Assessment of the flux of mitochondrial acetyl-CoA in liver and kidney by using the differential production of $^{14}\text{CO}_2$ from tracers of (1- ^{14}C)- and (2- ^{14}C)-labelled 4-methyl-2-oxovalerate

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A procedure is described to convert rates of $^{14}\text{CO}_2$ production into rates of mitochondrial acetyl-CoA production from a ^{14}C -labelled substrate. The principle is illustrated in perfused rat liver and kidney by the differential yield of $^{14}\text{CO}_2$ from 4-methyl-2-oxo[1- ^{14}C]valerate and 4-methyl-2-oxo[2- ^{14}C]valerate.

The present paper describes a procedure whereby measurements of rates of $^{14}\text{CO}_2$ production from a ^{14}C -labelled substrate, can be used to assess the relationship between mitochondrial production and oxidation of acetyl-CoA. The transfer to $^{14}\text{CO}_2$ of label from mitochondrial [^{14}C]acetyl-CoA is not quantitative for three reasons. First, a substantial and variable fraction of $^{14}\text{CO}_2$ produced by the cell can be re-incorporated into products non-volatile in acid (Tomera *et al.*, 1982). Secondly, in addition to its oxidation in the tricarboxylic acid cycle, acetyl-CoA can be incorporated into ketone bodies (in the liver), or transferred to the cytosol via citrate (Watson & Lowenstein, 1970). Third, a fraction of the label entering the tricarboxylic acid cycle is lost (i) via exchange reactions when the cycle operates in the 'non-synthesis' mode, and (ii) via exchange and synthetic reactions when the cycle operates as a synthetic pathway (Katz & Chaikoff, 1955; Weinman *et al.*, 1957; Krebs *et al.*, 1966). As shown below, the yield of label from mitochondrial [1- ^{14}C]acetyl-CoA to $^{14}\text{CO}_2$ can be calculated from the yields of $^{14}\text{CO}_2$ from tracers of (1- ^{14}C)- and (2- ^{14}C)-labelled MOV.

Experimental

Theoretical considerations

To assess the yield of ^{14}C -label in CO_2 from mitochondrial acetyl-CoA, one needs a procedure (that is, a metabolic pathway) by which a known

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Abbreviations used: MOV, 4-methyl-2-oxovalerate (α -oxoisocaproate); $^{14}\text{CO}_2$ labelling ratio, ($^{14}\text{CO}_2$ production from [2- ^{14}C]MOV)/($^{14}\text{CO}_2$ production from [1- ^{14}C]MOV).

amount of label from a suitable precursor can be channelled into mitochondrial acetyl-CoA. The ideal 'test' pathway should meet the following criteria: (1) the pathway should lead to mitochondrial acetyl-CoA exclusively; (2) the pathway should be unbranched; (3) intermediates of the pathway should not accumulate; (4) the rate of acetyl-CoA production by the pathway should be small compared with the rate of turnover of mitochondrial acetyl-CoA; (5) one should be able to assess the exact amount of label channelled through the first irreversible step of the pathway.

As far as we can determine, no pathway catabolizing one singly labelled substrate satisfies these five conditions. One can nevertheless devise a suitable protocol based on the differential production of $^{14}\text{CO}_2$ from MOV labelled at C-1 or C-2. C-1 of MOV is liberated as CO_2 by mitochondrial branched-chain keto-acid dehydrogenase, which converts MOV into isovaleryl-CoA. C-2 of MOV becomes C-1 of mitochondrial acetyl-CoA. On this basis, we designed experiments in which an isolated organ is perfused in open circuit with a low concentration of unlabelled MOV. [1- ^{14}C]- and [2- ^{14}C]-MOV are then infused alternately with label-free intervals. As the specific activities of both tracers are known, the following calculations can be performed. The production of $^{14}\text{CO}_2$ from [1- ^{14}C]MOV represents the apparent rate of MOV catabolism. This rate multiplied by the specific radioactivity of [2- ^{14}C]MOV yields the calculated d.p.m. from [2- ^{14}C]MOV that are channelled into mitochondrial acetyl-CoA. Division of d.p.m. in $^{14}\text{CO}_2$ from [2- ^{14}C]MOV by this number yields the fraction of the label from mitochondrial acetyl-CoA that is converted into CO_2 . The same fraction can be obtained more directly by comparing the apparent

rates of $^{14}\text{CO}_2$ production from $[2\text{-}^{14}\text{C}]$ - and $[1\text{-}^{14}\text{C}]\text{-MOV}$.

