Pharmacology Biochemistry & Behavior, Vol. 18, Suppl. 1, pp. 285–288, 1983.

Rates of Catabolism Calculated from $^{14}CO_2$ Production: Artifacts and Realities

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TOMERA, J. F., P. G. McCAFFREY AND H. BRUNENGRABER. *Rates of catabolism calculated from $^{14}CO_2$ production: Artifacts and realities.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 285–288, 1983.—We present evidence that the metabolism of acetate and ethanol by the liver markedly decreases the yield of label from mitochondrial [^{14}C]acetyl-CoA to $^{14}CO_2$. The production of [^{14}C]acetyl-CoA from a [^{14}C]labeled substrate can be calculated from the production of $^{14}CO_2$ if one assesses (1) $^{14}CO_2$ reincorporation and (2) the yield of label from [^{14}C]acetyl-CoA to CO_2.

Ethanol Acetate Isotope exchange Citric acid cycle Liver

WHEN a [^{14}C]labeled substrate is oxidized via the Krebs cycle, one may wish to derive two metabolic rates from the production of $^{14}CO_2$: (1) the actual rate of oxidation in the Krebs cycle of [^{14}C]acetyl-CoA derived from the original [^{14}C]substrate and (2) the rate of [^{14}C]acetyl-CoA production. Neither of these rates can be derived directly from the measured production of $^{14}CO_2$ for reasons schematized in Fig. 1. Label from mitochondrial [^{14}C]acetyl-CoA has three main fates: ketogenesis (in the liver), lipogenesis (via citrate) and oxidation in the Krebs cycle. In addition, a fraction of the label entering the Krebs cycle is lost via exchange reactions when the cycle operates in the "no synthesis mode" and via exchange and synthetic reactions when the cycle operates as a synthetic pathway [5,9]. Lastly, a variable fraction of $^{14}CO_2$ produced by the cell is reincorporated into products nonvolatile in acid [7]. In other words, for a given amount of [^{14}C]-labeling of mitochondrial acetyl-CoA, the amount of label measured in $^{14}CO_2$ can vary over a wide range. We have developed methods to evaluate in perfused organs (a) the yield of label from mitochondrial acetyl-CoA to $^{14}CO_2$ and (b) the reincorporation of $^{14}CO_2$. As shown below, these parameters are influenced markedly by the redox shifts induced by ethanol metabolism and by the metabolism of acetate derived from ethanol oxidation.

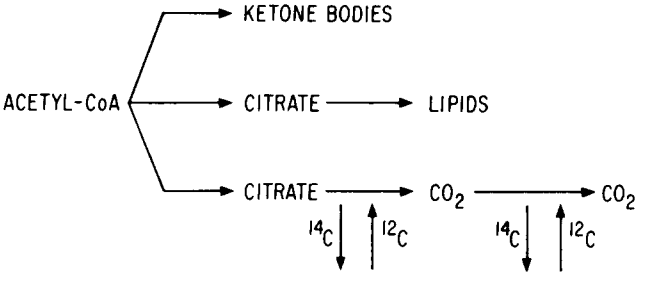

FIG. 1. Fates of mitochondrial acetyl-CoA in liver.

METHOD

Theoretical Considerations

To assess experimentally the fraction of label from mitochondrial acetyl-CoA transferred to $^{14}CO_2$, one needs to label the mitochondrial acetyl-CoA pool with a known number of ^{14}C. This can be achieved by perfusing a liver in open circuit with a low concentration of α-keto-isocaproate (KIC) labeled alternatively on carbons 1 and 2. C-1 of KIC is liberated as CO_2 by mitochondrial branched-chain ketoacid dehydrogenase. C-2 of KIC becomes C-1 of acetyl-CoA. The yield of label from [1-^{14}C]acetyl-CoA to $^{14}CO_2$ is equivalent to the ratio of the rates of $^{14}CO_2$ production from [2-^{14}C]KIC and [1-^{14}C]KIC [8].

