Formic acid is a product of the α-oxidation of fatty acids by human skin fibroblasts: deficiency of formic acid production in peroxisome-deficient fibroblasts

Alf POULOS,*‡ Peter SHARP,* Harmeet SINGH,* David W. JOHNSON,* William F. CAREY* and Christopher EASTON†

*Department of Chemical Pathology, Women's and Children's Hospital, 72 King William Road, North Adelaide, South Australia 5006, and †Department of Organic Chemistry, University of Adelaide, Adelaide, South Australia

Human skin fibroblasts in culture can oxidize β-methyl fatty acids, such as phytanic acid and 3-methylhexadecanoic acid, to CO_2 and water-soluble products. The latter are released largely into the culture medium. The major water-soluble product formed from [1-^{14}C]phytanic and [1-^{14}C]3-methylhexadecanoic acids is [^{14}C]formic acid. As phytanic acid and 3-methylhexadecanoic acids contain β-methyl groups and theoretically cannot be degraded by β-oxidation, we postulate that formic acid is formed from fatty acids by α-oxidation. The marked reduction in formic acid production from β-methyl fatty acids in peroxisome-deficient skin fibroblasts suggests that peroxisomes are involved in the generation of C_1 units.

INTRODUCTION

Fatty acid oxidation is an important biological process and takes place in most mammalian cells. Three separate oxidative pathways have been described, i.e. α-, β- and ω-oxidation. β-Oxidation has been studied in detail and is known to take place in mitochondria and peroxisomes. Different enzyme proteins are involved in mitochondrial and peroxisomal β-oxidation (Schulz, 1991). Acetyl-CoA is formed from fatty acids by β-oxidation in mitochondria and peroxisomes (Singh and Poulos, 1986), and is further metabolized in mitochondria via the citric acid cycle or is used in biosynthetic reactions in the cell. The intracellular site of α-oxidation is controversial. Subcellular fractionation studies carried out on rat, monkey and human liver suggest that α-oxidation takes place in mitochondria (Skjeldal and Stokke, 1987; Watkins et al., 1990; Wanders et al., 1991) but the occurrence of patients with abnormalities in peroxisomal assembly, whose skin fibroblasts show marked reductions in their ability to α-oxidize fatty acids, suggests that α-oxidation takes place in peroxisomes in human skin fibroblasts (Poulos et al., 1986a; Skjeldal et al., 1986). The primary non-lipid product of α-oxidation is believed to be carbon dioxide, formed by decarboxylation after the introduction of a hydroxy group at the α-position of the fatty acid (Herndon et al., 1969; Steinberg, 1972). These early studies were based on experiments performed with [U-^{14}C]phytanic acid. However, more recent studies carried out with [1-^{14}C]phytanic acid have failed to show the presence of the expected α-hydroxylated intermediate and have therefore raised doubts about the postulated pathway (Skjeldal and Stokke, 1988).

We report here on the production of formic acid from β-methyl fatty acids by human skin fibroblasts in culture. These findings suggest that formic acid is an α-oxidation product of fatty acids.

MATERIALS AND METHODS

Materials

Analytical grade solvents were purchased from May and Baker Pty. Ltd., Melbourne, Victoria, Australia, or from Ajax Chemicals, Sydney, Australia. Basal modified Eagle's medium (BME) was purchased from Flow Laboratories, Irvine, Scotland, U.K., and fetal calf serum was obtained from Gibco New Zealand Ltd. Formate dehydrogenase (from *Candida boidii*) was purchased from Boehringer Mannheim, Mannheim, Germany. Sodium [^{14}C]cyanide (47 mCi/mmol), [1-^{14}C]acetic acid (55 mCi/mmol), [^{14}C]formic acid (52.5 mCi/mmol), and NCS tissue solubilizer were purchased from Amersham Australia Pty. Ltd., Sydney, Australia. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Methods

