# On the quantitative importance of ω-oxidation of fatty acids

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**Abstract** The relative importance of ω-oxidation of fatty acids in livers of starved rats was studied by determination of the ratio between $^{14}C$ at C1 and at C2, C3, C4 in acetoacetate formed from [16-$^{14}C$]palmitic acid in vivo and in vitro and from [10-$^{14}C$]decanoic acid in vitro. The results are consistent with the possibility that at least a small fraction of the fatty acids may be subject to primary ω-oxidation prior to β-oxidation in the ketotic state. The maximal figure for this pathway was calculated to be of the magnitude 5–10%. The metabolic consequences of a pathway involving primary ω-oxidation of fatty acids are discussed.

**Supplementary key words** ketone bodies · ketosis · starvation · dicarboxylic acids

The relative importance of ω-oxidation of fatty acids under different conditions is not known. Antony and Landau (1) have studied the distribution of $^{14}C$ among the carbon atoms of liver glycogen-glucose after incubation of liver slices with long-chain fatty acids labeled with $^{14}C$ in the 1- or ω-position. From these studies it was concluded that ω-oxidation was of little quantitative importance as compared to β-oxidation under the conditions employed. No exact figure was given but it could be calculated that, of the acetate formed from the terminal carbons of stearate and incorporated into glucose, 25 times as much was formed via β-oxidation as was formed via ω-oxidation. In view of results of later in vitro studies, however, the experimental model used by Antony and Landau may not correspond to the conditions under which ω-oxidation is optimal. It has been shown that when using the 10,000 *g* or 20,000 *g* supernatant fluid of rat liver homogenate, starvation or diabetes increases ω-oxidation of fatty acids several fold (2–4). In the case of ω-oxidation of long-chain fatty acids, this increase is exclusively or almost exclusively due to decreased esterification, which increases the amount of fatty acids available for ω-oxidation (4). In the case of medium-chain fatty acids, at least part of the increased ω-oxidation is due to increased capacity of the microsomal fraction to hydroxylate in the ω-position (3).

That ω-oxidation is increased in the starved state is further evident from the fact that ketotic patients excrete considerable quantities of medium- and short-chain dicarboxylic acids in urine (5). These acids, which are almost absent in normal human urine, are most probably formed in the liver from long-chain monocarboxylic acids by ω-oxidation followed by β-oxidation. A low conversion in vivo of palmitic acid into dicarboxylic acids has been observed in ketotic rats (6). From all the above findings it appeared to be of interest to study the relative importance of ω-oxidation of fatty acids in the ketotic liver by using a technique similar to that used by Antony and Landau (1). A prerequisite for the study of Antony and Landau was a considerable exchange of $^{14}C$ between acetate and glycogen. In addition, the in vitro system used by Antony and Landau (1) may not be representative of the situation in vivo. In the present study the incorporation of $^{14}C$ into ketone bodies from 16-$^{14}C$-labeled palmitate was therefore studied in whole animals as well as in liver slices in a ketotic state. The fraction of the [16-$^{14}C$]palmitic acid that is ω-oxidized prior to β-oxidation will give rise to [1-$^{14}C$]acetate. The 1-$^{14}C$-label must be incorporated into 1- and 3-positions of acetoacetate. In the ketotic state the ratio between the $^{14}C$ incorporated in the 1- and 3-positions should be about 1.0 (7, 8). The fraction of the [16-$^{14}C$]palmitic acid that is β-oxidized will give rise to [2-$^{14}C$]acetate. The 2-$^{14}C$-label must be incorporated into the 2- and 4-positions of acetoacetate. Thus it may be possible to obtain some information concerning the relative importance of ω-oxidation by a determination of the ratio between $^{14}C$ incorporated into the 1-position and into the other positions in acetoacetate formed from [16-$^{14}C$]palmitic acid.

## EXPERIMENTAL PROCEDURE

### Materials

1-$^{14}$C-Labeled and 16-$^{4}$C-labeled palmitate as well as 1-$^{14}$C- and 2-$^{14}$C-labeled acetate (specific radioactivity 50–55 $\mu$Ci/$\mu$mol) were obtained from New England Nuclear (Frankfurt/Main, West Germany). The radiochemical purity of the fatty acids was more than 99% as judged by thin-layer chromatography using hexane–diethylether–acetic acid 18:2:1 (v/v/v) as solvent.

