A ^{13}C isotopomer n.m.r. method for monitoring incomplete β -oxidation of fatty acids in intact tissue

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An n.m.r. method is presented for monitoring the extent to which fatty acids undergo β -oxidation without release of shorter-chain intermediates. It is based upon a ^{13}C isotopomer analysis of glutamate from tissue presented with a mixture of [2,4,6,8- ^{13}C]octanoate and [1,2,3,4- ^{13}C]octanoate. The method does not require steady-state metabolic or isotopic conditions, so it may be applied during a variety of metabolic circumstances, including perfused tissue under stress and *in vivo*. We have tested the method in perfused rat hearts during anoxia, a model where previous work has shown that β -oxidation of palmitate is incomplete and shorter-chain intermediates are released [Rabinowitz and Hercker (1974) Arch. Biochem. Biophys. 161, 621–627].

Indeed, n.m.r. spectra of freeze-clamped, acid-extracted tissue show that octanoate undergoes complete β -oxidation in control normoxic rat hearts, but not in anoxic hearts. Complete β -oxidation of octanoate was observed under a number of other metabolic conditions in perfused rat hearts, including low-pressure-induced ischaemia, KCl arrest and in the presence of high concentrations of competing substrates. We also demonstrate that the technique is applicable in intact tissue by taking direct measurements in perfused rat hearts using a recently published [^{13}C]homonuclear decoupling technique and in *in vivo* heart and liver removed from rats after an intravenous infusion of a mixture of [2,4,6,8- ^{13}C]octanoate and [1,2,3,4- ^{13}C]octanoate.

INTRODUCTION

Recently there has been increasing interest in the control mechanisms involving β -oxidation of fatty acids because of diseases that are now known to result from genetic defects of one or more enzymes involved in this oxidative pathway [1,2]. β -oxidation of a fatty acyl-CoA occurs in mitochondria as a repetitive process of sequential dehydrogenation, hydration, oxidation to a β -oxo acid and finally condensation with a second molecule of CoA. Conversion of palmitoyl-CoA into acetyl-CoA normally takes place without detectable accumulation of medium- or short-chain acyl-CoA species [3,4], and it has therefore been suggested that the enzymes of β -oxidation exist as organized complexes, either in association with the mitochondrial inner membrane or in the mitochondrial matrix [5,6]. Carpenter et al. [7] provided recent direct evidence for organization of several β -oxidation enzymes by purifying to homogeneity the long-chain-specific 3-hydroxyacyl-CoA dehydrogenase from human infant liver and showing that the 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-oxoacyl-CoA thiolase activities are all part of the same, multifunctional, membrane-bound enzyme. Uchida et al. [8] confirmed that the long-chain 3-hydroxyacyl-CoA dehydrogenase from rat liver is also a trifunctional protein and is membrane-bound. The corresponding 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-oxoacyl-CoA thiolase activities involved with β -oxidation of short-chain and medium-chain acyl-CoA species and all of the acyl-CoA dehydrogenases (the enzymes which catalyse the first step in the β -oxidation spiral pathway) are all thought to be water-soluble matrix proteins, and here experimental evidence for functional organization is more elusive. Sumegi and Srere [5] have shown that several water-soluble β -oxidation enzymes, including the short-chain enoyl-CoA hydratase (crotonase), β -hydroxyacyl-CoA dehydrogenase, and β -ketothiolase, all bind to mitochondrial

inner membranes but not to phosphatidylcholine liposomes. More recently, Sumegi et al. [9] showed that respiration-linked oxidation of 3-hydroxybutyryl-CoA was significantly faster in gently sonicated mitochondria (where the presumed organization of matrix enzymes was partially intact) than in fully disrupted mitochondria (where any organized structure was certainly destroyed and diffusion becomes rate-limiting). Both experimental observations lend support to the view that the matrix soluble enzymes may be organized as well.

Deficiencies in acyl-CoA dehydrogenases have now been associated with numerous inborn errors of metabolism [2]. In particular, both short-chain [10] and medium-chain [11,12] acyl-CoA dehydrogenase deficiencies have been identified in humans. Although the functional significance of organization of the β -oxidation enzymes remains a question worthy of more study, it is possible that disruption of such organization could in principle play a role in some diseases associated with abnormal metabolism of fatty acids. Numerous other reports have appeared, for example showing that, in certain circumstances, hydroxy fatty acids and short-chain fatty acids can be detected *in vitro* [13–15]. Rabinowitz and Hercker [16] have also shown that palmitate does not undergo complete β -oxidation in intact, anoxic rat hearts and that shorter-chain intermediates do leak from cells.

