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Estimation of peroxisomal and mitochondrial fatty acid oxidation in rat hepatocytes using tritiated substrates

Robert ROGNSTAD
The Whittier Institute for Diabetes and Endocrinology, 9894 Genesee Avenue, La Jolla, CA 92037, U.S.A.

The pathways of peroxisomal and mitochondrial fatty acid oxidation were monitored with the use of substrates which produce NAD^{3}H. I used as marker substrates: D-[3-^{3}H]3-hydroxybutyrate for mitochondrial NAD^{3}H production, [2-^{3}H]glycerol for cytosolic NAD^{3}H production, and [2-^{3}H]acetate to measure carbon-bound ^{3}H which was also generated by the metabolism of the commercial 9,10-^{3}H-labelled fatty acids. The assumption that peroxisomal NAD^{3}H can be considered to be equivalent to cytosolic NAD^{3}H was supported using a specific inhibitor of mitochondrial fatty acid oxidation. The approach involves determination of the specific yields, and the relative distribution on carbons 4 and 6, of ^{3}H in glucose from the marker substrates and the labelled fatty acids. In hepatocytes from clofibrate-treated rats, the amount of palmitate or oleate oxidation which starts in the peroxisomes is comparable with that which starts in the mitochondria.

INTRODUCTION

Lazarow & de Duve (1976) discovered that rat liver peroxisomes can oxidize fatty acids. The acyl-CoA oxidation step in peroxisomes is coupled directly to O_2, and produces H_2O_2. The 3-hydroxyacyl-CoA dehydrogenase, however, is coupled to NAD$^+$, producing NADH, as does the mitochondrial enzyme. A great deal of work has been carried out on isolated peroxisomes, and the general conclusion has been that peroxisomes have a good deal of capacity for long chain fatty acid oxidation. However, approaches to the problem of peroxisomal versus mitochondrial fatty acid oxidation using intact cells have produced very different conclusions. Mannaerts *et al.* (1979) concluded that, under physiological conditions, the peroxisomal pathway was quite minor, even in hepatocytes from clofibrate-treated rats. Kondrup & Lazarow (1985), using a novel ^{14}C approach involving marker substrates for mitochondrial and cytosolic [^{14}C]acetyl-CoA generation, found that over 30% of palmitate oxidation was started in the peroxisomes. The reliability of this conclusion has in turn been criticized by Ochs & Harris (1986). Thus at present there seems to be little agreement as to the quantitative importance of the peroxisomal versus mitochondrial oxidation of various fatty acids. I present here an approach to the problem involving the use of specifically tritiated substrates to measure NAD^{3}H generation, based on earlier studies on pathways of NAD^{3}H metabolism in liver and kidney cortex (Rognstad & Clark, 1974).

METHODS

The preparation and incubation of hepatocytes has been described elsewhere (Katz *et al.*, 1978). Incubations were carried out in 5 ml of Krebs–Henseleit (1932) buffer in 25 ml Erlenmeyer flasks. When the tritiated fatty acids were used at tracer levels, the medium contained no albumin. However, at substrate levels of fatty acids, 1% albumin was present. Incubations were terminated by addition of 0.5 ml of 2 M-HClO$_4$, and the cells and medium were washed out to centrifuge tubes to a total of 10 ml. An aliquot (9 ml) of the centrifuged medium was put through ion-exchange resins [a 1 cm × 6 cm column of Dowex 50 (H$^+$) on top of a 1 cm × 8 cm column of Dowex 1 (acetate)] and the columns were washed with 30 ml of water. Aliquots of the eluate were counted for radioactivity both before and after (repeated) drying, the difference giving the H^{3}HO yield. The neutral fraction was brought to pH 7.5 and treated with MgATP and hexokinase, with the glucose 6-phosphate formed being trapped on a second 1 cm × 10 cm column of Dowex 1 (acetate), from which it was eluted with 40 ml of 4 M-formic acid. This was dried in air and made up to 5 ml with water, and a sample was counted for radioactivity to give the glucose yield. The glucose 6-phosphate was dephosphorylated with acid phosphatase, and the glucose formed was degraded with periodate to give the amount of ^{3}H on C-6 (Bloom, 1962).

Clofibrate treatment involved a daily subcutaneous injection of 50 mg/200 g body wt. for 1 week. Rats were fasted for 24 h before preparation of hepatocytes.

