.1, this would result in a rate of $^{14}CO_2$ exchange in livers from starved rats perfused with ethanol about 70 times the net rate of CO_2 production.

There is good evidence in the literature to ascribe the bulk of $^{14}CO_2$ exchange in the perfused liver to the reversal of reactions catalysed by malic enzyme and phosphoenolpyruvate carboxykinase. Hsu (1970) has shown that the rate of malate formation by pigeon liver malic enzyme is about 40% of the rate of pyruvate production. Chang *et al.* (1966) have reported that the relative rates of carboxylation, decarboxylation and oxaloacetate–$H^{14}CO_3^-$ exchange by pig liver phosphoenolpyruvate carb-

oxykinase are in the proportions 1.0:11.3:34 respectively. Rognstad (1981) has shown that, in hepatocytes converting lactate into glucose in the presence of $H^{14}CO_3^-$, activation of phosphoenolpyruvate carboxykinase by Mn^{2+} (Colombo *et al.*, 1978; Brinkworth *et al.*, 1981) increases labelling but not production of glucose.

One can question the validity of using a tracer of exogenous [^{14}C]bicarbonate to estimate the rate of reincorporation of $^{14}CO_2$ generated inside the cell. In another study (S. B. Weinstock, R. R. Kopito & H. Brunengraber, unpublished work), we found that, in two livers from fed rats perfused with 40μM-*R*-[1-^{14}C]mevalonate, the production of $^{14}CO_2$ was 58% and 60% of the amount of label taken up by the liver. In the present study, the corresponding recovery of [^{14}C]bicarbonate label was $63 \pm 3.9\%$ (Table 1). As C-1 of *R*-[1-^{14}C]mevalonate is quantitatively released as CO_2 by pyrophosphomevalonate decarboxylase, we conclude that, as far as recovery of label is concerned, a tracer of [^{14}C]bicarbonate reflects the fate of intracellularly generated $^{14}CO_2$.

Our data show that not taking into account the processes of $^{14}CO_2$ incorporation in the perfused liver can underestimate rates of oxidation of ^{14}C-labelled substrates by up to 50%. Proper evaluation of $^{14}CO_2$ production from a ^{14}C-labelled substrate requires duplicate experiments with unlabelled substrate and a tracer of [^{14}C]bicarbonate to assess reincorporation of labelled CO_2.

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References

Brinkworth, R. I., Hanson, R. W., Fullin, F. A. & Schramm, V. L. (1981) *J. Biol. Chem.* **256**, 10795–10802

Brunengraber, H., Boutry, M. & Lowenstein, J. M. (1973) *J. Biol. Chem.* **248**, 2656–2669

Brunengraber, H., Boutry, M. & Lowenstein, J. M. (1978) *Eur. J. Biochem.* **82**, 373–384

Chang, H.-C., Maruyama, H., Miller, R. S. & Lane, M. D. (1966) *J. Biol. Chem.* **241**, 2421–2430

Colombo, G., Carlson, G. M. & Lardy, H. A. (1978) *Biochemistry* **17**, 5321–5329

Endemann, G., Goetz, P. G., Edmond, J. & Brunengraber, H. (1982) *J. Biol. Chem.* **257**, 3434–3440

Forsander, D. A. (1968) in *Stoffwechsel der isoliert perfundierten Leber* (Staib, W. & Scholtz, R., eds.), pp. 210–215, Springer-Verlag, New York

Good, C., Kramer, H. & Somogyi, M. (1933) *J. Biol. Chem.* **100**, 485–491

Hellwig, J. T. & Council, K. A. (1979) *SAS Users Guide*, pp. 317–329, SAS Institute, Cary

Hoberman, H. D. & d'Adamo, A. F., Jr. (1960) *J. Biol. Chem.* **235**, 934–936

Hsu, R. Y. (1970) *J. Biol. Chem.* **245**, 6675–6682

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Mallette, L. E., Exton, J. H. & Park, C. R. (1969) *J. Biol. Chem.* **244**, 5713–5723

Müllhofer, G., Müller, C., von Stetten, C. & Gruber, E. (1977) *Eur. J. Biochem.* **75**, 331–341

Rognstad, R. (1981) *J. Biol. Chem.* **256**, 1608–1610

Utter, M. F. & Wood, H. G. (1951) *Adv. Enzymol. Relat. Subj. Biochem.* **12**, 41–151

Williamson, J. R., Scholz, R. & Browning, E. T. (1969) *J. Biol. Chem.* **244**, 4617–4627