We have been interested in developing a non-invasive n.m.r. method for evaluating the extent to which a fatty acid is committed to complete β -oxidation once it enters this spiral of reactions in intact tissues. We recently introduced a ^{13}C isotopomer technique to measure relative substrate utilization in perfused hearts by simple inspection of the glutamate C-4 resonance [17] and have adapted this method to study the extent of β -oxidation of the medium-chain fatty acid octanoate in perfused hearts during differing physiological states and in the presence of various substrates which can contribute to acetyl-CoA entering the citric acid cycle in this tissue. Forsey et al. [18]

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have shown that octanoate is avidly used by perfused hearts and that other carbohydrate fuels and ketone bodies do not compete effectively with this medium-chain fatty acid. Since octanoate can enter mitochondria without transport by carnitine [18], we hypothesized that this medium-chain fatty acid might accumulate in mitochondria and hence increase the probability of release of one or more shorter-chain intermediates from the β -oxidation pathway. Although octanoate is not considered to be physiological substrate, it has been used in perfused-heart experiments [18,19]. Also, the commercial availability of ^{13}C -enriched octanoates with multiple labelling patterns made this medium-chain fatty acid a reasonable choice to use in developing the n.m.r. method and to illustrate the principles involved. Our n.m.r. results verify that glucose and other glycolytic substrates do not compete effectively with octanoate for oxidation by heart tissue, while higher concentrations of ketone bodies suppress octanoate oxidation substantially. Data from anoxic hearts verify that, like palmitate [16], octanoate does not undergo complete β -oxidation in rat heart mitochondria. However, n.m.r. data from hearts examined under a variety of other physiological conditions, including low-pressure-induced ischaemia, KCl arrest and in the presence of competing substrates, indicate that every molecule of octanoate which enters the β -oxidation spiral pathway is fully committed to three complete rounds of oxidation.

MATERIALS AND METHODS

[2,4,6,8- ^{13}C]Octanoate and [1,2,3,4- ^{13}C]octanoate were purchased from Tracer Technologies (Somerville, MA, U.S.A.) as sodium salts. All other reagents from commercially available sources were of the highest quality available.

Male Sprague-Dawley rats weighing 300–350 g were anaesthetized under a diethyl ether atmosphere and their hearts were rapidly exercised and placed in an ice-cold Krebs-Henseleit buffer. The aorta was immediately cannulated, and retrograde perfusion of the coronary arteries was initiated at a normal pressure of 70 cm of water. In some hearts, the pressure was

decreased to 25 cm of water to reduce coronary flow and induce partial ischaemia, while in others anoxia was induced by switching the gas bubbled through the perfusate from the usual O_2/CO_2 (19:1) to N_2/CO_2 (19:1). The coronary flow rates were 12–18 ml/min in hearts perfused at normal pressures and 5–7 ml/min at low pressure. All hearts were perfused with a modified Krebs-Henseleit buffer containing 119.2 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl_2 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 and 10 mM glucose. After addition of a ^{13}C -enriched substrate, each heart was perfused with 125 ml of recirculating buffer to isotopic and metabolic steady state (30 min for normal pressure, both normoxic and anoxic; 45 min for low pressure) before freeze-clamping. The time required to reach steady state was determined by freeze-clamping hearts at various time points after introduction of a ^{13}C -enriched octanoate and comparing their spectra. The tissue was judged to be at isotopic and metabolic steady state when the glutamate ^{13}C -multiplet peak areas no longer changed with longer perfusion periods. Freeze-clamped hearts were extracted with 7% (w/v) cold HClO_4 , neutralized with KOH, freeze-dried and dissolved in 0.6 ml of $^2\text{H}_2\text{O}$ for n.m.r. analysis.

For the *in vivo* experiments, a 1:1 (54 mg each) mixture of [2,4,6,8- ^{13}C]octanoate and [1,2,3,4- ^{13}C]octanoate was dissolved in 2 ml of deionized water. Rats (180–200 g) were anaesthetized by intramuscular injection of a bolus mixture of 500 μl of ketamine (100 mg/ml) and 70 μl of xylazine (100 mg/ml). The juglar vein was cannulated, and the mixture of octanoates was infused continuously for 1 h (2 ml/h) before freeze-clamping the heart and liver. The freeze-clamped tissue samples were extracted as described above.

