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A Comparison of [9,10-^{3}H]Palmitic and [9,10-^{3}H]Myristic Acids for the Detection of Defects of Fatty Acid Oxidation in Intact Cultured Fibroblasts

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Summary: The production of tritiated water from [9,10-^{3}H]myristic acid can be used as a screening assay for the detection of medium-chain acyl-CoA dehydrogenase deficiency, multiple acyl-CoA dehydrogenation defects (glutaric aciduria type 2 and ethylmalonic-adipic aciduria types), and some types of hydroxydicarboxylic aciduria. Comparison with the release of tritiated water from [9,10-^{3}H]palmitic acid may give an indication of the chain-length specificity of the metabolic defect. In a case of ethylmalonic-adipic aciduria such a prediction has been confirmed by examination of accumulated intermediates in the affected fibroblasts.

Defects of the mitochondrial β-oxidation pathway are emerging as relatively common conditions, often associated with considerable morbidity and mortality (Vianey-Liaud *et al.*, 1987). They are difficult to diagnose on the basis of metabolic findings (Pollitt, 1989a). The individual acyl-CoA dehydrogenases (long-, medium- and short-chain), electron-transfer flavoprotein, electron-transfer flavoprotein dehydrogenase and the 3-hydroxyl-CoA dehydrogenases may now be measured in small amounts of tissue including cultured fibroblasts. However, all these assays are time-consuming and technically demanding and none are routinely available for diagnostic use. Thus, simple global assays of fatty acid oxidation in intact cultured fibroblasts using radioactively-labelled substrates continue to have a major role in the routine investigation of children suspected to have (or to have died because of) β-oxidation defects.

The most commonly used assays of fatty acid oxidation in fibroblasts rely on the trapping of $^{14}CO_2$ released from [^{14}C]labelled fatty acids. Such assays have been used by Kølvraa *et al.* (1982), Saudubray *et al.* (1982), Rhead *et al.* (1983) and many others. [1-^{14}C]Fatty acids, usually butyric, octanoic and palmitic, are used to examine the activities of individual sections of the β-oxidation spiral. [U-^{14}C]palmitic or oleic acids or [16-^{14}C]palmitic acid assess overall activity. While such assays may in some

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instances indicate defective fatty acid oxidation quite clearly, there are frequently problems in interpretation due to the wide normal range and large interassay variability generally experienced. Only a small proportion (approximately 20%) of the acetyl residues produced by β-oxidation of fatty acids are converted immediately to carbon dioxide, the remainder being incorporated into non-volatile metabolic intermediates (Veerkamp *et al.*, 1986a), and CO_2-release assays have been much criticized on this point. Alternative approaches based on the determination of acid-soluble oxidation products are available for long-chain substrates. An analogous method (Veerkamp *et al.*, 1986b) for use with octanoic acid is not really practicable for routine use but the incorporation of ^{14}C into cellular protein (Rhead *et al.*, 1985) seems a viable alternative.

Tritium-labelled substrates offer a number of advantages over [^{14}C]substrates for this type of assay, particularly the higher specific activities available and lower cost. For fatty acids with ^3H distributed equally between two adjacent carbon atoms, at least 75% of the label is converted to ^3H_2O during the initial β-oxidation and further release occurs during metabolism of the acetyl-CoA produced. Tritiated water is readily separated from unreacted substrate and acidic metabolites. Such assays may require only small amounts of fibroblasts. Moon and Rhead (1987) have exploited these advantages in a study of complementation analysis of various fatty acid oxidation defects using [9,10(n)-^3H]palmitic acid. Amongst the cell lines studied were three from patients with medium-chain acyl-CoA dehydrogenase deficiency (McKusick 20145). These showed rates of tritiated water production of 15, 18 and 22% of normal, which compare favourably with the residual activities usually observed with CO_2-release assays. However, in our hands results with medium-chain acyl-CoA dehydrogenase-deficient fibroblasts were much less clear-cut. This led us to examine the theoretical basis for this assay and the nature of accumulated intermediates in medium-chain acyl-CoA dehydrogenase-deficient and other abnormal fibroblasts. We propose [9,10(n)-^3H]myristic acid, a C_{14} saturated fatty acid, as an alternative substrate to the C_{16} saturated palmitic acid for the detection of fatty acid oxidation defects. Preliminary results were presented at the Society for the Study of Inborn Errors of Metabolism meeting at Glasgow in September 1988.