[1-^{14}C]Phytanic acid (47 mCi/mmol) and [1-^{14}C]pristanic acid (47 mCi/mmol) were synthesized as described by Poulos et al. (1980) and by Johnson and Poulos (1989) respectively. [1-^{14}C]3-Methylhexadecanoic acid (47 mCi/mmol) was synthesized from 2-methylpentadecanoic acid via a reaction sequence involving a Favorsky rearrangement (Johnson and Poulos, 1989). The 2-methyl acid was then converted to [1-^{14}C]3-methylhexadecanoic acid by a chain elongation procedure involving methylation, reduction, mesylation, conversion to a [^{14}C]nitrile and alkaline hydrolysis. The structures of all radiolabelled fatty acids used in these studies was confirmed by g.c./m.s. (Poulos et al., 1988). The radiochemical purities of [1-^{14}C]phytanic acid, β-methylhexadecanoic acid and pristanic acid were determined by reverse-phase t.l.c. essentially as described by Street et al. (1989), except that the non-esterified fatty acids rather than their corresponding methyl esters were chromatographed. Briefly, the fatty acids (200000–300000 d.p.m.) were applied to reverse phase KC-18 thin layer plates (Whatman Inc., Clifton, NJ, U.S.A.) and chromatograms were developed twice in the same direction in acetonitrile/tetrahydrofuran/acetic acid (90:10:1, by vol.). Under these conditions fatty acids were separated according to carbon chain length and degree of unsaturation. Autoradiographs were prepared by exposing the plates to Hyperfilm-^{3}H (Amersham) for 5 days. After development of autoradiographs, the chromatograms were divided into 1–2 cm zones and each

Abbreviation used: BME, basal modified Eagle's medium.

‡ To whom correspondence should be addressed.

zone was scraped from the plate and into scintillation vials containing 1 ml of water. The radioactivity content was determined after adding 10 ml of Optiphase Hisafe 3 (LKB, Loughborough, Leics., U.K.) and scintillation counting. The radiochemical purities of [1-^{14}C]phytanic acid, β-methylhexadecanoic acid and pristanic acid were > 99.3%, 99.9% and 98.0% respectively.

Skin fibroblast cultures were established in the Department of Chemical Pathology, Adelaide Children's Hospital, from skin biopsies of patients with peroxisomal disorders, and from individuals with no prior evidence of abnormality. The diagnoses of Zellweger's syndrome, infantile Refsum's disease and classical Refsum's disease were based on case histories and on clinical and biochemical investigations (Poulos et al., 1986a). Human skin fibroblasts were routinely grown under sterile conditions in 25 cm^2 culture flasks in BME containing 10% fetal calf serum until confluent. The medium was then removed and replaced with 3 ml of BME containing 0.5% fetal calf serum. Endogenous phytanic acid, as determined by gas chromatographic analysis (Poulos et al., 1988), could not be detected either in the BME or in the fetal calf serum used for cell culture. After 24 h the medium was replaced with BME containing 0.5–1.0% fetal calf serum and 0.05–0.06 μCi of [1-^{14}C]phytanic acid. $^{14}CO_2$ production was measured after 4 days as reported earlier (Poulos, 1981; Poulos et al., 1986b). After collection of $^{14}CO_2$, the acidified culture medium was extracted by the Bligh and Dyer (1959) method. The radioactivity in the upper aqueous phase, which contained the water-soluble metabolites, was determined by liquid scintillation counting. For studies involving the characterization of the water-soluble product, larger amounts of radiolabelled substrate were added to the skin fibroblast cultures (0.33 μCi of [1-^{14}C]phytanic acid, 0.33 μCi of [1-^{14}C]pristanic acid and 0.30 μCi of [1-^{14}C]3-methylhexadecanoic acid) and incubated at 37 °C for 4 days, except for the cultures containing [1-^{14}C]pristanic acid which were incubated for 1 day (Singh et al., 1990). Fatty acid analysis of the culture medium was carried out as described by Poulos et al. (1988).

Characterization of radiolabelled water-soluble product

Ion-exchange chromatography

The cell culture medium after incubation with radiolabelled substrates was acidified and partitioned according to the Bligh and Dyer (1959) method. The radioactive product present in the upper aqueous phase was applied to a Dowex 50W-X8 (200–400 mesh; hydrogen form) column. The column was eluted with 5 column vol. of 0.1 M HCl. The non-retained material was neutralized and applied to a column of Bio-Rad AG1 X8 (100–200 mesh; propionate form) and washed first with 6 column vol. of water and then eluted with 4 vol. of 1.5 M HCl.