N.m.r. methods

Proton-decoupled ^{13}C n.m.r. spectra of the heart extracts were obtained on a GN-500 spectrometer in a 5 mm probe using 16000 data points, a 45° observe pulse every 6 s, and WALTZ bilevel proton decoupling. The temperature was maintained at 25°C using a standard General Electric variable temperature accessory. All spectra were zero-filled to 32000 points before Fourier transformation, and the relative areas of the multiplet components in each glutamate resonance were determined by using the deconvolution package in n.m.r.-286 (SoftPulse Software, Guelph, Ontario, Canada).

Spectra of intact, perfused hearts were collected in a 18 mm thin-walled n.m.r. tube (Wilma) with the heart bathed in perfusate using a 30° observe pulse every 1.3 s, with WALTZ bilevel proton decoupling, and 16000 data points, as previously described [17]. Shimming was performed on the ^{23}Na free induction decay; typical linewidth was 17–22 Hz. Homonuclear ^{13}C decoupling was accomplished by using single-frequency decoupling generated by the spectrometer's F3 channel [20]. The relative areas of the multiplet components in each glutamate resonance were quantified as described above.

Isotopomer methods and theory

The glutamate C-4 resonance from a ^{13}C -n.m.r. spectrum of an extract of a rat heart perfused with a 1:1 molar mixture (0.25 mM each) of [1,2,3,4- ^{13}C]- and [2,4,6,8- ^{13}C]-octanoate is shown in Figure 1. This resonance has four multiplet components: a singlet (S), two doublets (D34 and D45) and a quartet (Q). The singlet (S) represents those isotopomers which have ^{13}C at C-4, but not at C-3 or C-5. One of the doublets, D34, corresponds to those isotopomers with ^{13}C at both C-3 and C-4, but not at C-5. The second doublet, D45, has a different coupling constant and

Figure 1 Proton-decoupled ^{13}C -n.m.r. spectrum of an extract of a rat heart showing the glutamate C-4 resonance

The heart was supplied with a 1:1 molar mixture (0.25 mM each) of [1,2,3,4- ^{13}C]- and [2,4,6,8- ^{13}C]-octanoate. Abbreviations: S, singlet; D34, doublet due to J34; D45, doublet due to J45; Q, quartet or doublet of doublets.

represents isotopomers having ^{13}C at both C-4 and C-5, but not at C-3. Finally, a quartet (Q, or, more precisely, a doublet of doublets) corresponds to those isotopomers with carbon enriched at all three glutamate carbons (C-3, C-4 and C-5). The areas of the glutamate C-4 multiplet resonances reflect the uptake of acetyl-CoA enriched at different carbon atoms. The ^{13}C fractional enrichment of the acetyl-CoA carbon atoms entering the citric acid cycle is defined according to Malloy et al. [21]. In this notation, the fraction of acetyl-CoA enriched with ^{13}C in the carbonyl carbon only (C-1), the methyl carbon only (C-2), or in both carbons, is denoted as F_{C1} , F_{C2} or F_{C3} respectively [21]. The fraction of unenriched acetyl-CoA (natural abundance levels of ^{13}C) is denoted as F_{C0} . Note that this latter definition does not distinguish between unenriched acetyl-CoA carbon atoms derived from the unenriched 'end' of [1,2,3,4- ^{13}C]octanoate versus those derived from other unenriched, endogenous substrates. For this reason we define here the fraction of unenriched acetyl-CoA derived from [1,2,3,4- ^{13}C]octanoate as $F_{\text{C0'}}$ and the fraction of acetyl-CoA derived from other endogenous unlabelled sources as $F_{\text{C0''}}$. Given these definitions, our previous equations then apply to the present experimental conditions, where:

$$F_{\text{C0'}} + F_{\text{C0''}} = F_{\text{C0}}$$

and

$$F_{\text{C0}} + F_{\text{C2}} + F_{\text{C3}} = 1$$

Furthermore, when [1,2,3,4- ^{13}C]octanoate and [2,4,6,8- ^{13}C]octanoate are presented to tissue in equal concentrations, it may be shown that:

$$F_{\text{C2}} - F_{\text{C3}} = F_{\text{C0'}} \text{ (see below)}$$

Statistical analysis

All results are presented as the mean $\pm$ 1 S.D. Results were compared by analysis of variance with use of the Student-Newman-Keuls [22] test where applicable (Table 2 below).