In the in vitro experiments, the labeled fatty acids were diluted with unlabeled palmitic acid to give a specific radioactivity of 2.3 $\mu$Ci/$\mu$mol. [10-$^{14}$C]Decanoic acid was prepared by electrolytic coupling of [2-$^{14}$C]acetic acid with methyl hydrogen sebacate according to a method described for the preparation of [10-$^{2}H_3$]decanoic acid from [2-$^{2}H_3$]acetate (9). The product (0.7 $\mu$Ci/$\mu$mol) was purified by silicic acid chromatography, and was shown to be more than 99% pure as judged by thin-layer chromatography as above. Decarboxylation of [16-$^{14}$C]palmitic acid and [10-$^{14}$C]decanoic acid showed that less than 0.1% of the radioactivity was present in the carboxyl group. Enzymes and cofactors (cf. below) were obtained from Sigma Chemical Co, (St. Louis, MO).

### Methods

White male rats of the Sprague-Dawley strain weighing about 200 *g* were used. The rats were starved for 72 hr prior to the experiments. In the in vivo experiments, 2 ml of a solution of the sodium salt of the labeled fatty acid, 50 $\mu$Ci, in 2 ml of a sodium chloride solution (0.9%, w/v) containing serum albumin (1%, w/v) was injected into the femoral vein under ether anesthesia. Blood was collected from the aorta after 3–5 min and the liver was removed. The blood was diluted with 5 ml of isotonic sodium chloride solution containing heparin. The liver was homogenized in 20 ml of isotonic sodium chloride solution.

A solution of $CuSO_4$ (10 ml, 25% w/v) was added to the combined liver and blood samples (8). The mixture was centrifuged and the pH of the supernatant was corrected to 8.0 with solid calcium chloride. The solution was recentrifuged and the clear supernatant was incubated at 37°C for 40 min together with sodium pyruvate (10 $\mu$mol), $NAD^+$ (10 $\mu$mol), serum albumin (10 mg), lactate dehydrogenase (4 U), and $\beta$-hydroxybutyrate dehydrogenase (2.4 U) (8). The acetoacetate formed was stabilized by addition of 200 $\mu$mol of acetoacetate. The mixture was then centrifuged. Half of the supernatant was used for determination of radioactivity at C1 in acetoacetate and half for determination of radioactivity at C2–C4 in acetoacetate. The portion to be tested for radioactivity at C1 in acetoacetate was acidified to pH 4 with a 50% solution of citric acid. Excess $CO_2$ was removed by gassing with $CO_2$-free nitrogen for 30 min. Decarboxylation was performed with aniline citrate according to Edson (10) in a sealed vessel for 3 hr. The labeled $CO_2$ liberated was absorbed in 5 ml of carbonate-free 0.1 M sodium hydroxide solution. Both the reaction mixture and the sodium hydroxide solution were stirred continuously. The radioactive $CO_2$ was precipitated as barium carbonate by the addition of 6 ml of 0.1 M $NH_4Cl$ and 2.25 ml of 0.5 M $BaCl_2$. After centrifugation and washing of the precipitate with $CO_2$-free water, the barium carbonate was dried overnight at 120°C and weighed. Exact portions of barium carbonate, 1–2 mg, were then treated with 0.1 M HCl and the liberated carbon dioxide was absorbed in 20 ml of ethanolamine–ethyleneglycol monomethyl ether 1:2 (v/v) by gassing with $CO_2$-free nitrogen for 30 min. In some experiments decarboxylations were performed with 0.1 M acetic acid instead of 0.1 M HCl.

A 15-ml portion of scintillation solution, consisting of toluene–ethyleneglycol monomethyl ether 2:1 (v/v) and 83 mg of PPO, was added to the solution with the trapped carbon dioxide. Radioactivity was assayed with a SL 30 Intertechnique liquid spectrophotometer. The maximal counting error in any of the experiments was 5%. Quench corrections and corrections for differences in counting efficiency between the two different scintillants (cf. below) were performed with the use of $^{14}$C-labeled internal standards. The specific radioactivity of the carboxyl group was calculated from the mean of three independent decarboxylations of the same preparation of barium carbonate.