MATERIALS AND METHODS

The cultured fibroblasts examined were from patients with a variety of known or suspected disorders of fatty acid oxidation. The six patients with medium-chain acyl-CoA dehydrogenase deficiency were diagnosed on the basis of the characteristic urinary organic acid pattern (including the excretion of hexanoylglycine and suberylglycine) and in some cases diagnosis was confirmed by phenylpropionic acid loading tests or specific electron-transfer flavoprotein-linked assay of medium-chain acyl-CoA dehydrogenase. One patient with a severe, glutaric aciduria type 2, multiple acyl-CoA dehydrogenation disorder was originally reported by Bennett *et al.* (1984), the other is unpublished. Two patients had the milder, ethylmalonic-adipic aciduria, variant of multiple acyl-CoA dehydrogenation disorder. One showed a marked clinical response to riboflavin supplementation (Green *et al.*, 1985), the other is

unresponsive. Five patients with 'hydroxydicarboxylic aciduria' were investigated. One of these, with progressive cirrhosis of the liver, has been previously reported (Pollitt *et al.*, 1987) and another showed a very similar clinical course. Of the other three hydroxydicarboxylic aciduria patients, two had presented with Reye-like illnesses which proved fatal and the last was classified as a sudden infant death (Pollitt, 1989b). The patient with defective long-chain fatty acid oxidation (now known to be due to deficiency of carnitine palmitoyltransferase 1) was described by Layward *et al.* (1987).

Fibroblasts were routinely cultured in HEPES buffered Ham's F10 or Eagle's minimum essential media with glutamine, 10–15% fetal bovine serum, penicillin and streptomycin. Carbon dioxide-release experiments were performed with cell monolayers in Krebs phosphate buffer, pH 6.9 at 37°C using the general conditions described by Bennett *et al.* (1984). Riboflavin-depleted medium was prepared as before (Green *et al.*, 1985). [16-^{14}C]Palmitic acid was produced by CEA, France, and [9,10-(n)^{3}H]palmitic and [9,10-(n)^{3}H]myristic acids were from Amersham (UK).

Tritiated water release experiments were performed in 24-well microplates. In some preliminary experiments the original method of Moon and Rhead (1987) was followed, the cells being cultured in a bicarbonate buffered system and assayed in Swim's medium. It was necessary to add calcium salts to the Swim's medium for the 20-h incubation following plating out in order to maintain cell viability. [9,10-^{3}H]Palmitic acid was used at a concentration of 22 μmol/L. In later experiments the culture and assay media were based on those used for carbon dioxide release as described above. In all assays palmitic and myristic acids for use as substrates were complexed with defatted bovine serum albumin at a concentration in the final medium of 0.45 mg/ml. It proved to be unnecessary to deproteinize the assay medium prior to ion-exchange treatment after the incubation. Fibroblast protein was determined by the Lowry method.

For the investigation of accumulated intermediates, fibroblasts in monolayer culture were incubated for 3.5 h in Krebs–Ringer phosphate buffer pH 6.9, containing 0.5 mmol/L L-malate, with [16-^{14}C]palmitic acid (1 μCi/ml, 10 μmol/L) complexed to bovine serum albumin. At the end of this period the entire contents of the incubation flask were adjusted to 0.67 mol/L sodium hydroxide and heated at 60°C for 0.5 h. The mixture was then acidified with 2 mol/L hydrochloric acid and the acids were extracted with ethyl acetate and diethyl ether. Unlabelled even-chain monocarboxylic acids (C_6–C_{16}) and 3-hydroxycarboxylic acids (C_6–C_{16}) were added as carriers and chromatographic markers. The extracted acids were converted to trimethylsilyl derivatives using bis(trimethylsilyl)trifluoro-acetamide containing 1% chlorotrimethylsilane and pyridine (1 : 1) and examined by gas chromatography on a packed OV-11 column (3% loading, 9 m long), trapping the combustion products from the flame ionization detector (Lee *et al.*, 1987) in Soluene (Canberra-Packard) and using Hionic Fluor (Canberra-Packard) as scintillant. On this column the lower 3-hydroxy-monocarboxylic acids were poorly resolved from the corresponding n + 2 unsubstituted monocarboxylic acids. However, separate experiments, using higher substrate concentrations and detecting metabolites by selected-ion-monitoring GC–MS, showed only traces of 3-hydroxy-acids.