RESULTS

Hearts perfused with either [2,4,6,8- ^{13}C]octanoate or [1,2,3,4- ^{13}C]octanoate

Representative ^{13}C spectra of hearts perfused to steady state with either 0.5 mM [2,4,6,8- ^{13}C]octanoate (top) or [1,2,3,4- ^{13}C]octanoate (bottom) are shown in Figure 2(a) and Figure 2(b) respectively. A complete steady-state isotopomer analysis of each spectrum was performed as described by Malloy et al. [21] using the individual multiplet areas of the glutamate C-2, C-3 and C-4 resonances. An analysis of the top spectrum indicated that F_{C2} was 0.96 and $F_{\text{C0'}}$ was, by difference, 0.04. These F_{C2} and $F_{\text{C0'}}$ values indicate that 96% of the total acetyl-CoA oxidized by this heart was derived from [2,4,6,8- ^{13}C]octanoate, while only 4% was derived from unidentified endogenous sources (Table 1).

The spectrum shown at the bottom of Figure 2 from a heart perfused to steady-state with [1,2,3,4- ^{13}C]octanoate appears quite different from the top spectrum, reflecting the different labelling pattern of the acetyl-CoA which entered the citric acid cycle in this heart. An isotopomer analysis of this spectrum indicated that F_{C3} was 0.49, with unenriched acetyl-CoA contributing the difference ($F_{\text{C0}} = 1 - 0.49 = 0.51$). Since the contribution from unenriched endogenous sources must equal that in the heart perfused with [2,4,6,8- ^{13}C]octanoate ($F_{\text{C0''}} = 0.04$), the contribution from the unenriched 'end' of [1,2,3,4- ^{13}C]octanoate ($F_{\text{C0'}}$) must have been $0.51 - 0.04 = 0.47$. This value was not significantly different from F_{C3} in these hearts (Table 1), indicating that each octanoate which entered the β -oxidation spiral was

committed to three complete turns, yielding only four acetyl-CoA species (i.e., there was no experimental evidence for preferential entry of the ^{13}C -enriched 'end' of the fatty acid into the citric acid cycle).

Also shown in Table 1 are results of similar experiments where the perfusion pressure was decreased from a normal column height of 70 cm of water to 25 cm of water. We have shown previously that substrate utilization changes quite dramatically in hearts perfused with a mixture of lactate and acetate during similar low-pressure perfusions (C. R. Malloy, L. Alvarez, J. R. Thompson, F. M. H. Jeffrey, A. D. Sherry and A. J. Liedtke, unpublished work). However, this was not observed in hearts perfused with octanoate. As shown in Table 1, the contribution made by octanoate to acetyl-CoA either decreased only slightly (F_{C2} decreased from 0.96 to 0.93 in hearts perfused with [2,4,6,8- ^{13}C]octanoate) or not at all (F_{C3} remained unchanged, 0.49 versus 0.50, in hearts perfused with [1,2,3,4- ^{13}C]octanoate) during low-pressure perfusion. A determination of $F_{\text{C0'}}$ (the contribution to acetyl-CoA from the unenriched 'end' of [1,2,3,4- ^{13}C]octanoate) in these spectra using the same assumptions as outlined above showed that $F_{\text{C0'}}$ appeared to be somewhat lower than F_{C3} (see Table 1). This indicates that every [1,2,3,4- ^{13}C]octanoate molecule may not undergo complete β -oxidation in partially ischaemic heart tissue.

Upon examining these data more closely it became clear that the data from the two different enriched octanoate substrates were not internally consistent. For example, F_{C2} appeared to

Figure 2 ^{13}C -n.m.r. spectrum of an extract of a rat heart which had been perfused to steady state with (a) 0.5 mM [2,4,6,8- ^{13}C]octanoate or (b) 0.5 mM [1,2,3,4- ^{13}C]octanoate

The insets show the glutamate C-4 resonance expanded. A different heart was used for each perfusion. Resonances labelled G2, G3 and G4 represent glutamate C-2, C-3 and C-4 respectively.

Table 1 Steady-state ^{13}C isotopomer analysis of hearts perfused with either 0.5 mM [2,4,6,8- ^{13}C]octanoate or 0.5 mM [1,2,3,4- ^{13}C]octanoateData are means $\pm$ S.D.