H.p.l.c

After incubation with the radiolabelled fatty acids, the culture medium was acidified and evaporated to dryness under vacuum, and the volatile material was collected in an alcohol/solid CO_2 trap. The volatile fractions were made alkaline with the addition of 50 μl of 1 M NaOH and lyophilized. The residue was dissolved in 250 μl of 0.1 M HCl, the pH was adjusted to pH < 3, and 100 μl was injected into a Beckman single pump h.p.l.c. apparatus fitted with a Bio-Rad Aminex ion-exclusion HPX-87H column (300 mm × 7.8 mm). The column was eluted with 6 mM H_2SO_4 at a flow rate of 0.5 ml/min and the eluate was monitored at 210 nm using a Beckman 163 variable wavelength detector. The various absorbing fractions, as well as the fractions in between, were collected manually and their radioactivity content was determined by liquid scintillation counting. For some experiments reverse-phase h.p.l.c. was also carried out using an SGE ODS C18 column (250 mm × 4 mm), both on its own and in tandem with the ion-exclusion column (Buchanan and Thoene, 1982), using 6 mM H_2SO_4 as the elution solvent.

Recovery of formic acid was assessed by adding [^{14}C]formic acid (83500 d.p.m.; 52.5 mCi/mmol) and unlabelled formic acid (40 nmol) to 2 ml of culture medium (1% fetal calf serum in BME), trapping the volatile fractions and counting the radioactivity. Under the conditions described, > 90% of the added label was recovered in the volatile fraction and < 5% of the remaining label was lost after lyophilization. More than 85% of the [^{14}C]formic acid applied to the ion-exclusion column was recovered in a peak eluting with unlabelled formic acid. The overall recovery was 65–70%.

Enzymic analysis

Enzymic analysis of the water-soluble products was carried out by a modification of the method outlined by Morand et al. (1988). The incubation mixtures contained an aliquot of the volatile fraction (containing 16000–19000 d.p.m. of volatile product) or [^{14}C]formic acid (78800 d.p.m.; 52.5 mCi/mmol), KCl (110 mM), NaCl (550 mM), unlabelled formic acid (400 μM), NAD^+ (7.5 mM) and formate dehydrogenase (0.5 mg; 0.47 unit/mg) in 2.0 ml of 100 mM sodium phosphate buffer, pH 6.8. Incubations were carried out for 2 h at 37 °C in 25 cm^2 culture flasks sealed with a rubber stopper (Suba seal). At the end of this period 1 ml of 1 M H_2SO_4 was added and $^{14}CO_2$ released was collected by passing nitrogen through the culture flask via a syringe needle and trapping in 2 ml of NCS. $^{14}CO_2$ release was also measured in a control incubation from which formate dehydrogenase was omitted. Under these conditions $^{14}CO_2$ was released from [^{14}C]formic acid by formate dehydrogenase, but not from [1-^{14}C]acetic acid; 77.5% of the label from the volatile product (mean of duplicate incubations) was recovered as $^{14}CO_2$.

Table 1 Oxidation of [1-^{14}C]phytanic acid by skin fibroblasts in culture

The production of radiolabelled CO_2 and water-soluble products from [1-^{14}C]phytanic acid (0.05–0.06 μCi) by skin fibroblasts in culture was measured as outlined in the Materials and methods section. The data represent the means of duplicate assays. The protein content of the cell pellet from individual culture flasks varied from 179 to 451 μg. Endogenous non-labelled phytanic acid (i.e. esterified and non-esterified) could not be detected in the culture medium added to the cells.

	Radiolabelled products formed (pmol/h per mg of protein)	
Cell line	CO_2	Water-soluble metabolites
Controls		
1	0.30	3.1
2	0.43	2.8
3	0.46	2.7
4	0.67	7.7
Refsum's disease	0.02	0.02
Infantile Refsum's disease	0.02	0.04
Zellweger's syndrome		
1	0.01	0.05
2	0.01	0.05