RESULTS AND DISCUSSION

Medium-chain acyl-CoA dehydrogenase deficiency

Moon and Rhead (1987) observed a low rate of tritiated water production from [9,10-^{3}H]palmitate by medium-chain acyl-CoA dehydrogenase-deficient cells which they explained in terms of retention of the label as [1,2-^{3}H]octanoyl-derivatives. We were unable to achieve useful discrimination between medium-chain acyl-CoA dehydrogenase-deficient and normal fibroblasts using [9,10(n)-^{3}H]palmitic acid under either of the conditions described above (detailed results not shown). When incubated with palmitic acid, medium-chain acyl-CoA dehydrogenase-deficient fibroblasts do accumulate substantial amounts of octanoate-containing intermediates (Figure 1b), as was envisaged by Moon and Rhead (1987). However, the argument that label from [9,10-^{3}H]palmitate will largely be retained in such derivatives is incorrect, as there is no hydrogen directly attached to the 1-carbon in octanoic acid or its derivatives. In cells where β-oxidation proceeds only as far as C_8, 50% of the label from [9,10-^{3}H]palmitic acid will be released compared to between 75 and 100% in cells where β-oxidation goes to completion (Table 1). This, taken with the degree of residual activity shown by medium-chain acyl-CoA dehydrogenase-deficient cells in carbon dioxide-release assays (anything up to 40% is usual with [1-^{14}C]octanoate as substrate), accounts for the lack of clear distinction between normal and medium-chain acyl-CoA dehydrogenase-deficient lines. A low level of tritiated water production from [9,10-^{3}H]palmitate would require the β-oxidation spiral to be halted at C_{10} (see Table 1). Possibly the marked kinetic isotope effect observed with medium-chain acyl-CoA dehydrogenase (Pohl *et al.*, 1986) may, under some assay conditions, make the intermediate [3,4-^{3}H]decanoyl-CoA a sufficiently poor substrate for medium-chain acyl-CoA dehydrogenase to explain the observations of Moon and Rhead (1987). We were not able to reproduce this effect but did have to slightly modify the original Moon and Rhead (1987) protocol to achieve adequate cell viability.

With [9,10-^{3}H]myristic acid, which is also commercially available, there should be no production of tritiated water until β-oxidation reaches the C_6 stage (Table 1) and this provides the basis for an alternative assay for medium-chain acyl-CoA dehydrogenase deficiency. Optimum discrimination between normal and medium-chain acyl-CoA dehydrogenase-deficient cell lines was achieved at nominal myristate concentration of approximately 110 μmol/L (Figure 2). A concentration of 100 μmol/L (at approximately 10 μCi/ml) was chosen for routine assays and some cell lines were also assayed using [9,10-^{3}H]palmitic acid at this concentration (Table 2). With

Table 1 Percentage of label released as tritiated water from [9,10-^{3}H]myristic and [9,10-^{3}H]palmitic acids at different stages of chain shortening

Acyl residue (carbon atoms)	4	6	8	10
[9,10-^{3}H]Myristic acid	100 (75)	50	0	0
[9,10-^{3}H]Palmitic acid	100 (75)	100 (75)	50	0

The open figures are based on the assumption that all label in [^{3}H]acetyl-CoA is rapidly released by further metabolism. Figures in parentheses assume no release of label from the acetyl-CoA formed by β-oxidation and no exchange at the 4-position of 3-oxoacyl-CoA intermediates. It is assumed that in both substrates the ^{3}H is equally distributed between the 9 and 10 positions and the *R* and *S* configurations