The supernatant for the determination of radioactivity at C2–C4 of acetoacetate was boiled for 30 min with 11 ml of 50% (w/v) sulfuric acid, 38 ml of a 10% (w/v) mercuric sulfate solution, and 50 ml of water (8). The precipitated complex was collected and treated with 15 ml of 3 M HCl. The acetone formed was isolated by steam distillation and converted into 2,4-dinitrophenylhydrazone. The hydrazone was purified by chromatography on aluminium oxide (Woelm III). Pure hydrazone was eluted with toluene and crystallized twice from ethanol prior to assay of radioactivity. The purity of the crystallized material was also ascertained by thin-layer chromatography (with benzene–ethyl acetate 1:1 (v/v) as solvent).

In the in vitro experiments, 300 mg of liver slices (wet weight) were incubated in a closed incubation

flask together with 5 ml of Krebs-Henseleit bicarbonate buffer and 1 ml of a solution of albumin-bound labeled fatty acid in an atmosphere of 95% $O_2$ and 5% $CO_2$ for 90 min at 37°C (11). The final concentration of fatty acid was 0.25 mM, corresponding to 2.24 $\mu$Ci in the case of 1- and 16-$^{14}$C-labeled palmitic acid and 0.5 $\mu$Ci in the case of [10-$^{14}$C]decanoic acid. The incubations were terminated by heating to 80°C in a water bath for 10 min. The $\beta$-hydroxybutyrate formed was immediately oxidized into acetoacetate using the same conditions as above. Analysis of radioactivity in the carboxyl group and in the carbonyl group of acetoacetate was performed as above.

## RESULTS

**Table 1** summarizes the results. In accordance with previous work (7, 8) acetoacetate formed from [1-$^{14}$C]acetate as well as from [1-$^{14}$C]palmitate in vivo had a ratio between $^{14}$C label at C1 and at C2, C3, C4 of about 1. The ratio between $^{14}$C label at C1 and at C2, C3, C4 in acetoacetate formed from [2-$^{14}$C]acetate in four different experiments was 0.001 ± 0.001 (mean ± SEM). The ratio between $^{14}$C label at C1 and at C2, C3, C4 in acetoacetate formed from [16-$^{14}$C]-palmitate in vivo in four different experiments was 0.010 ± 0.003 (mean ± SEM) which was significantly higher than the corresponding value obtained with [2-$^{14}$C]acetate ($P < 0.05$, Student's $t$ test). In view of the considerable variation obtained in the in vivo experiments, in vitro experiments were also performed. The ratio between $^{14}$C label at C1 and at C2, C3, C4 in acetoacetate formed from [16-$^{14}$C]palmitate in vitro varied between 0.008 and 0.010 in the three experiments performed. A higher ratio, about 0.02, was obtained in two in vitro experiments with [10-$^{14}$C]-decanoic acid. In similarity with the in vivo experiments, the ratio between $^{14}$C label at C1 and at C2, C3, C4 in acetoacetate formed from [1-$^{14}$C]palmitate was about 1.

It was not possible to perform conclusive experiments with 1-$^{14}$C- and 2-$^{14}$C-labeled acetate in vitro due to the high concentration of unchanged substrate in the buffer medium. Thus, to some extent, volatile acetic acid from the medium always contaminated the carbon dioxide in the decarboxylation experiments. Carbon dioxide generated from $BaCO_3$ obtained in experiments with [2-$^{14}$C]acetate therefore contained small amounts of radioactivity. That this label mainly corresponds to 2-$^{14}$C-labeled acetate was evident from the finding that the radioactivity in the carbon dioxide decreased when hydrochloric acid was substituted for acetic acid in the decarboxylation procedure. This was not the case, however, in the decarboxylations performed with materials from any of the other experiments in vivo or in vitro.