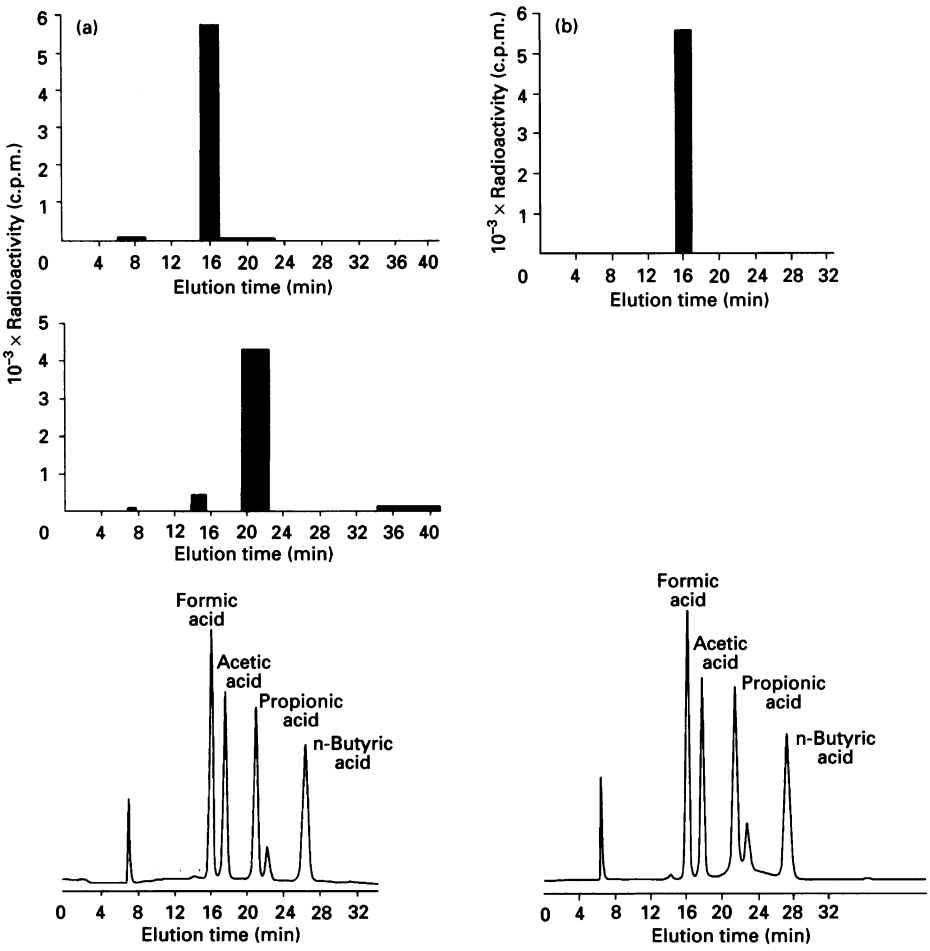

Figure 1 Volatile ^{14}C-labelled metabolites produced from [1-^{14}C]fatty acids by skin fibroblasts

(**a**) The volatile fatty acids produced from [1-^{14}C]fatty acids were subjected to h.p.l.c. as described in the Materials and methods section. The top two panels show the ion-exclusion h.p.l.c. radioactivity profile obtained with the volatile fractions produced from [1-^{14}C]phytanic acid (0.33 μCi) (top panel) and [1-^{14}C]pristanic acid (0.33 μCi) (middle panel). Chromatography was carried out on a Bio-Rad Aminex ion-exclusion HPX-87H column as outlined in the Materials and methods section. The bottom panel shows the profile obtained by chromatography of a mixture of unlabelled short-chain acids under the same conditions, with monitoring of the eluate at 210 nm. (**b**) The top panel shows the radioactivity profile obtained with the volatile products obtained from [1-^{14}C]3-methylhexadecanoic acid (0.30 μCi), while the bottom panel shows the profile obtained from a standard mixture of unlabelled short-chain acids. The chromatographic conditions were the same as those used in (**a**).

Chemical analysis

Chemical oxidation of the radiolabelled water-soluble product present in non-acidified medium was carried out as described by Hefetz and Blum (1978), except for the substitution of mercuric acetate for mercuric chloride. For these experiments 0.5 ml of culture medium (obtained after incubation of control skin fibroblasts with [1-^{14}C]phytanic acid) was treated with mercuric acetate and released $^{14}CO_2$ was trapped in NCS (as above). $^{14}CO_2$ was formed from [^{14}C]formic acid, but not from [1-^{14}C]phytanic acid, under these conditions.

Mass spectrometry

Mass spectrometric analysis of the volatile fraction isolated from the culture medium of cells incubated in the presence of [1-^{14}C]phytanic acid was performed on a JEOL DX-303 mass spectrometer operating in electron impact mode with the gas chromatograph inlet blanked off. The source pressure was 1.2×10^{-4} Pa (9×10^{7} Torr). Aqueous samples (5 μl; equivalent to the volatile products from a single culture flask) were injected into the heated inlet system with a reservoir temperature of 70 °C. The mass spectra of the various solutions were obtained at an electron multiplier setting of 1 kV, and the intensities of the common ions were measured relative to m/z 40 (argon). A 0.9% aqueous solution of formic acid in water afforded ions at m/z 48 [$(M+2)^+$, 0.000], 47 [$(M+1)^+$, 0.050], 46 [M^+, 1.83] and 45 [$(M-1)^+$, 1.50]. [^{14}C]Formic acid gave a series of ions that were 2 mass units higher than that observed for the unlabelled acid.