Methods

L-Amino acid oxidase, catalase and sodium MOV were purchased from Sigma. $\text{L-[1-}^{14}\text{C]Leucine}$, $[^{14}\text{C}]\text{toluene}$ counting standard and Oxyfluor- CO_2 liquid-scintillation counting fluid were obtained from New England Nuclear. $\text{L-[2-}^{14}\text{C]Leucine}$ was purchased from Research Products Inc. $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]\text{-leucine}$ were converted into $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]\text{-MOV}$ by L-amino acid oxidase and catalase (Rudiger *et al.*, 1972). The product of the reaction was run through a small column ($0.4\text{ cm} \times 1\text{ cm}$) of Dowex AG-50 (X8; H^+ form) to remove unchanged $[^{14}\text{C}]\text{leucine}$. $[^{14}\text{C}]\text{MOV}$ was further purified by chromatography on Dowex AG-1 (X8; Cl^- form) as shown in Fig. 7 of Sabourin & Bieber (1981). Treatment of $[1\text{-}^{14}\text{C}]\text{MOV}$ and $[2\text{-}^{14}\text{C}]\text{MOV}$ with acidic H_2O_2 (Odessey & Goldberg, 1979) evolved 99% and less than 1% of the radiolabel as CO_2 respectively. This confirms the radiochemical purity of the tracers.

Livers from schedule-fed Sprague-Dawley rats were perfused with Krebs-Ringer bicarbonate buffer with or without 4% dialysed bovine serum albumin (fraction V; fatty acid-poor; Miles) and glucose (15 mM or 4 mM 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). Kidneys from fed rats were perfused with buffer containing 6.8% albumin and 10 mM-glucose. The technique and apparatus were those of Nishiitsutsuji-Uwo *et al.* (1967). After 15 min of recirculating perfusion, the perfusion circuit was opened and unlabelled

MOV was infused into the perfusion line via a syringe pump to achieve a final concentration of 0.05 or 0.2 mM. After 20 min, $[1\text{-}^{14}\text{C}]\text{MOV}$ was infused for 5 min at a specific radioactivity of $4.0 \times 10^4\text{ d.p.m./}\mu\text{mol}$. The effluent perfusate was collected from 20 to 30 min into a glass cylinder containing 2 ml of 5 M-NaOH to prevent loss of $^{14}\text{CO}_2$. At 30 min, $[2\text{-}^{14}\text{C}]\text{MOV}$ was infused for 5 min at a specific radioactivity of $8.0 \times 10^4\text{ d.p.m./}\mu\text{mol}$ and the effluent perfusate was collected from 30 to 40 min. The order of infusion of $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]\text{-MOV}$ was changed between experiments. Portions (100 ml) of effluent perfusate were transferred to wide-mouth 1-litre Erlenmeyer flasks fitted with rubber stoppers from the bottoms of which were suspended liquid-scintillation vials containing 2 ml of ethanolamine. $^{14}\text{CO}_2$ in the perfusate was released by injecting 150 ml of 1 M-citric acid through the stopper into the flasks. The flasks were agitated for 3 h, after which 15 ml of Oxyfluor- CO_2 was added to the counting vial. Counting efficiencies of all samples were determined with internal standards of $[^{14}\text{C}]\text{toluene}$.