Substrate	F_{C_2}	F_{C_3}	$F_{\text{C}_0'}$	$F_{\text{C}_0''}$
[2,4,6,8- ^{13}C]Octanoate				
Normal pressure (70 cm) ($n = 6$)	0.96 ± 0.01	N/A†	N/A	0.04 ± 0.01
Low pressure (25 cm) ($n = 4$)	$0.93 \pm 0.01^*$	N/A	N/A	$0.07 \pm 0.01^*$
[1,2,3,4- ^{13}C]Octanoate				
Normal pressure (70 cm) ($n = 4$)	N/A	0.49 ± 0.01	0.47 ± 0.01	0.04 ± 0.01
Low pressure (25 cm) ($n = 4$)	N/A	0.50 ± 0.02	$0.43 \pm 0.01^*$	$0.07 \pm 0.01^*$

*Different from normal perfusion pressure ($P < 0.005$).

†N/A, not applicable.

Figure 3 An illustration of how F_{C_2} , F_{C_3} and $F_{\text{C}_0'}$ would change if there were incomplete oxidation of an equimolar mixture of [1,2,3,4- ^{13}C]- and [2,4,6,8- ^{13}C]octanoate

decrease significantly between high and low pressure conditions in hearts perfused with [2,4,6,8- ^{13}C]octanoate, while F_{C_3} did not change significantly between these same groups of hearts perfused with [1,2,3,4- ^{13}C]octanoate (see Table 1). We surmised that these parameters might be quite sensitive to small changes in concentration and, because of small variations in perfusion volume for each heart, it was possible that the actual concentrations of the two enriched octanoate samples were different by a few per cent. Thus we sought to test the hypothesis that these molecules may not be fully oxidized by ischaemic heart tissue using experimental conditions where the amount of octanoate presented to hearts could be more precisely controlled.

Hearts perfused with a 1:1 mixture of [2,4,6,8- ^{13}C]octanoate and [1,2,3,4- ^{13}C]octanoate

The model shown in Figure 3 illustrates how F_{C_2} , F_{C_3} and $F_{\text{C}_0'}$ may be determined from a single spectrum. Assuming [2,4,6,8- ^{13}C]octanoate and [1,2,3,4- ^{13}C]octanoate are utilized in equal amounts and each molecule that enters the β -oxidation spiral completes three complete turns of the spiral to generate four acetyl-CoA species, the $F_{\text{C}_2}/F_{\text{C}_3}$ ratio should equal 2.00. This ratio may be obtained by direct analysis of the glutamate C-4

resonance as $(\text{S} + \text{D}34)/(\text{Q} + \text{D}45)$ (see Figure 1). If, however, only three acetyl groups, on average, were removed from each octanoate molecule (starting from the carboxy end), then the $F_{\text{C}_2}/F_{\text{C}_3}$ ratio would be 1.5; if only two acetyl groups were removed on average, then $F_{\text{C}_2}/F_{\text{C}_3}$ would be 1.0. Thus any deviation of the $F_{\text{C}_2}/F_{\text{C}_3}$ ratio from 2 would be an indication of incomplete β -oxidation of octanoate. Since this analysis can be done by simple inspection of the glutamate C-4 resonance, it does not require steady-state metabolic or isotopic conditions and hence is applicable under a wide variety of physiological conditions, including those *in vivo*. If, in addition, one obtained absolute values for F_{C_2} and F_{C_3} using either a steady-state analysis [21] or non-steady-state analysis [24], then one could also determine the fraction of unenriched acetyl-CoA derived from the unenriched 'end' of [1,2,3,4- ^{13}C]octanoate or $F_{\text{C}_0'}$. Inspection of the examples in Figure 3 shows that $F_{\text{C}_0'}$ is simply equal to $F_{\text{C}_2} - F_{\text{C}_3}$.