RESULTS AND DISCUSSION

Human skin fibroblasts in culture converted [1-^{14}C]phytanic acid to $^{14}CO_2$ (Table 1). In addition, up to 20% of the radioactivity from added [1-^{14}C]phytanic acid was converted into radiolabelled water-soluble products and released into the culture medium (Table 1). Most of the water-soluble products were volatile under acidic conditions, as around 75% of the radioactivity was lost under a nitrogen stream at 40 °C. However, little loss occurred if the evaporation was carried out under alkaline conditions. The

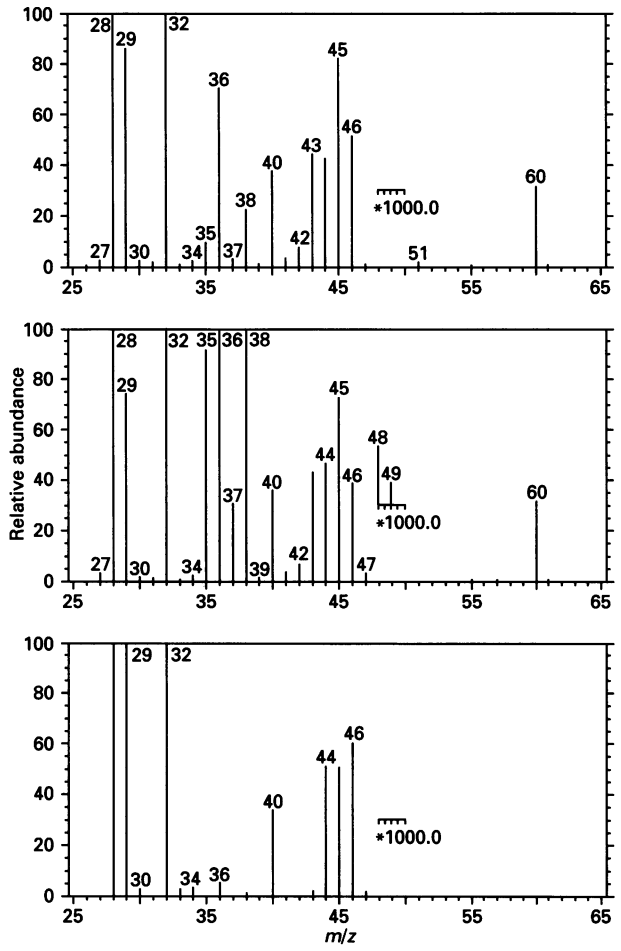

Figure 2 Mass spectrometric identification of [^{14}C]formic acid in the volatile fraction isolated from the cell culture medium

Normal cells were incubated with culture medium containing unlabelled phytanic acid (7 nmol) and [1-^{14}C]phytanic acid (0.33 μCi) and the volatile products were isolated (see the Materials and methods section). Mass spectra of volatile fractions produced from unlabelled phytanic acid (upper panel), [1-^{14}C]phytanic acid (middle panel), and an unlabelled formic acid standard (lower panel) are shown. Identical source conditions and electron multiplier settings were used.

amount of water-soluble products formed from [1-^{14}C]phytanic acid by fibroblasts of patients with disorders of peroxisomal assembly (Zellweger's syndrome and infantile Refsum's disease), and of patients with Refsum's disease, was markedly depressed compared with control cells (Table 1). These cells showed the expected reductions in $^{14}CO_2$ production, an indicator of abnormal phytanic acid oxidase activity.

These observations suggested that the volatile water-soluble product was a short-chain acid and was not formed in peroxisome-deficient cells. Further supporting evidence for this hypothesis was provided by the demonstration that most (70–80%) of the water-soluble product was retained on AG1 X8 (propionate form) anion-exchange columns and was eluted with 1.5 M HCl. Because of the difficulties inherent in the characterization of the volatile water-soluble product in the presence of the relatively large concentrations of hydrophilic substances, the culture medium was evaporated to dryness under vacuum and the volatile products were recovered in an alcohol/solid CO_2 trap. Of the total radioactivity added to the cells, 21.6% (mean of four incubations) was recovered in the volatile fraction isolated from the acidified culture medium. This figure correlated closely with the amount of volatile material (determined by counting aliquots of the acidified culture after air drying). Between 85 and 90% of the volatile radioactivity eluted from ion-exclusion (Figure 1) and reverse-phase h.p.l.c. (results not shown) columns as a single peak which co-eluted with formic acid.