TABLE 1. Distribution of radioactivity between carbonyl and carboxyl part of ketone bodies derived from 1- and 2-$^{14}$C-labeled acetate as well as 1- and $\omega$-$^{14}$C-labeled fatty acids

| Ketone Bodies Derived From | Radioactivity at C2, C3, C4 | Radioactivity at C1 | Ratio C1 / C2, C3, C4 |
|---|---|---|---|
| | *cpm/μmol* | *cpm/μmol* | |
| [1-$^{14}$C]Acetate in vivo | 408 | 420 | 1.03 |
| [1-$^{14}$C]Acetate in vivo | 293 | 354 | 1.20 |
| [1-$^{14}$C]Acetate in vivo | 885 | 717 | 0.81 |
| [2-$^{14}$C]Acetate in vivo | 1011 | 0 | 0.000 |
| [2-$^{14}$C]Acetate in vivo | 326 | 0 | 0.000 |
| [2-$^{14}$C]Acetate in vivo | 1337 | 4 | 0.003 |
| [2-$^{14}$C]Acetate in vivo | 487 | 1 | 0.002 |
| [1-$^{14}$C]Palmitate in vivo | 282 | 306 | 1.08 |
| [1-$^{14}$C]Palmitate in vivo | 343 | 295 | 0.86 |
| [1-$^{14}$C]Palmitate in vivo | 32 | 22 | 0.69 |
| [16-$^{14}$C]Palmitate in vivo | 2491 | 37 | 0.015 |
| [16-$^{14}$C]Palmitate in vivo | 1954 | 31 | 0.016 |
| [16-$^{14}$C]Palmitate in vivo | 1648 | 6 | 0.004 |
| [16-$^{14}$C]Palmitate in vivo | 1547 | 6 | 0.004 |
| [1-$^{14}$C]Palmitate in vitro | 399 | 352 | 1.13 |
| [1-$^{14}$C]Palmitate in vitro | 119 | 113 | 1.05 |
| [1-$^{14}$C]Palmitate in vitro | 23 | 29 | 0.79 |
| [16-$^{14}$C]Palmitate in vitro | 1191 | 11 | 0.009 |
| [16-$^{14}$C]Palmitate in vitro | 761 | 6 | 0.008 |
| [16-$^{14}$C]Palmitate in vitro | 298 | 3 | 0.010 |
| [10-$^{14}$C]Decanoate in vitro | 532 | 12 | 0.023 |
| [10-$^{14}$C]Decanoate in vitro | 99 | 2 | 0.020 |

## DISCUSSION

The finding that the ratio between radioactivity at C1 and at C2, C3, C4 in acetoacetate formed from [16-$^{14}$C]palmitate was significantly higher than the corresponding ratio in acetoacetate formed from [2-$^{14}$C]-acetate can be taken as evidence for an $\omega$-oxidation in vivo in ketotic rats.

In order to make a rough estimation of the quantitative importance of this pathway, several different assumptions must be made:

1. Steady-state conditions are obtained during the experiments and there is no large unlabeled dicarboxylic acid pool in the tissues.
2. The dicarboxylic acid formed in the $\omega$-oxidation may be $\beta$-oxidized from either end of the molecule with as much likelihood for one end as the other to be oxidized. If so, half of the $^{14}$C-label from each labeled dicarboxylic acid molecule will

end up in [1-$^{14}$C]acetate and half in either [1-$^{14}$C]succinate or a higher 1-$^{14}$C-labeled dicarboxylic acid.

3. Radioactivity at C1 = C3 and at C2 = C4 in the acetoacetate formed during the different experimental conditions (cf. ref. 8).
4. The relative incorporation of $^{14}$C from [1-$^{14}$C]-acetate into acetoacetate is the same as that from [2-$^{14}$C]acetate under the conditions employed.
5. There is no ω-oxidation of the CoA derivative of fatty acids.
6. The acetyl CoA pool formed from acetate is common to that formed from the fatty acids.

The maximal ratio between radioactivity at C1 and at C2, C3, C4 in acetoacetate formed from [16-$^{14}$C]-palmitate in any of the in vivo experiments was 0.016. According to assumption 3 this corresponds to a ratio between acetoacetate generated from [1-$^{14}$C]acetate and [2-$^{14}$C]acetate of about 0.032. Assumption 2 will give a correction factor of 2. Thus the maximal figure for ω-oxidation is about 6%. If the mean value for ratio between radioactivity at C1 and at C2, C3, C4 in acetoacetate formed from [16-$^{14}$C]palmitate in vivo and in vitro is used in the calculation, about 4% of palmitic acid should be ω-oxidized under the conditions employed. The corresponding figure for decanoic acid is higher, about 8%. This may be expected in view of the previous finding that medium-chain fatty acids are ω-oxidized more efficiently than long-chain fatty acids in vitro (14).