Results

Table 1 shows that the ratio of $^{14}\text{CO}_2$ production from $[2\text{-}^{14}\text{C}]\text{-}$ and $[1\text{-}^{14}\text{C}]\text{-MOV}$ ($^{14}\text{CO}_2$ labelling ratio) in liver is markedly affected by the presence of albumin in the perfusate and whether the organ donor is fed, starved or diabetic. In liver perfusions without albumin, the $^{14}\text{CO}_2$ labelling ratio was the same as the two MOV concentrations used (0.2 and 0.05 mM). On the other hand, in the same perfusions, the absolute rates of $[1\text{-}^{14}\text{C}]\text{-}$ and $[2\text{-}^{14}\text{C}]\text{-MOV}$ catabolism decreased 17–18-fold when the sub-

Table 1. $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{-}$ and $[2\text{-}^{14}\text{C}]\text{-MOV}$ in perfused livers and kidneys
The glucose concentration in the perfusate was 15 mM and 4 mM for livers from fed and starved rats, and 10 mM for kidneys. For additional details, see under 'Methods'. Results are means $\pm$ S.E.M.

	Sex (n)	Status	Additions to perfusion medium		$^{14}\text{CO}_2$ production (nmol/g dry wt. per min) from		$^{14}\text{CO}_2$ from $[2\text{-}^{14}\text{C}]\text{MOV}$
			Albumin (g/litre)	MOV (mM)	$[1\text{-}^{14}\text{C}]\text{MOV}$	$[2\text{-}^{14}\text{C}]\text{MOV}$	$^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{MOV}$
Liver	M(6)	Fed	0	0.2	1550 ± 413	489 ± 205	0.29 ± 0.05
	F(5)	Fed	0	0.2	1380 ± 270	487 ± 108	0.35 ± 0.06
	F(3)	Fed	0	0.05	75 ± 10	28 ± 4	0.37 ± 0.02
	M(6)	Fed	4.0	0.05	93 ± 1.3	21 ± 0.7	0.25 ± 0.04
	F(6)	Fed	4.0	0.05	$59 \pm 5^*$	$14 \pm 1.4^*$	0.25 ± 0.01
	M(5)	Starved	4.0	0.05	114 ± 19	$13 \pm 1.4^*$	$0.12 \pm 0.01^*$
	M(6)	Fed diabetic	4.0	0.05	$26 \pm 10^*$	$0.62 \pm 0.08^*$	$0.042 \pm 0.01^*$
	M(6)	Fed	6.8	0.2	116 ± 9	39 ± 4	0.34 ± 0.03
Kidney	F(4)	Fed	6.8	0.2	$146 \pm 8^*$	53 ± 6	0.37 ± 0.03

* Significantly different from the corresponding fed male control ($P < 0.05$).

strate concentration decreased only 4-fold. This is in contrast with findings of other groups, who reported that the rate of $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{MOV}$ by perfused livers (Patel *et al.*, 1981) or incubated hepatocytes (Corkey *et al.*, 1982) was proportional to the concentration of MOV in the range 0.05–0.2 mM. Note that in these other studies the perfusion or incubation medium did not contain glucose.

Addition of albumin to the perfusate decreased slightly the rate of $[1\text{-}^{14}\text{C}]\text{MOV}$ decarboxylation. This effect might be explained by the binding of MOV to albumin (Livesey & Lund, 1982; Nissen *et al.*, 1982). In perfusions with albumin, the absolute rate of MOV catabolism was greater in livers from male rats compared with females. An inverse sex difference in rates of MOV catabolism was observed in perfused kidneys. There was no sex difference in the $^{14}\text{CO}_2$ labelling ratio in liver or kidney.

Discussion

The production of $^{14}\text{CO}_2$ from low concentrations of $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]\text{-MOV}$ as an index of the yield of label from mitochondrial acetyl-CoA to $^{14}\text{CO}_2$ rests on the assumption that liver mitochondria contain one single pool of acetyl-CoA. If this is so, the five criteria listed under 'Theoretical considerations' are met. The experimental protocol allows cancellation of three systematic errors that affect equally the production of $^{14}\text{CO}_2$ from both tracers. First, although the rates of $^{14}\text{CO}_2$ production are not corrected for $^{14}\text{CO}_2$ re-incorporation into compounds non-volatile in acid, it is reasonable to assume that $^{14}\text{CO}_2$ derived from $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]\text{-MOV}$ are re-incorporated in the same proportions. The re-incorporation factors would then cancel out in the final calculation. Secondly, the transamination of labelled MOV to leucine affects equally the concentration of both tracers. Lastly, production of unlabelled MOV by transamination of leucine affects equally the specific activities of both tracers. In other words, although it is not possible to calculate precisely the number of ^{14}C d.p.m. derived from $[2\text{-}^{14}\text{C}]\text{MOV}$ that enter the mitochondrial acetyl-CoA pool, one can calculate precisely the yield of ^{14}C -label from mitochondrial $[1\text{-}^{14}\text{C}]\text{acetyl-CoA}$ to CO_2 . Our data on the fraction of $[1\text{-}^{14}\text{C}]\text{acetyl-CoA}$ derived from $[2\text{-}^{14}\text{C}]\text{MOV}$ that is converted into $^{14}\text{CO}_2$ in livers from fed and starved rats are similar to those reported by Lund (1978) and by Corkey *et al.* (1982).