Given this derivation, two other groups of hearts (normal pressure and low pressure) were perfused with 0.25 mM each of the two enriched octanoates, prepared from an exact 1:1 stock solution. The stoichiometry of this 1:1 stock solution was verified by ^1H n.m.r. (the methyl resonance of octanoate was a triplet in one case and a doublet of triplets in the other). An analysis of the ^{13}C n.m.r. spectra of hearts perfused with this mixture is found in Table 2. As shown in the first two lines, the $F_{\text{C}_2}/F_{\text{C}_3}$ ratio was equal to 2 in both groups, indicating that complete β -oxidation of both labelled substrates occurred at normal perfusion pressure and at reduced flow (low pressure). A third group of hearts were arrested with 20 mM KCl and then perfused with this same mixture of octanoates for 30 min before freeze-clamping. Again, the $F_{\text{C}_2}/F_{\text{C}_3}$ ratio was equal to 2 and $F_{\text{C}_0'}$ was equal to F_{C_3} within experimental error. Finally, a fourth group was examined during anoxia. In this case, the $F_{\text{C}_2}/F_{\text{C}_3}$ ratio clearly deviated from 2, reflecting incomplete oxidation of octanoate. Interestingly, total utilization of octanoate decreased considerably in anoxic hearts (both F_{C_2} and F_{C_3} are smaller), while the contribution of unenriched endogenous sources to acetyl-CoA ($F_{\text{C}_0'}$) increased about 8-fold over normoxic hearts.

Since octanoate contributed essentially all of the acetyl-CoA in the first three groups of hearts shown in Table 2 ($F_{\text{C}_2} + F_{\text{C}_3} + F_{\text{C}_0'}$ was 96–100% in all three groups), we decided to add additional unenriched substrates which might compete with octanoate and hence limit its oxidation. Surprisingly, when 10 mM lactate was co-added with the mixture of octanoates, the results were no

Table 2 Steady-state ^{13}C isotopomer analysis of hearts perfused with a mixture of $[1,2,3,4\text{-}^{13}\text{C}]$ - and $[2,4,6,8\text{-}^{13}\text{C}]$ -octanoate

Hearts were perfused with a mixture of 0.25 mM $[1,2,3,4\text{-}^{13}\text{C}]$ octanoate and 0.25 mM $[2,4,6,8\text{-}^{13}\text{C}]$ octanoate. $F_{\text{C}0'}$ was determined by the difference between $F_{\text{C}2}$ and $F_{\text{C}3}$ for each heart; the fractional contribution to acetyl-CoA from other endogenous sources, $F_{\text{C}0''}$, was determined by subtracting $F_{\text{C}0'}$ from the $F_{\text{C}0}$ value obtained by a steady-state analysis. Results are means $\pm$ S.D.

Perfusion conditions	$F_{\text{C}2}$	$F_{\text{C}3}$	$F_{\text{C}0'}$	$F_{\text{C}0''}$	$F_{\text{C}2}/F_{\text{C}3}$
No competing substrates					
Normal pressure	0.48 ± 0.03	0.25 ± 0.02	0.23 ± 0.02	0.05 ± 0.01	1.96 ± 0.09
(70 cm) ($n = 4$)					
Low pressure	0.50 ± 0.02	0.25 ± 0.02	0.25 ± 0.04	0.01 ± 0.01	2.00 ± 0.05
(25 cm) ($n = 4$)					
KCl arrest	0.48 ± 0.01	0.25 ± 0.01	0.23 ± 0.02	0.05 ± 0.01	1.96 ± 0.11
(20 mM) ($n = 6$)					
Anoxia	0.28 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.43 ± 0.01	$1.74^* \pm 0.05$
($n = 4$)					
With competing unlabelled substrates					
10 mM Acetate	0.43 ± 0.01	0.21 ± 0.00	0.22 ± 0.00	0.14 ± 0.02	2.02 ± 0.01
($n = 4$)					
5 mM β -Hydroxybutyrate	0.31 ± 0.03	0.16 ± 0.02	0.15 ± 0.01	0.39 ± 0.06	1.97 ± 0.03
($n = 4$)					

*Significantly different ($P < 0.05$) from the other five groups in this Table by analysis of variance using the Student–Newman–Keuls test.

different. Addition of 10 mM acetate decreased octanoate utilization, but only to about 86%. β -Hydroxybutyrate at 5 mM had the most pronounced effect, reducing the total octanoate contribution to acetyl-CoA to about 60%. However, with both competing substrates, the $F_{\text{C}2}/F_{\text{C}3}$ ratio remained near 2 and $F_{\text{C}3}$ was equal to $F_{\text{C}0'}$ in both groups.