[9,10-^{3}H]Palmitate and [9,10-^{3}H]oleate were obtained from New England Nuclear (Boston, MA, U.S.A) or from Research Products International (Mount Prospect, IL, U.S.A.). [2-^{3}H] Acetate was from ICN (Irvine, CA, U.S.A.). The synthesis of the other tritiated substrates has been described (Rognstad & Clark, 1974).

RESULTS AND DISCUSSION

We have previously used tritiated substrates in order to determine whether a given substrate is oxidized in the mitochondria or in the cytosol (Rognstad & Clark, 1974). As in cellular fractionation studies, this approach requires the use of markers, in this case substrates of NAD$^+$-linked dehydrogenases whose locus is assumed to be known. D-[3-^{3}H]3-Hydroxybutyrate is used as a substrate of a mitochondrial NAD$^+$-linked dehydrogenase. Various substrates are possible for use as cytosolic markers, but [2-^{3}H]glycerol has a possible advantage in the present studies. L-[2-^{3}H]Glycerol 3-phosphate is a substrate for the cytosolic NAD$^+$-linked glycerol-3-phosphate dehydrogenase. This enzyme has B-type specificity with regard to the hydrogen on C-4 of the nicotinamide moiety of NADH (Levy *et al.*, 1962). D-3-Hydroxybutyrate dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase are also B-type enzymes (Bentley, 1970). At present, few tritiated fatty acids are commercially available. The probable fate of ^{3}H from (chemically synthesized) [9,10-^{3}H]palmitate in hepatocytes can be outlined. One of the ^{3}H atoms on C-9 will be transferred to NAD^{3}H in the 3-hydroxyacyl-CoA dehydrogenase reaction. The other ^{3}H atom on C-9 and one ^{3}H atom on C-10 will be released to water. The second ^{3}H atom on C-10 will be retained on C-2 of the acetyl-CoA generated. [2-

Table 1. Fraction of peroxisomally initiated fatty acid oxidation estimated from the use of tritiated substrates

Hepatocytes (30–45 mg dry wt.) from 24 h faster rats (controls or with prior treatment with clofibrate for 1 week) were incubated for 30–45 min in 5 ml of Krebs–Henseleit buffer with 10 mM-dihydroxyacetone plus the tritiated substrates shown (10–40 μCi per flask). Tracer levels were used, except that in expt. 4 the [9,10-^{3}H]oleate was 1 mM. TDGA, tetradecylglycidate. A and M are obtained from [2-^{3}H]glycerol, B and N from D-[3-^{3}H]3-hydroxybutyrate, D and P from [2-^{3}H]acetate, and F and Q from the 9,10-^{3}H-labelled fatty acid shown (see the Appendix).

Expt.	Conditions	Tritiated fatty acid	Specific yields in glucose				Fraction of ^{3}H on C-6 of glucose				Fraction of peroxisomally initiated fatty acid oxidation
			M	N	P	Q	A	B	D	F	(C)
1a	Control	[9,10-^{3}H]Palmitate	0.153	0.0056	0.020	0.014	0.035	0.257	0.267	0.145	0.18
1b	Clofibrate-treated	[9,10-^{3}H]Palmitate	0.148	0.0055	0.023	0.020	0.042	0.265	0.276	0.107	0.45
2a	Control	[9,10-^{3}H]Palmitate	0.150	0.0060	0.022	0.015	0.034	0.286	0.292	0.138	0.26
2b	Clofibrate-treated	[9,10-^{3}H]Palmitate	0.144	0.0058	0.023	0.024	0.040	0.291	0.293	0.111	0.46
3a	Control	[9,10-^{3}H]Palmitate	0.156	0.0066	0.025	0.019	0.033	0.166	0.174	0.097	0.23
		[9,10-^{3}H]Oleate	0.156	0.0066	0.025	0.045	0.033	0.166	0.174	0.074	0.44
3b	Clofibrate-treated	[9,10-^{3}H]Palmitate	0.151	0.0051	0.021	0.027	0.036	0.193	0.199	0.071	0.56
		[9,10-^{3}H]Oleate	0.151	0.0051	0.021	0.058	0.036	0.193	0.199	0.066	0.66
4a	Control	[9,10-^{3}H]Oleate	0.250	0.0142	0.056	0.057	0.053	0.190	0.201	0.125	0.27
	50 μM-TDGA added	[9,10-^{3}H]Oleate	0.185	0.0093	0.042	0.101	0.043	0.184	0.197	0.073	0.95

^{3}H]Acetate is used as a marker to monitor the fate of this second ^{3}H atom on C-10.