To assess the reincorporation of $^{14}CO_2$ into nonvolatile compounds, the production of $^{14}CO_2$ by the liver is simulated by a constant infusion of $NaH^{14}CO_3$ into the perfusate [7]. The recovery of the tracer is defined as [($^{14}CO_2$ evolved from the oxygenator) + ($^{14}CO_2$ in HCO_3 of final perfusate) × 100]/($NaH^{14}CO_3$ infused).

Perfusions

Livers from Sprague-Dawley rats were perfused with Krebs-Ringer bicarbonate buffer containing 4% bovine serum albumin, glucose (15 mM or 4 mM in the case of livers from fed and two-day starved rats, respectively) and other substrates where indicated. The surgical technique and the perfusion apparatus have been described previously [1].

Production of $^{14}CO_2$ by the perfused liver was simulated by a constant infusion of $NaH^{14}CO_3$ in the recirculating perfusate. $^{14}CO_2$ in the gas evolved from the oxygenator and

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TABLE 1

RECOVERY OF A TRACER OF $NaH^{14}CO_3$ INFUSED TO LIVE RATS

Sex (n)	weight (g)	age (d) range	% recovery of tracer
Female (12)	213 ± 4	(75–90)	67.2 ± 3.1
Male (6)	390 ± 7	(75–90)	60.7 ± 4.3
Male (6)	230 ± 6	(55–60)	71.6 ± 4.6

Two groups of male rats were either age-matched or nearly weight-matched to one group of female rats. All rats were infused with 2.5 μCi $NaH^{14}CO_3$/hr for 4 hr. Breath $^{14}CO_2$ was collected for 6 hr. The data are presented as mean ± SE.

present in the bicarbonate pool of the final perfusate was collected as described previously [3].

Experiments with labeled KIC were conducted as follows. After a 15 min period of equilibration with recirculating perfusate, the perfusion circuit was opened and 0.05 mM unlabeled KIC was added to the perfusate. At 20 min [1-^{14}C]KIC (8000 dpm/μmol) was infused into the perfusion line for 5 min. The effluent perfusate was collected from 20 to 30 min. At 30 min, [2-^{14}C]KIC (12,000 dpm/μmol) was infused into the perfusion line for 5 min. The effluent perfusate was collected for 30 to 40 min. $^{14}CO_2$ present in the effluent perfusate was evolved by acidification, trapped in ethanolamine and counted in a liquid scintillation spectrometer.

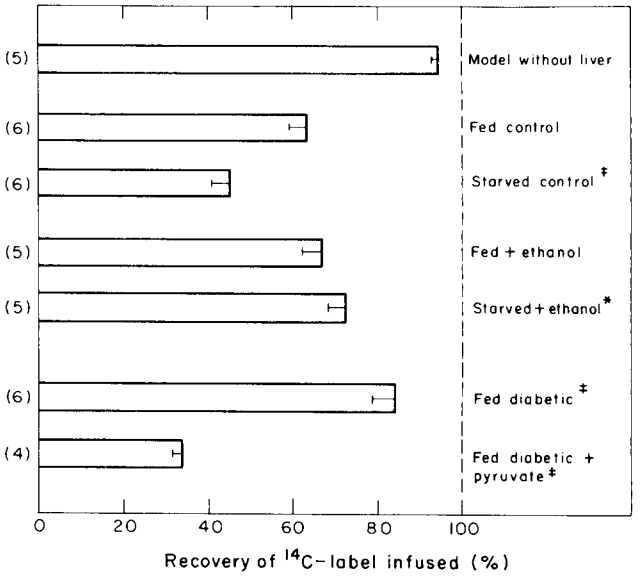

FIG. 2. Recovery of tracer amounts of the $NaH^{14}CO_3$ infused into the perfusate of isolated livers. Significant differences ($p<0.05$) refer to fed controls (‡) or starved conrols (*).