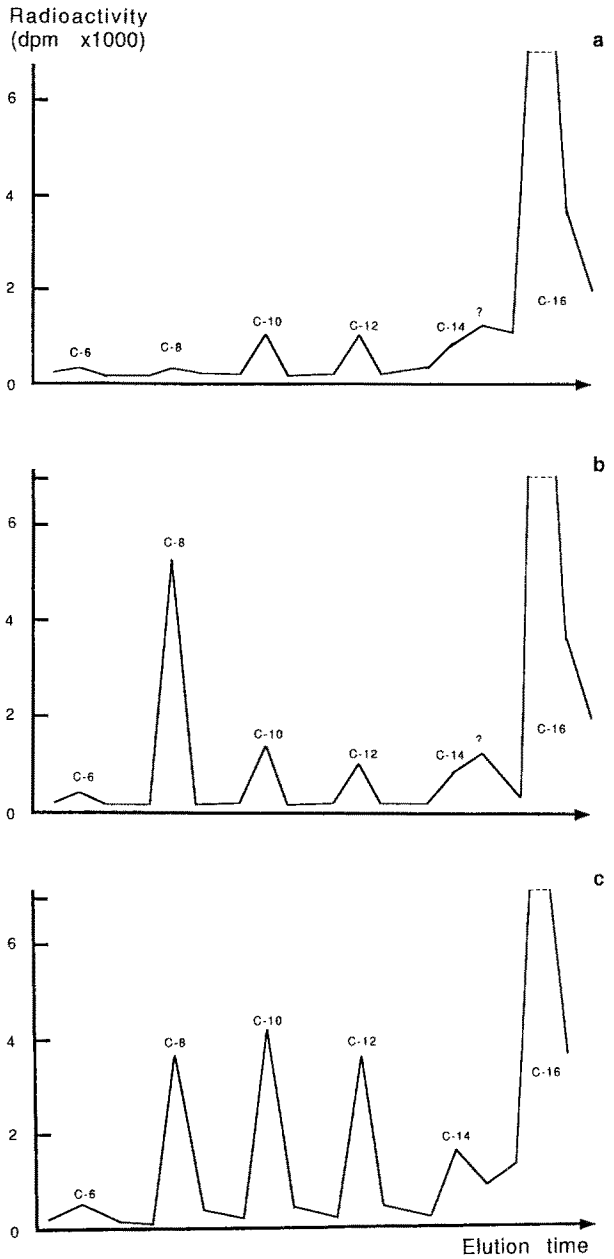

Figure 1 Labelling of fatty acid intermediates in cultured fibroblast incubated with [16-^{14}C]palmitic acid. For details see Materials and Methods section. Hexanoic acid is likely to be underestimated by this method due to evaporative loss. (a) Normal fibroblasts; (b) medium-chain acyl-CoA dehydrogenase deficiency; (c) patient RM, ethylmalonic-adipic aciduria unresponsive to riboflavin

normal cells production of tritiated water from [9,10-^{3}H]myristic acid proved to be linear with time from 1–4 h and with fibroblast protein in the range 15–90 μg per well. Ten normal fibroblast lines, each assayed in triplicate at least twice, gave 3180–6184 pmol $H_2O\,h^{-1}$ (mg protein)$^{-1}$, mean 3840, SD 880. Intra-assay variability based on triplicates was 5%. Between-assay variabilities for two normal cell lines, each assayed in triplicate ten times over a period of 4 weeks, were 10.2 and 19.0%. By comparison, the carbon dioxide-release assay with [1-^{14}C]octanoate with 17 normal fibroblast lines assayed at least in duplicate gave 114–846 pmol CO_2 released h^{-1} (mg protein)$^{-1}$, mean 373, SD 210. Four cell lines, each assayed twice in duplicate, gave a mean intra-assay variability of 4% but an inter-assay variability of 23%.

With the [9,10-^{3}H]myristate assay six medium-chain acyl-CoA dehydrogenase-deficient lines gave average values of 180–1190 pmol $H_2O\,h^{-1}$ (mg protein)$^{-1}$, mean 510, SD 380. Despite the good overall discrimination, there was considerable inter-assay variation with individual medium-chain acyl-CoA dehydrogenase-deficient cell lines, as there is in carbon dioxide-release assays. In the worst case, patient NM, tritiated water release rates in five separate assays ranged from 20 to 40% of simultaneous normal controls (Table 2).