In previous studies we have used two criteria to categorize tritiated substrates on the basis of whether they are cytosolic or mitochondrial, or are mixed substrates which were oxidized partly in the mitochondria and partly in the cytosol. The criteria were: (i) the relative specific yields in glucose, and (ii) the distribution of ^{3}H on carbons 6 and 4 of glucose. As described previously, mitochondrial substrates produce considerably more labelling on C-6 of glucose than do cytosolic substrates, the reason being that the fumarase exchange is more closely involved in the conversion of mitochondrial NAD^{3}H to glucose. Substrates which generate mitochondrial NAD^{3}H and substrates which generate mitochondrial carbon-bound ^{3}H, such as [2-^{3}H]acetate or [2,3-^{3}H]succinate, produce quite similar patterns of ^{3}H distribution in glucose. However, the relative labelling of glucose versus water is considerably higher from the substrates containing carbon-bound ^{3}H (Rognstad & Clark, 1974).

The Appendix describes in detail the procedure used to calculate the fraction of fatty acid oxidation which originates in the peroxisomes versus that in the mitochondrial fraction. The key assumption required is that peroxisomal NAD^{3}H is equivalent to cytosolic NAD^{3}H. The high permeability of the peroxisomal membrane to certain low-molecular-mass compounds such as sucrose was established long ago by de Duve and co-workers (Beaufay *et al.*, 1964; du Duve & Baudhuin, 1966). More recently, Van Veldhoven *et al.* (1987) have shown that the peroxisome is highly permeable to all low-molecular-mass (up to 800 Da) molecules by virtue of a pore-forming protein. Also, the results below, showing that under certain conditions there is essentially complete NAD^{3}H formation in the cytosolic space, suggest that our assumption is justified.

Table 1 presents the results of a number of experiments, using hepatocytes from normal and clofibrate-treated rats, with dihydroxyacetone as the gluconeogenic substrate. In most of the experiments the tritiated fatty acids were added at tracer levels, and thus the pathway of endogenous fatty acid oxidation is being chiefly measured. In some experiments, substrate levels of fatty acids were added. More experiments will be required with a greater range of added fatty acid concentrations to see how changes in overall rates of fatty acid oxidation may affect the proportions of the peroxisomal and mitochondrial pathways. There are too few repetitions of conditions to make any very quantitative conclusions. However, it can be seen that (*a*) peroxisomal fatty acid oxidation is not necessarily a minor pathway; (*b*) as is the case with isolated peroxisomes, more oleate than palmitate is oxidized peroxisomally; (*c*) clofibrate treatment causes a definite increase in the fraction of the peroxisomal pathway; and (*d*) as expected, an inhibitor (tetradecylglycidate) of mitochondrial long chain fatty acid oxidation increased the fraction of peroxisomal fatty acid oxidation. In preliminary experiments with L-lactate as the gluconeogenic substrate, I have found in general a somewhat increased contribution of peroxisomal fatty acid oxidation compared with the experiments with dihydroxyacetone. Indeed, with L-lactate as the gluconeogenic substrate, results were obtained that were very comparable with those of Kondrup & Lazarow (1985), who estimated about 30% peroxisomal palmitate metabolism with their ^{14}C approach. Both approaches require the use of marker substrates. Kondrup & Lazarow used [^{14}C]butyrate and [^{14}C]acetate as specific generators of [^{14}C]acetyl-CoA in the mitochondria and cytosol respectively, and used the ^{14}C yield in cholesterol as a measure of the cytosolic acetyl-CoA path.