In Vivo *Experiments*

Groups of age-matched or nearly weight-matched male and female rats were used to assess the recovery of $^{14}CO_2$ generated *in vivo*. After an overnight fast, the rats were re-

TABLE 2

DERIVATION OF FACTORS USED TO CONVERT MEASURED RATES OF $^{14}CO_2$ PRODUCTION TO RATES OF MITOCHONDRIAL [1-^{14}C]ACETYL-CoA PRODUCTION FROM [^{14}C]LABELED SUBSTRATES

Group	$^{14}CO_2$ Production from [1-^{14}C]KIC*	$^{14}CO_2$ Production from [2-^{14}C]KIC*	A: $\frac{^{14}CO_2 \text{ from [2-}^{14}\text{C]KIC}}{^{14}CO_2 \text{ from [1-}^{14}\text{C]KIC}}$	B: $^{14}CO_2$ Recovery	$\frac{1}{A \times B}$ Combined factor
Fed Control	93 ± 1.3	21 ± 0.7	0.25 ± 0.04	0.63 ± 0.04	6.3
Fed + Acetate	77 ± 2.5†	5.2 ± 0.5†	0.067 ± 0.0005†	0.54 ± 0.03†	28
Fed + Ethanol	99 ± 4.4‡	9.0 ± 0.4†‡	0.096 ± 0.004†‡	0.67 ± 0.04‡	16
Starved Control	114 ± 19	13 ± 1.4†	0.12 ± 0.01†	0.45 ± 0.05†	19
Fed Diabetic	26 ± 10	0.62 ± 0.08†	0.042 ± 0.01†	0.85 ± 0.06†	28

In the first set of experiments, livers were perfused in open circuit with 0.05 mM of [1-^{14}C] and [2-^{14}C]KIC during alternate 5-min periods [8]. Ratio A, i.e., ($^{14}CO_2$ from [2-^{14}C]KIC/$^{14}CO_2$ from [1-^{14}C]KIC) represents the yield of label from mitochondrial [1-^{14}C]acetyl-CoA to CO_2 [8].

In a second set of experiments, livers were perfused in closed circuit while a tracer of $NaH^{14}CO_3$ was infused continuously from 30 to 90 min [7]. The $^{14}CO_2$ recovery (B) represents the fraction of the infused tracer recovered as $^{14}CO_2$ evolved from the oxygenator plus $^{14}CO_2$ present in the perfusate bicarbonate at 120 min. The combined factor, $1/A \times B$, is the number by which the measured production of $^{14}CO_2$ is to be multiplied to obtain the rate of mitochondrial [1-^{14}C]acetyl-CoA production from a [^{14}C]substrate under each metabolic condition.

*nmol/g dry weight × min; n=6 in all groups.

†Significantly different from controls ($p<0.05$ using two-sided t-test).

‡Significantly different from acetate group ($p<0.05$ using two-sided t-test).