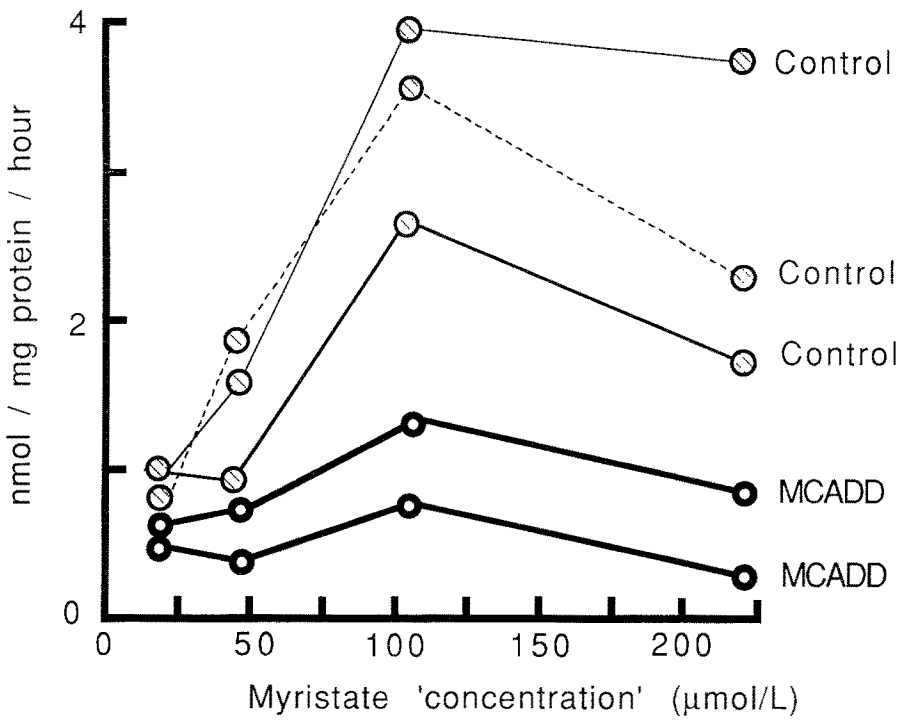

Figure 2 Effect of nominal myristate concentration on release of tritiated water from [9,10-^{3}H]myristic acid by normal fibroblasts and fibroblasts from two medium-chain acyl-CoA dehydrogenase-deficient patients, NM and JR

Table 2 Comparison of production of tritiated water (pmol h^{-1} (mg protein)$^{-1}$ from [9,10-^{3}H]myristic and [9,10-^{3}H]palmitic acids (100 μmol/L) by individual fibroblast lines. Each result is a mean of triplicate assays and is expressed as a percentage of the activity of normal lines run in the same batch

Fibroblast type	*Myristate*	*Palmitate*
Medium-chain acyl-CoA dehydrogenase deficiency		
patient NM	20; 22; 30; 38; 40	86
patient JR[a]	12; 14; 25	67
Multiple acyl-CoA dehydrogenation disorders		
patient BA[b]	0.5; 0.6; 1.3	7
patient DH[c]	8.6; 15	46
patient RM[d]	3.4; 15; 21	23, 28
'Hydroxydicarboxylic aciduria'		
patient HB	22; 24; 41; 49	31; 41
patient SP	17; 26; 27; 43	30; 74
patient DR	26; 41; 44; 49	25
Carnitine palmitoyltransferase deficiency 1		
patient DM[e]	28; 29	3.6

[a]Electron-transfer protein-linked assay showed 1.5% of normal activity with octanoyl-CoA; $^{14}CO_2$ release from [1-^{14}C]octanoate was 26% of normal (Bennett *et al.*, 1987)
[b]Glutaric aciduria type 2 (Bennett *et al.*, 1984)
[c]Ethylmalonic-adipic aciduria type, clinically responsive to riboflavin (Green *et al.*, 1985)
[d]Ethylmalonic-adipic aciduria type, riboflavin unresponsive
[e]Layward *et al.* (1987). $^{14}CO_2$ release from [1-^{14}C]palmitate 0.3% of simultaneous normal controls