Spectra of intact, perfused hearts and tissue extracts from live animals after infusion of a 1:1 mixture of $[2,4,6,8\text{-}^{13}\text{C}]$ octanoate and $[1,2,3,4\text{-}^{13}\text{C}]$ octanoate

We have recently introduced a $\{^{13}\text{C}\}$ homonuclear decoupling method which simplifies the nine-line glutamate C-4 resonance (see Figure 1) into a three-line multiplet without significant loss of metabolic isotopomer information [20]. The spectrum shown in Figure 4 is that of an intact, functioning rat heart oxidizing a 1:1 mixture of the two enriched octanoates. No other unlabelled substrates were present. Upon homonuclear decoupling of glutamate C-3, the C-4 resonance appeared as an overlapping singlet (C-4S') and doublet (C-4D'), with the doublet having a coupling constant of 52 Hz characteristic of J_{45} coupling [20]. As shown in Figure 4, the C-4S'/C-4D' directly reported $F_{\text{C}2}/F_{\text{C}3}$, which again was found equal to 2. This demonstrates that direct information about β -oxidation of fatty acids can be obtained non-invasively, without tissue biopsies and high-resolution spectra of tissue extracts.

To test whether octanoate is also highly utilized *in vivo*, where numerous other endogenous substrates are also present, we infused an equimolar mixture of the two enriched octanoates (54 mg each, intravenously) into 180–200 g rats over a period of 60 min before killing the animals and freeze-clamping the heart and liver. ^{13}C spectra of acid extracts of a heart and a liver are shown in Figure 5 and a summary of the $F_{\text{C}x}$ variables derived from such spectra is given in Table 3. $F_{\text{C}3}$ was again found equal

Figure 4 ^{13}C -n.m.r. spectrum of an intact rat heart

The heart was supplied with 1:1 molar mixture of $[1,2,3,4\text{-}^{13}\text{C}]$ - and $[2,4,6,8\text{-}^{13}\text{C}]$ octanoate. The bottom plot (b) shows the glutamate C-2, C-4 and C-3 resonances before homonuclear decoupling was turned on, while the top spectrum (a) shows the three-line multiplets of glutamate C-2 and C-4 during homonuclear decoupling of glutamate C-3. Resonances labeled G2, G3 and G4 represent glutamate C-2, C-3 and C-4 respectively.

Figure 5 Proton-decoupled ^{13}C -n.m.r. spectra of extracts of a heart (a) and liver (b) from a rat after infusing 54 mg each of $[2,4,6,8\text{-}^{13}\text{C}]$ - and $[1,2,3,4\text{-}^{13}\text{C}]$ octanoate

Resonances labelled G2, G3 and G4 represent glutamate C-2, C-3 and C-4 respectively. Gln-C-2, Gln-C-3 and Gln-C-4 in the liver spectrum are from glutamine C-2, C-3 and C-4 respectively.

Table 3 Steady-state ^{13}C isotopomer analysis of tissue removed from rats after infusion of a mixture of [1,2,3,4- ^{13}C]- and [2,4,6,8- ^{13}C]octanoate

Results are means $\pm$ S.D. Rats were infused with 54 mg each of [1,2,3,4- ^{13}C]- and [2,4,6,8- ^{13}C]octanoates for 60 min before removing and freeze-clamping the heart and liver. F_{Co} and F_{Co} were determined as described in Figure 3.

Tissue	F_{C2}	F_{C3}	F_{Co}	F_{Co}
Heart ($n = 3$)	0.29 $\pm$ 0.07	0.13 $\pm$ 0.04	0.16 $\pm$ 0.03	0.42 $\pm$ 0.14
Liver ($n = 3$)	0.26 $\pm$ 0.07	0.11 $\pm$ 0.03	0.15 $\pm$ 0.04	0.48 $\pm$ 0.15

to F_{Co} in both tissues, indicating there was no preferential β -oxidation of the carboxy end of octanoate *in vivo*. The value of F_{Co} was considerably higher in these samples (42 and 48 % in the *in vivo* heart and liver respectively), indicating that endogenous substrates contributed significantly more to the acetyl-CoA pool in these *in vivo* tissues than typically observed in perfused hearts.