Note that in experiments with $[2\text{-}^{14}\text{C}]\text{MOV}$, acetoacetate produced by MOV catabolism is not labelled. Therefore, utilization of acetoacetate in liver (Endemann *et al.*, 1982) or kidney (Buchanan *et al.*, 1945) does not label the acetyl-CoA pool, and thus acetoacetate utilization need not be taken into account in the interpretation of our data.

To calculate the rate of conversion of a substrate such as $(R)\text{-}[5\text{-}^{14}\text{C}]\text{mevalonate}$ into mitochondrial $[1\text{-}^{14}\text{C}]\text{acetyl-CoA}$ (Edmond & Popják, 1974), the rate of $^{14}\text{CO}_2$ production is divided by the product of two recovery factors: (i) the yield of label from $[1\text{-}^{14}\text{C}]\text{acetyl-CoA}$ to $^{14}\text{CO}_2$ (Table 1) and (ii) recovery of a tracer of $\text{NaH}^{14}\text{CO}_3$ to correct for $^{14}\text{CO}_2$ re-incorporation (Tomera *et al.*, 1982). Under our experimental conditions, the recovery of a tracer of $\text{NaH}^{14}\text{CO}_3$ is 63, 45 and 85% in livers from fed, starved and diabetic rats respectively. In kidneys from fed rats, the recovery of a tracer of $\text{NaH}^{14}\text{CO}_3$ is 92% (R. R. Kopito & H. Brunengraber, unpublished work). In perfused livers from fed, starved and diabetic rats, the combined correction factors are $1/(0.25 \times 0.63) = 6.3$, $1/(0.12 \times 0.45) = 18.5$ and $1/(0.85 \times 0.041) = 28$ respectively. In perfused kidneys from fed male rats, the combined correction factor is $1/(0.34 \times 0.92) = 3.2$.

The calculated flux of acetyl-CoA generated from a ^{14}C -labelled substrate combines all the fates of mitochondrial acetyl-CoA, including the rate of acetyl-CoA oxidation in the tricarboxylic acid cycle. It is assumed that the cycle operates in a steady state and that the intermediates of the cycle and acetyl-CoA are in isotopic equilibrium. This is achieved after a few turns of the cycle (Katz & Chaikoff, 1955; Weinman *et al.*, 1957). Note that the production of $^{14}\text{CO}_2$, even when corrected for $^{14}\text{CO}_2$ re-incorporation, gives only a minimal value for the rate of $[1\text{-}^{14}\text{C}]\text{acetyl-CoA}$ oxidation. Even when the cycle operates in the 'no synthesis' mode, label from $[1\text{-}^{14}\text{C}]\text{acetyl-CoA}$ is lost from the cycle via exchange processes.

When both carbon atoms in the acetyl moiety of acetyl-CoA are labelled, the yields of label from C-1 and C-2 are markedly different when the tricarboxylic acid cycle operates in the 'synthetic' mode (Weinman *et al.*, 1957). Therefore, for substrates generating $[2\text{-}^{14}\text{C}]$ - or $[1,2\text{-}^{14}\text{C}]\text{-acetyl-CoA}$, the yield of label from acetyl-CoA to CO_2 should be determined in experiments with $[1\text{-}^{14}\text{C}]\text{MOV}$ and either $[3\text{-}^{14}\text{C}]$ - or $[2,3\text{-}^{14}\text{C}]\text{-MOV}$.

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