Multiple acyl-CoA dehydrogenation disorders

Fibroblasts from patient BA, who had severe, glutaric aciduria type 2, multiple acyl-CoA dehydrogenation disorders, showed a very low rate of tritiated water release from [9,10-^{3}H]myristic acid, averaging 24 pmol $H_2O\,h^{-1}$ (mg protein)$^{-1}$. (A second glutaric aciduria type 2 patient showed 26 pmol $H_2O\,h^{-1}$ (mg protein)$^{-1}$.) The somewhat higher rate of tritiated water production observed with [9,10-^{3}H]palmitic acid (Table 2) suggests that, while long-chain substrates are oxidized quite slowly by the BA fibroblasts, the block is more severe at the C_8 stage. This agrees with the results of carbon dioxide-release assays with these fibroblasts previously reported (Bennett *et al.*, 1984) where the rate of production of $^{14}CO_2$ from [1-^{14}C]substrates followed the order palmitate > oleate > butyrate > > octanoate and residual activity with octanoate was 1.4% of normal.

In the fibroblasts from the two patients with the ethylmalonic-adipic aciduria type of multiple acyl-CoA dehydrogenase deficiency, a significant reduction in the release of label from [9,10-^{3}H]palmitate was observed, and a somewhat greater reduction from [9,10-^{3}H]myristate. This finding is consistent with the pattern of metabolite accumulation observed in cultured fibroblasts (Figure 1c) and can be again explained in terms of a moderate reduction in activity of long-chain acyl-CoA dehydrogenase

and a greater reduction in medium-chain activity. The release of tritiated water from [9,10-^{3}H]myristate was not altered by culturing DH cells for 12 days in medium supplemented with 4 mg/L of riboflavin.

Fibroblasts from DH have been studied by carbon dioxide release assays with erratic and confusing results. Initial investigations with [1-^{14}C]butyrate suggested a significant reduction in oxidation, but when the assays were repeated a further nine times over a period of months the pooled patient values finished in the lower part of the normal range (Green *et al.*, 1985). Other substrates such as [1-^{14}C]oleate, [1-^{14}C]octanoate and [6-^{14}C]lysine also gave erratic low–normal results. However, cultivation in a riboflavin-deficient medium produced a significant reduction in the rate of butyrate oxidation by DH cells but not by normal cells (Green *et al.*, 1985). In the present study the degree of reduction in carbon dioxide release seen after 18–20 days in riboflavin-depleted medium was greatest with octanoate, followed by oleate, with the effect on butyrate being insignificant (Table 3). The large reduction in octanoate oxidation is consistent with the metabolite findings (Figure 1c) and the interpretation of the tritiated water release experiments. It may also explain the strong resemblance of some of the urinary organic acid profiles seen in DH to those in medium-chain acyl-CoA dehydrogenase deficiency (Green *et al.*, 1985).

Hydroxydicarboxylic aciduria

Hydroxydicarboxylic aciduria is defined as an intermittent hypoketotic dicarboxylic aciduria in which the unsubstituted even-chain dicarboxylic acids are accompanied by comparable amounts of the corresponding 3-hydroxydicarboxylic acids. In some patients this hydroxydicarboxylic aciduria seems to be a transient phenomenon associated with severe dysfunction of hepatic mitochondria, as in Reye syndrome. In others there is clearly some hereditary condition precipitating such attacks (Pollitt, 1989b). We have studied fibroblasts of affected children from two families where hydroxydicarboxylic aciduria was associated with severe progressive hepatic cirrhosis.