DISCUSSION

We have demonstrated a unique ^{13}C n.m.r. method for determining whether the medium-chain fatty acid octanoate undergoes complete β -oxidation in perfused rat hearts and *in vivo* hearts and liver. No evidence could be found for incomplete β -oxidation of octanoate in the perfused heart under high and low perfusion pressures, during KCl arrest or in the presence of high concentrations of competing substrates. This suggests that the enzymes of the β -oxidation spiral in heart tissue are not structurally disrupted during partial ischaemia (low-pressure perfusion), during membrane depolarization (KCl arrest) or in the presence of other substrates which produce excess acetyl-CoA. Only during an anoxic event could we find evidence for disruption of the β -oxidation spiral of reactions. The latter observation is in agreement with a prior study [16] which reported that palmitate was not fully oxidized in Langendorff perfused hearts during anoxia. The consistency of these two observations, one using a long-chain fatty acid that requires carnitine for transport into mitochondria and another using a medium-chain fatty acid that does not require carnitine, suggests that disruption of the β -oxidation spiral which occurs during anoxia does not involve a problem associated with transport of fatty acids across the mitochondrial inner membrane. Rather, we assume this reflects a disruption of the short- and/or medium-chain β -oxidation spiral of reactions, either by direct inhibition of one or more enzymes or by indirect disruption of their functional organization. This interesting result supports data presented previously by Sumegi et al. [9] which showed a significant kinetic advantage of organization of the soluble, short-chain β -oxidation enzymes in partially disrupted, isolated mitochondria. Although Cornish-Bowden [25,26] has used computer models to argue that there is no kinetic advantage to "channeling" of intermediates along a sequential series of enzyme-catalysed reactions, one oversimplification of his model is the assumption of a well-stirred reactor, i.e., he assumes there are no steep local concentration gradients. This is most certainly an oversimplification for the β -oxidation spiral of reactions which take place in the highly concentrated mitochondrial matrix.

Our observation of complete β -oxidation of octanoate did extend to the *in vivo* rat heart and liver, but may or may not extend to isolated cells. Chatzidakis and Otto [27] found that $^{14}\text{CO}_2$ release in hepatocytes oxidizing palmitate, expressed as

a percentage of total oxidized products, was greatest from [1- ^{13}C]palmitate, less from [U- ^{13}C]palmitate, and least from [16- ^{13}C]palmitate and suggested this might reflect incomplete mitochondrial β -oxidation. We do not know whether this result differs from ours in the *in vivo* liver because of organizational differences of the enzymes of β -oxidation in isolated cells versus intact tissue, but it is certainly consistent with this hypothesis.

In hearts perfused with 0.5 mM octanoate, the virtual lack of competition by 10 mM lactate (20-fold molar excess), the relatively minor competition by 10 mM acetate (20-fold molar excess), and the substantial competition by 5 mM β -hydroxybutyrate (10-fold molar excess) suggests that these substrates may compete with octanoate by different mechanisms. Latipaa et al. [19] have shown that 2 mM octanoate decreases the % active form of pyruvate dehydrogenase from 48 % in control hearts to 17 % in octanoate perfused hearts, so the observed non-competition by 10 mM lactate may reflect low activity of pyruvate dehydrogenase in octanoate-perfused hearts. Forsey et al. [18] have also shown that 5 mM pyruvate, lactate or glucose do not compete with 0.1 mM octanoate as a metabolic fuel in the isolated perfused heart, while 5 mM acetoacetate or β -hydroxybutyrate do compete effectively. The greater tendency for hearts to oxidize 0.5 mM octanoate (2 mM acetyl-CoA equivalents) over either 10 mM acetate or 5 mM β -hydroxybutyrate (both 10 mM acetyl-CoA equivalents) may reflect the ease by which this medium-chain fatty acid is able to diffuse into the mitochondrial matrix. β -Hydroxybutyrate is thought to be transported via a non-specific monocarboxylate-transport protein [27], acetate transport is thought to occur via the HCO_3^- -transport system [28], while octanoate apparently freely diffuses across the mitochondrial inner membrane [18].

In summary, the ^{13}C method presented here for monitoring incomplete β -oxidation of fatty acids can be applied to any tissue and under a variety of metabolic circumstances. The method is simple, quantitative, non-invasive and therefore easily applied *in vivo*. It offers advantages over traditional radiolabelling methods for interrogating the β -oxidation spiral of reactions *in vivo* because the ^{13}C spectrum of glutamate C-4 directly reports the ^{13}C isotopomer population of acetyl-CoA entering the citric acid cycle in each tissue. It thus offers the possibility of examining the extent to which the enzymes of β -oxidation are functionally intact *in vivo* so that, once committed, an acyl-CoA remains in the β -oxidation spiral until it is completely oxidized to acetyl-CoA.

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