It must be pointed out that the above calculations are rough estimates and that all of the assumptions may be open to criticism.

The first assumption seems reasonable, at least in the in vivo experiment. It may be mentioned that combined gas–liquid chromatography/mass spectrometry has failed to demonstrate any significant pool of medium-chain dicarboxylic acids in liver preparations.[1]

The second assumption also seems reasonable, but there is no experimental proof for it. An alternative assumption is that both ends of the same dicarboxylic acid molecule may be β-oxidized. If so, one molecule of [1-$^{14}$C]acetate is obtained from every molecule of oxidized 1-$^{14}$C-labeled dicarboxylic acid. The present assumption may give an overestimation of ω-oxidation and the alternative assumption may give an underestimation. It may be mentioned that Antony and Landau (1) did not use the present assumption in their calculation.

Concerning the third assumption, it may be argued that radioactivity at C4 eventually may be somewhat higher than at C2. The reason for this is the possibility of an incomplete cleavage of the four last carbon atoms of palmitate during β-oxidation. If so, a subsequent condensation of the acetoacetyl CoA formed with acetyl CoA may lead to a different degree of dilution of radioactivity at C2 and C4 in acetoacetate formed from [16-$^{14}$C]palmitate.

Since [1-$^{14}$C]acetate as well as [2-$^{14}$C]acetate must be fed into the acetyl CoA pool from which acetoacetate is formed, the fourth assumption also seems reasonable. Theoretically, there may be a small dilution of radioactivity at C1 relative to C2 in the acetyl CoA pool. The reason for this is that if acetyl CoA is fed into the Krebs cycle, radioactivity at C1 is lost as $CO_2$. Radioactivity at C2 of acetyl CoA fed into the Krebs cycle might, however, to some extent be reconverted into [1,2-$^{14}$C]acetate via [2,3-$^{14}$C]succinate and [2,3-$^{14}$C]pyruvate. That such a pathway is of little or no importance under the conditions of the present experiments is evident from the fact that there was little or no radioactivity in the carboxyl group of acetoacetate derived from [2-$^{14}$C]acetate in vivo. A significant amount of radioactivity would have been expected if a significant recycling had occurred. In spite of the above considerations, there was in fact a higher incorporation of radioactivity from [2-$^{14}$C]-acetate than from [1-$^{14}$C]acetate into acetoacetate in the in vitro experiments. It was not possible to make such comparisons in the in vivo experiments because of the great variations in the different individual experiments. Since it is difficult to explain a difference between incorporation from [1-$^{14}$C]acetate and [2-$^{14}$C]acetate into acetoacetate by the known pathways, the results obtained in the in vitro experiments must be taken with caution. Thus it may not be justified to use the difference in relative incorporation of radioactivity from [1-$^{14}$C]acetate and [2-$^{14}$C]-acetate in the in vitro experiments as a correction factor in the calculation of the relative importance of ω-oxidation in vivo.

Concerning the fifth assumption, there is as yet no experimental evidence for ω-oxidation of the CoA derivative of fatty acids. In a previous communication it was shown that if the CoA derivative of stearic acid is incubated with a 20,000 *g* supernatant fluid of homogenate of liver from a starved rat, a significant amount of ω-oxidized product could be isolated (3). Later studies, however, showed that at least the major part of the CoA derivative had been hydrolyzed prior to ω-oxidation in the system used (4). If there is some ω-oxidation of the CoA derivative of ω-$^{14}$C-labeled fatty acids prior to β-oxidation, the major part of the label must be trans-

[1] Björkhem, I. Unpublished observations.

formed into [1-$^{14}$C]succinate in the subsequent metabolism. This radioactivity is incorporated into acetoacetate only to a very small extent. Thus, if the fifth assumption is not valid, the above calculations will underestimate ω-oxidation.

Concerning the sixth assumption, there is no experimental evidence that the acetyl CoA pool formed from acetate is different from that formed from fatty acids in the liver. It would have been safer, however, to use 2-$^{14}$C-labeled palmitic acid in the experiments.