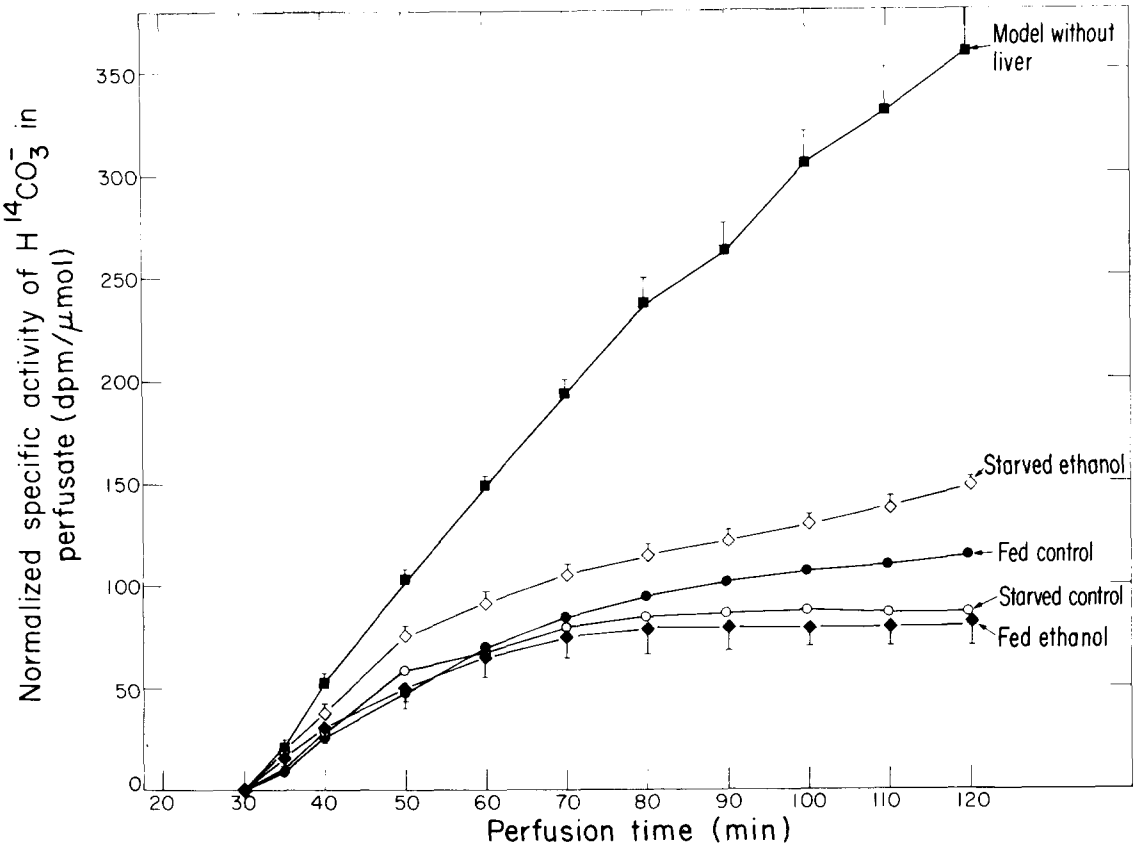

FIG. 3. Specific activity of bicarbonate in the perfusate. The data are normalized for 1 μCi of $NaH^{14}CO_3$ infused over 90 min.

strained in Plexiglas cages equipped with two openings located near the head and the tail of the animal. $NaH^{14}CO_3$ dissolved in saline was infused for 4 hr (2.5 μCi/hr) into a lateral tail vein. This was followed by a 2-hr infusion of saline. The atmosphere of the cage was drawn through the front opening into a NaOH trap for 6 hr. Carbon dioxide was evolved by acidification of the alkaline solution, retrapped in Oxifluor-CO_2 (New England Nuclear) and counted as described previously [3].

RESULTS

The recovery of a tracer of $NaH^{14}CO_3$ injected intravenously in the form of $^{14}CO_2$ exhaled in the breath of live rats varied from 61 to 72% (Table 1). The fraction of label incorporated into non-volatile compounds was not affected by the sex of the animal and by the type of matching system used to compare males and females (by age or by weight).

In perfusion experiments, the recovery of tracer amounts of $NaH^{14}CO_3$ infused into the perfusate was 95% in control experiments without a liver (Fig. 2). In the presence of a liver, 10 to 50% of the infused label was incorporated into compounds nonvolatile in acid (Fig. 2 and Table 2). Ethanol and oleate significantly decreased the incorporation of $^{14}CO_2$ in livers from starved but not from fed rats. Note that in the two gluconeogenic states of starvation and diabetes, the recoveries of infused label were markedly different. In livers from fed rats, the recovery of label was not affected by the presence of 20 μM acetazolamide, an inhibitor of carbonic anhydrase (not shown). The specific activity of bicarbonate in the perfusate varied roughly in parallel with the percentage recovery of $^{14}CO_2$ (Fig. 3).

The yield of label from mitochondrial [1-^{14}C]acetyl-CoA to CO_2, calculated as the ratio of $^{14}CO_2$ production from [2-^{14}C] and [1-^{14}C]KIC was 25% in livers from fed rats (Table 2). This yield of label was decreased markedly by starvation and diabetes as well as by the presence of acetate or ethanol in the perfusate.