The estimated values of peroxisomal fatty acid oxidation in the present paper are higher than those of Mannaerts *et al.* (1979). However, I have estimated peroxisomally initiated fatty acid oxidation, and total peroxisomal fatty acid oxidation may be only about 60% of this, since peroxisomal fatty acid oxidation does not go to completion. Even with this adjustment, however, I calculate there to be a considerably higher proportion of peroxisomal fatty acid oxidation than do Mannaerts *et al.* (1979) who estimated about 5% in control and 7.5% in clofibrate-treated rats. Mannaerts *et al.* (1979) used formaldehyde production from methanol as a measure of the H_2O_2 produced in the peroxisomal fatty acid oxidation pathway. One possible problem with the method (as used) was that semicarbazide, at 10 mM, was added to trap the formaldehyde. We have found that even 5 mM-semicarbazide inhibited gluconeogenesis from lactate by 65%

under our conditions. Using our approach I found that 5 mM-semicarbazide caused a marked decrease in the fraction of peroxisomal palmitate oxidation (results not shown). On the other hand, Mannaerts *et al.* (1979) showed that the presence of semicarbazide had no effect on the overall rate of oleate oxidation under their conditions.

The present results suggest the equivalence of peroxisomal and cytosolic NAD^{3}H, and suggest that peroxisomal metabolism increases the supply of NADH to the cytosol, rather than that peroxisomes are a sink for cytosolic NADH oxidation (de Duve & Baudhuin, 1966; Masters & Holmes, 1977). Cytosolic NADH must in turn be transferred into the mitochondria (by the glycerol phosphate or malate–aspartate shuttles), and it is likely that the gluconeogenic substrates used here, by raising intermediate levels (Rognstad, 1981), increase the capacity of these shuttle systems. It is conceivable that the rate of NADH oxidation by mitochondrial shuttle systems may affect the rate of peroxisomal fatty acid oxidation if the 3-hydroxyacyl-CoA dehydrogenase reaction is at all rate-limiting. Indeed, another known effect of clofibrate, in addition to peroxisomal proliferation, is an increase in mitochondrial glycerol 3-phosphate oxidase activity (Westerfeld *et al.*, 1968), which may be especially required if NADH generation by peroxisomal fatty acid metabolism becomes large.

The approach described in the present paper could be materially improved by using fatty acids specifically tritiated on the odd-numbered carbons, since no correction for carbon-bound ^{3}H would then be required. Data from specifically tritiated fatty acids (3-^{3}H, 5-^{3}H, 7-^{3}H, 9-^{3}H, etc.) would also give the exact degree to which peroxisomal oxidation may terminate at the C-10, C-8, C-6 level, etc. The assumption in the present approach, that peroxisomal palmitate oxidation proceeds at least as far as octanoate, will then no longer be required.

The present approach has assumed a uniform population of hepatocytes. However, it is possible that pathways of fatty acid oxidation may be somewhat different quantitatively in periportal and perivenous regions (Olson & Thurman, 1987). Further studies with tritiated substrates on hepatocytes from periportal and perivenous zones (Quistorff, 1985) will be required to establish whether peroxisomal contributions to overall fatty acid oxidation are different between these zones.

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APPENDIX

Calculation of relative amounts of peroxisomally initiated versus mitochondrial fatty acid oxidation using tritiated substrates

D-[3-^{3}H]3-Hydroxybutyrate is oxidized solely by the NAD$^+$-linked D-3-hydroxybutyrate dehydrogenase reaction in the mitochondria. [2-^{3}H]Glycerol, after phosphorylation to [2-^{3}H]glycerol 3-phosphate, is oxidized in the cytosol by the NAD$^+$-linked glycerol-3-phosphate dehydrogenase. It is also oxidized by the mitochondrial glycerol-3-phosphate oxidase reaction, but this is of no importance here, since H^{3}HO is the only product of the reaction. As mentioned in the main text, 9,10-^{3}H-labelled fatty acids such as palmitate or oleate will generate NAD^{3}H from one ^{3}H atom on C-9 and [2-^{3}H]acetyl-CoA from one ^{3}H atom on C-10. This is the fate of the two ^{3}H atoms in [9,10-^{3}H]oleate. With [9,10-^{3}H]palmitate, the other two ^{3}H atoms on carbons 9 and 10 are released to water. To monitor the fate of the [2-^{3}H]acetyl-CoA, I have used the marker compound [2-^{3}H]acetate. The approach thus requires the use of three marker compounds: [2-^{3}H]glycerol to measure cytosolic NAD^{3}H; D-[3-^{3}H]3-hydroxybutyrate to measure mitochondrial NAD^{3}H; and [2-^{3}H]acetate to measure mitochondrial carbon-bound ^{3}H. As mentioned previously, the approach assumes that peroxisomal NAD^{3}H is equivalent to cytosolic NAD^{3}H.