To summarize, two of the assumptions used in the calculation of the importance of ω-oxidation may lead to an underestimation and one may lead to an overestimation. The final figure obtained for ω-oxidation of palmitic acid in vivo under ketotic conditions, about 5%, is however very similar to the figure obtained by Antony and Landau (1) with their independent in vitro technique.

After completion of the present work, a publication appeared by Wada and Usami (15) in which attempts had been made to quantitate ω-oxidation of fatty acids in vivo in rats. It was shown that label from injected U-$^{14}$C-labeled palmitic acid was incorporated into blood glucose more efficiently than label from [1-$^{14}$C]palmitic acid. It was also shown that label from [U-$^{14}$C]hexadecanedioate was incorporated into glucose more efficiently than label from [1-$^{14}$C]hexadecanedioate. From the results obtained, it was calculated that about 15% of palmitic acid was subjected to ω-oxidation and then β-oxidation. This calculation was based upon the assumption that if palmitate is metabolized by β-oxidation without ω-oxidation, there should be no difference in the incorporation of $^{14}$C into glucose from [1-$^{14}$C]- and [U-$^{14}$C]palmitate. This assumption may, however, be erroneous. During the metabolism most of the label in [U-$^{14}$C]palmitate is transferred into [1,2-$^{14}$C]acetate. It is well established that $^{14}$C is incorporated into glucose considerably more efficiently from [2-$^{14}$C]acetate than from [1-$^{14}$C]-acetate (1). This is due to the fact that carbon 1 is lost as $CO_2$ to a greater extent than is carbon 2 in the Krebs cycle (cf. above). As a consequence carbon 2 is retained more in succinate and glucose than is carbon 1. In view of this, the results by Wada and Usami (15) may have been expected even if there was no ω-oxidation at all. In the calculation by Wada and Usami, it was also assumed that intraperitoneally injected palmitate and hexadecanedioate are equally well transported to the liver and that the metabolic fate of administered dicarboxylic acid is the same as that of dicarboxylic acid formed in the hepatocytes from palmitic acid. In view of the different degree of dilution with endogenous material, however, the latter assumption may also be open to criticism. Most probably, the calculation by Wada and Usami (15) overestimates ω-oxidation.

The fraction of the fatty acids that is primarily ω-oxidized gives succinyl CoA in subsequent β-oxidations. This succinyl CoA may be incorporated into glucose and by this means a net conversion into glucose would occur (2, 12). For each molecule of ω-oxidized acid, a maximum of four carbon atoms are then incorporated into glucose. Using the present calculated figure for primary ω-oxidation of palmitic acid, about 5%, the maximal transfer of carbon from fatty acid metabolism into biosynthesis of glucose would be about 0.2 carbon atom per molecule of palmitic acid oxidized. Such small net conversion is difficult or impossible to demonstrate experimentally. In a study on fatty acid metabolism in perfused rat liver, Krebs and Hems (12) did not observe any increase in glucose formation in the liver after addition of medium- and long-chain fatty acids to the perfusion medium. Use of livers of rats treated with phenobarbital did not affect the rate of glucose formation or rate of formation of ketone bodies. It was concluded that ω-oxidation is of little quantitative importance since treatment with phenobarbital was believed to increase ω-oxidation of fatty acids and such increase would affect formation of glucose and ketone bodies. Later studies have shown, however, that phenobarbital has no stimulatory effect on ω-oxidation of long-chain fatty acids (13). The latter finding makes the results of the study by Krebs and Hems (12) less conclusive. Wada et al. (2) have shown that administration of long-chain dicarboxylic acid to diabetic rats decreases the concentration of ketone bodies. On the basis of this experiment it was suggested that ω-oxidation may be of importance under conditions of increased utilization of fatty acids due to increased formation of succinate. The experimental conditions used by Wada et al. (2) were, however, highly unphysiological since more than 80% of the diet consisted of long-chain dicarboxylic acid. From the results of the present work and the previous work by Krebs and Hems (12) and by Antony and Landau (1), it can be concluded that ω-oxidation is of little importance from a quantitative metabolic point of view. The possibility has not been excluded, however, that products of ω-oxidation of fatty acids may have some regulatory importance.

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