DISCUSSION

Our studies show that the reincorporation of $^{14}CO_2$ into compounds nonvolatile in acid is neither negligible nor constant in the liver. There is good evidence that the bulk of $^{14}CO_2$ reincorporation occurs at the level of phosphoenolpyruvate carboxykinase (PEP-CK). Chang *et al.* [2] have shown that the oxaloacetate-HCO_3^- exchange catalyzed by PEP-CK is three times faster than the net rate of phos-

phoenolpyruvate production. This explains the high $^{14}CO_2$ incorporation in livers from starved rats in which PEP-CK is activated. In livers from diabetic rats, only a small fraction of the $H^{14}CO_3^-$ infused is incorporated in spite of the high activity of PEP-CK present in these livers. This can be explained by the very low production of pyruvate by these livers. The incorporation of $^{14}CO_2$ by PEP-CK is probably limited by the availability of oxaloacetate generated by pyruvate carboxylation. When livers from diabetic rats were perfused with 5 mM pyruvate, the recovery of infused $H^{14}CO_3^-$ decreased from 85 to 40% (Fig. 2).

In their 1957 review, Weinman *et al.* emphasized the importance of isotopic exchange from the Krebs cycle even when the cycle operates in the "no synthesis mode"—that is, when the only species feeding into the cycle is acetyl-CoA [9]. The stoichiometry of the conversion of 1 acetyl-CoA to 2 CO_2 is not paralleled by a quantitative transfer of the label carried by acetyl-CoA to CO_2. Substrates of the cycle are in partial isotopic equilibrium with amino acids (such as glutamate or aspartate) and glycolytic intermediates. An equal but variable fraction of the label from both carbons of acetyl-CoA is lost and the "CO_2 ratio" defined by Weinman *et al.* as the ratio of $^{14}CO_2$ production from [2-^{14}C] acetate and [1-^{14}C]acetate is equal to 1.0.

The process of loss of label from the Krebs cycle is further complicated when the cycle operates in the "synthetic mode" [5], that is when an unlabeled gluconeogenic substrate such as glutamate is fed into the cycle together with [^{14}C]acetyl-CoA. When carbon from glutamate leaves the cycle as oxaloacetate, it carries part of the label from [^{14}C]acetyl-CoA [6]. Furthermore, unequal fractions of the label from the two carbons of acetyl-CoA are lost in this synthetic process and Weinman *et al.*'s "CO_2 ratio" is greater than 1.0 [5,9].

The factor derived from (1) the differential yield in $^{14}CO_2$ of tracers of [1-^{14}C] and [2-^{14}C]KIC, and from (2) the recovery of a tracer of $NaH^{14}CO_3$ (Table 2) correct for all the processes which prevent label from mitochondrial [1-^{14}C]acetyl-CoA from being liberated as free $^{14}CO_2$. In other words, these factors allow one to calculate the production of mitochondrial [1-^{14}C]acetyl-CoA from a [^{14}C]labeled substrate. When the Krebs cycle operates in the "no synthesis mode", the recovery factors of Table 2 are valid for substrates generating either [1-^{14}C] or [2-^{14}C]-acetyl-CoA. When the Krebs cycle operates in the "synthetic mode", in order to trace the yield of label from [2-^{14}C]acetyl-CoA to CO_2, one should perfuse the liver with [1-^{14}C] and [3-^{14}C]KIC as the latter generates [2-^{14}C]acetyl-CoA.

The data from Table 2 show that the presence of ethanol or acetate decreases markedly the production of $^{14}CO_2$ from a given amount of [^{14}C]acetyl-CoA derived from a [^{14}C]labeled substrate. The mechanisms by which ethanol and acetate exert this effect are certainly different because ethanol, but not acetate, inhibits the operation of the Krebs cycle [4]. Although the fates of the carbon and of the label of [^{14}C]acetyl-CoA are affected markedly by ethanol or acetate, it is still possible, using the methods described above, to assess a rate of catabolism defined as the production of [^{14}C]acetyl-CoA from a labeled substrate.

In the whole animal, about one-third of the production of $^{14}CO_2$ is reincorporated into non volatile compounds (Table 1). The yield of label from mitochondrial [^{14}C]acetyl-CoA to CO_2 has not yet been assessed *in vivo*.

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