^{3}H from NAD^{3}H produced in either the mitochondria or the cytosol causes labelling essentially only on carbons 4 and 6 of glucose (Hoberman & D'Adamo, 1960; Rose *et al.*, 1969). Tritium labelling on C-4 of glucose occurs as the result of NAD^{3}H reduction of 1,3-bisphosphoglycerate. ^{3}H labelling on C-6 of glucose is more involved. Reduction of (mitochondrial plus cytosolic) oxalacetate with NAD^{3}H produces L-[2-^{3}H]malate; the fumarase reaction randomizes this extensively to L-[2,3-^{3}H]malate. ^{3}H on C-3 of malate yields [6-^{3}H]glucose in the gluconeogenic pathway. The rates of the mitochondrial and cytosolic malate dehydrogenase reactions, and the rate of isotopic exchange of malate between the mitochondria and cytosol, are not slow, but they are by no means fast enough to isotopically equilibrate the mitochondrial and cytosolic NAD^{3}H pools. Thus there is considerably more ^{3}H incorporation in glucose from substrates which generate cytosolic NAD^{3}H than from substrates which generate mitochondrial NAD^{3}H.

Using the mitochondrial and cytosolic marker substrates described, I have obtained data for the specific yields [here used as the ratio glucose/(glucose + water)] of ^{3}H in glucose and the

fractional amount of this ^{3}H on C-6 of glucose. In the initial derivation here, involving the use of commercial 9,10-^{3}H-labelled fatty acids, we require an assumption as to how far peroxisomally initiated fatty acid oxidation proceeds. In order for NAD^{3}H to be generated in the peroxisomes, [9,10-^{3}H]palmitate oxidation must proceed at least up to the octanoate stage (four cycles of acetyl-CoA formation), while [9,10-^{3}H]oleate must be oxidized at least to the decanoate stage (again four cycles of acetyl-CoA formation). Whether the oxidation proceeds farther than this in the peroxisomes before the shortened fatty acid is transferred to the mitochondria is of no consequence to the present approach. However, if for example a significant fraction of peroxisomally initiated [9,10-^{3}H]palmitate oxidation proceeded only to the decanoate stage, the ^{3}H would all be retained (as [3,4-^{3}H]-decanoate). Thus the current approach would underestimate peroxisomally initiated oxidation, since further mitochondrial metabolism of [3,4-^{3}H]decanoate would produce mitochondrial NAD^{3}H in the next cycle of acetyl-CoA formation. I discuss in the main text the approach needed to quantify exactly how much peroxisomal oxidation may terminate at any stage (e.g. C-10, C-8, C-6) before shuttling the shortened fatty acid into the mitochondria.

I have considered a fatty acid which is metabolized partly in the mitochondria and partly in the peroxisomes (equivalent to the cytosol). Let C equal the fractional amount of oxidation which is initiated in the peroxisomes, and $1-C$ is the fractional amount which is initiated (and here of course goes to completion) in the mitochondria. C is the unknown value which we wish to estimate. The symbols used for the derivation are given in Table A1. The specific yield from the fatty acid will not be used in the present derivation. The total ^{3}H specific yield in glucose, from [9,10-^{3}H]palmitate, is $\frac{1}{4}C(M+P)$ in the peroxisomes plus $\frac{1}{4}(1-C)(N+P)$ in the mitochondria. The ^{3}H specific yield on C-6 of glucose is $\frac{1}{4}C(MA+PD)$ in the peroxisomes plus $\frac{1}{4}(1-C)(NB+PD)$ in the mitochondria. The ^{3}H yield on C-6, versus total ^{3}H yield in glucose, from [9,10-^{3}H]palmitate is denoted as F, and is thus given by (the $\frac{1}{4}$ terms cancelling):

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Table A1. Symbols used in calculation of peroxisomal fatty acid oxidation

Tritiated substrate	Fraction of ^{3}H on C-6 of glucose	Specific yield in glucose
[2-^{3}H]Glycerol	A	M
D-[3-^{3}H]3-Hydroxybutyrate	B	N
[2-^{3}H]Acetate	D	P
[9,10-^{3}H]Palmitate	F	Q

$$F=\frac{C(MA+PD)+(1-C)(NB+PD)}{C(M+P)+(1-C)(N+P)}$$

Solving this equation for the unknown, C, gives:

$$C=\frac{P(D-F)+N(B-F)}{M(F-A)+N(B-F)}$$

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