Small amounts of unlabelled formic acid and acetic acid, together with a number of other unidentified substances, were detected by ion-exclusion h.p.l.c. of the volatile fractions isolated from the culture medium (results not shown).

The mass spectra, obtained under identical conditions, of the volatile fractions recovered from human skin fibroblasts which had been cultured in the presence of unlabelled phytanic acid and [1-^{14}C]phytanic acid respectively, were compared (Figure 2). In both cases, formic acid (M^+; m/z 46) and acetic acid (M^+; m/z 60) were the major identifiable components (present in the culture medium). The mass spectrum derived from the [1-^{14}C]phytanic acid culture also showed a small but significant ion at m/z 48 which had been observed in the mass spectrum of [1-^{14}C]formic acid. Formate dehydrogenase treatment of the radioactive water-soluble product released $^{14}CO_2$. Also, treatment of the radiolabelled water-soluble product with mercuric acetate resulted in the formation of $^{14}CO_2$. These observations provide evidence that an α-oxidation product of phytanic acid is formic acid.

To determine whether the [^{14}C]formic acid produced was specific for phytanic acid, we compared the volatile radioactive products formed from [1-^{14}C]phytanic acid and two other branched-chain fatty acids, [1-^{14}C]pristanic acid and [1-^{14}C]3-methylhexadecanoic acid. The data shown in Figure 1 demonstrate that there are differences in the short-chain fatty acids produced from the three substrates. The major volatile radiolabelled product formed from the fatty acids containing a β-methyl group, i.e. [1-^{14}C]phytanic and [^{14}C]3-methylhexadecanoic acids, was [^{14}C]formic acid. In contrast, [^{14}C]propionic acid was the major volatile product formed from [1-^{14}C]pristanic acid (a fatty acid that can undergo β-oxidation). As [^{14}C]formic acid was also detected in cultures incubated with [1-^{14}C]3-methylhexadecanoic acid, this suggests that any fatty acid which cannot be degraded by β-oxidation is degraded by α-oxidation, and one oxidation product formed by this pathway is formic acid.

To our knowledge, this is the first report showing that formic acid can be produced from fatty acids by human skin fibroblasts. Activated single-carbon units, normally linked to tetrahydrofolic acid, are formed during the catabolism of purines and amino acids (Rabinowitz and Pricer, 1956; Hefetz and Blum, 1978), and enzymes which can cleave these compounds to release free formic acid have been described in bacterial, insect and mammalian tissues (Whiteley, 1960; Rader and Huenneken, 1973; Hefetz and Blum, 1978), although the synthesis of free formic acid in high concentrations is more a feature of certain specialized tissues such as the poison glands of ants (Hefetz and Blum, 1978). Formic acid can also be produced by irradiation of 12-(1-pyrene)dodecanoic acid-photosensitized mammalian cells (Morand et al., 1988). Our data indicate that formic acid production is linked to the α-oxidation of β-methyl fatty acids. Whether it is formed directly, or indirectly from CO_2, is not known. As formic acid production is several-fold greater than CO_2 production, we speculate that CO_2 is formed from formic acid. This hypothesis is supported by preliminary experiments indicating that fibroblasts in culture, or in suspension, can generate $^{14}CO_2$ from [^{14}C]formic acid. Early studies on the α-oxidation of phytanic acid indicated that the first step involved is an α-hydroxylation of phytanic acid, followed by a subsequent

decarboxylation of the hydroxylated product to give pristanic acid (Herndon et al., 1969). CO_2 is believed to be the sole non-lipid product of α-oxidation. Whether a single enzyme is responsible for the hydroxylation and decarboxylation, or whether more than one enzyme is involved in the process, is not clear. The production of formic acid from phytanic acid raises some doubts as to the proposed sequence of reactions (Herndon et al., 1969).

The oxidation of fatty acids is a major peroxisomal function. There is now good evidence that the acetate released by peroxisomal β-oxidation is utilized in biosynthetic reactions (Street et al., 1990). Our data showing the generating of formic acid via the α-oxidation of fatty acids believed to be oxidized predominantly in peroxisomes suggest that peroxisomes are involved in the production of C_1 units which can be utilized for biosynthetic processes within the cell.

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