Table 3 Production of $^{14}CO_2$ (pmol h^{-1} (mg protein)$^{-1}$) from [1-^{14}C]fatty acids by cells from patient DM after growth in riboflavin-depleted medium. The results of two sets of assays, performed after 18 and 20 days' growth in depleted medium, are averaged

Substrate	*Normal medium*	*Riboflavin-depleted medium*
[1-^{14}C]butyrate (10 μmol/L)		
normal lines (n = 3)	124	101
DH cells	64	50
[1-^{14}C]octanoate (10 μmol/L)		
normal lines (n = 6)	254	259
DH cells	142	23
[1-^{14}C]oleate (20 μmol/L)		
normal lines (n = 3)	16	14
DH cells	19	6

The results of all assays ([9,10-^{3}H]myristate, [1-^{14}C]octanoate, [1-^{14}C]palmitate and [16-^{14}C]palmitate) have been normal in both. However, in three other unrelated cases, with Reye-like or sudden infant death presentations, significant abnormalities in fatty acid oxidation were found using both [9,10-^{3}H]myristic and [9,10-^{3}H]palmitic acids (Table 2). These results suggest that the portion of the β-oxidation pathway affected lies above C_8. Release of $^{14}CO_2$ from [1-^{14}C]palmitate with these cells was normal or marginally low (results not shown). Thus the metabolic lesion may be restricted rather specifically to the C_{14}–C_{10} section of the β-oxidation spiral or, more likely, it extends to all long-chain substrates but has a relatively mild effect which needs to be magnified by several turns of the spiral before it can reliably be detected.

Carnitine palmitoyltransferase 1 deficiency

The clinical history and the results of carbon dioxide-release assays in patient DM were compatible with carnitine palmitoyltransferase 1 deficiency (Demaugre *et al.*, 1988) and this diagnosis has now been confirmed by the Paris group. DM's fibroblasts gave lower tritiated water release with [9,10-^{3}H]palmitic acid than with [9,10-^{3}H]myristic acid, suggesting that the latter substrate may to some extent enter mitochondria in a carnitine-independent manner or may act as a substrate for other carnitine acyltransferases.

CONCLUSIONS

Assays of fatty acid oxidation based on the formation of tritiated water from [9,10-^{3}H]myristic acid or [9,10-^{3}H]palmitic acids compare favourably with $^{14}CO_2$-release assays in several repects. Oxidation releases a large proportion of the label directly and secondary metabolic disturbances, leading for example to increased re-utilization of acetate residues, are less likely to influence results. These substrates have the further theoretical advantage of presenting intermediates to the enzymes lower down the β-oxidation pathway in a physiological manner. Because such substrates, labelled in the mid- or distal portions of the chain, require the action of several turns of the oxidation spiral before any label is released, they are particularly useful for detecting disorders such as mild multiple acyl-CoA dehydrogenation disorder and possibly some hydroxydicarboxylic acidurias where the individual steps are not too severely affected. These last two arguments can also be used in favour of [16-^{14}C]palmitic acid as a substrate but this compound is a 100-fold more expensive than the [9,10-^{3}H]substrates. The relatively low cost of the latter also allows higher concentrations to be used in the assay medium, stressing the β-oxidation pathway and thus emphasizing any reduction in V_{max} of the constituent enzymes. This higher substrate concentration, together with the greater recovery of label from the metabolized fatty acid, gives measured activities with [9,10(n)-^{3}H]palmitic acid many times greater than those observed under usual conditions with [1-^{14}C]palmitate. Thus smaller quantities of cells are required and assays may conveniently be performed in microtitre plates. On the debit side, the isolation of the tritiated water prior to counting is time-consuming.

[9,10-^{3}H]Myristic acid has clear advantages over [9,10-^{3}H]palmitic acid for the detection of disorders such as medium-chain acyl-CoA dehydrogenase deficiency and the milder forms of multiple acyl-CoA dehydrogenation disorders where β-oxidation is halted at the C_8 stage but the advantage is less clear-cut for severe multiple acyl-CoA dehydrogenation disorders and hydroxydicarboxylic aciduria. Myristate is expected to be less sensitive than palmitate for disorders specific to long-chain fatty acids. Thus the two assays are to some extent complementary and, when performed together, give useful additional indications of the nature of the metabolic defect. Finally, we have the impression that with both assays there is a narrower normal range than seen with carbon dioxide-release assays. This would have great advantages in routine diagnosis in obviating the need to run large numbers of normal controls with each assay.

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