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De novo arachidonic acid synthesis in *Perkinsus marinus*, a protozoan parasite of the eastern oyster *Crassostrea virginica*

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Abstract

The capability of synthesizing fatty acids de novo in the meront stage of the oyster protozoan parasite, *Perkinsus marinus*, was investigated employing stable-isotope-labeled precursors (1,2 ^{13}C-acetate and palmitic-d_{31} acid). Fatty acid methyl esters derived from 1,2 ^{13}C-acetate and palmitic-d_{31} acid were analyzed using gas chromatography/mass spectrometry and gas chromatography/flame ionization detection. Results revealed that in vitro cultured *P. marinus* meronts utilized ^{13}C-acetate to synthesize a range of saturated and unsaturated fatty acids. The saturated fatty acids 14:0, 16:0, 18:0, 20:0, 22:0, 24:0 and the unsaturated fatty acids, 18:1(*n*-9), 18:2(*n*-6), 20:1(*n*-9), 20:2(*n*-6), 20:2(*n*-9), 20:3(*n*-6), 20:4(*n*-6) were found to contain ^{13}C, after 7, 14, and 21 days incubation with the precursor. This indicates that meronts can synthesize fatty acid de novo using acetate as a substrate. Meronts efficiently elongated 16:0-d_{31} to 18:0, 20:0, 22:0, 24:0, but desaturation activity was limited, after 7 and 14 days cultivation. Only a small quantity of 18:1-d_{29} was detected. This suggests that meronts cannot directly convert exogenous palmitic acid or its products of elongation to unsaturated counterparts. The ability to synthesize 20:4(*n*-6) from acetate is particularly interesting. No parasitic protozoan has been reported to be capable of synthesizing long chain essential fatty acids, such as 20:4(*n*-6) de novo. Future study will be directed to determine whether the observed in vitro activities indeed reflect the in vivo activities, when meronts are associated with the host.

Keywords: Fatty acid synthesis; Parasitic protozoan; *Perkinsus marinus*; Eastern oyster; *Crassostrea virginica*; Lipids

1. Introduction

Lipids play a vital role for long term survival and life cycle completion of endogenous parasites [1,2]. Generally, parasitic protozoans require an exogenous source of essential lipids for replication, differentiation, and life cycle completion. Thus, they scavenge lipids from the host environment not only for energy supply, but also for membrane synthesis during proliferation, growth and development to next life history stage. Interconversion of incorporated phospholipids, cholesterol, fatty acids and lysophospholipids from culture media and/or host to constitutive lipids has been reported in several protozoan parasites [2–5]. However, only limited capacities for chain elongation and desaturation of fatty acids have been reported in the *Giardia lamblia*, *Leishmania* spp, *Cryptosporidium parvum*, and *Plasmodium* spp [3,6–9]. *Trypanosoma cruzi* (a tsetse fly-transmitted pathogenic protozoan that lives in the blood and body fluids of the mammalian host), *Leishmania donovani* (a parasitic protozoa that infects the viscera of vertebrates including human), and *Plasmodium falciparum* (a pathogen from the Phylum Apicomplexa that causes malaria disease), are the three parasitic protozoans reported to be able to use acetate as a substrate for fatty acid synthesis [10–12].

Abbreviations: AA, arachidonic acid ((20:4(*n*-6)); BSA, bovine serum albumin; CI, positive chemical ionization; DHA, docosahexaenoic acid ((22:6(*n*-3)); EPA, eicosapentaenoic acid ((20:5(*n*-3)); FAME, fatty acid methyl ester; FTM, fluid thioglycollate medium; PUFAs, polyunsaturated fatty acids; GC/FID, gas chromatography/flame ionization detection; GC/MS, gas chromatography/mass spectrometry; PG, prostaglandin.

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The protozoan, *Perkinsus marinus*, is presently the most prevalent parasite of the eastern oyster *Crassostrea virginica* in mid-Atlantic waters. It has caused severe oyster mortality on the East Coast of the United States since the 1950s. The disease caused by *P. marinus* is infectious and can be transmitted from infected to uninfected oysters [13]. Four life stages, meront, prezoosporangium (hypnospores), zoosporangium, and biflagellated zoospore have been identified and described [14,15]. Three life stages, meront, prezoosporangium, and biflagellated zoospore are infective [13]. The uninucleate meront is also termed 'trophozoite' [16]. Meronts are often found in the phagosomes of hemocytes, and in tissues, intercellular and intracellular, of infected oysters [15] and comprise the majority of the parasite load in tissues of heavily infected oysters. Prezoosporangia, developed from meronts, are sometimes observed in moribund and dead oyster tissues. When tissue-associated meronts are placed in fluid thioglycollate medium (FTM) for 4–5 days, they develop into prezoosporangia. Zoosporulation (production of biflagellated zoospores) usually occurs after incubating FTM-cultured prezoosporangia in estuarine or sea water (20–22 ppt) for 5–6 days. Disease caused by *P. marinus* is transmitted through water-borne infective cells released from infected live and deceased oysters and the meronts are primary agents for disease transmission [13,15]. Temperature and salinity are two important factors controlling the rate of proliferation, development and the progression of the disease in the host [13,14,17–20]. *P. marinus* proliferates and develops rapidly between 20 and 30 °C in both in vitro and in vivo. *P. marinus* disease prevalence and intensity are positively correlated with temperature and salinity.

Recent advances in techniques for culturing the meront stage in vitro in defined media [21–23] provide the opportunity to characterize this life stage biochemically and physiologically. Our previous study [24] demonstrated that both meront and prezoosporangium stages of *P. marinus* incorporated and modified fluorescent lipid analogs from the media. Also, in a separate study, we found that lipid metabolism of in vitro cultured meronts extended beyond the modification of exogenous lipids and fatty acids. They are capable of synthesizing fatty acids and lipids [25]. We found that meronts cultivated in two media with different fatty acid composition showed similar fatty acid profiles. The quantities of total lipids and fatty acids in meronts increased as the number of meronts increased and far exceeded the initial amounts in the media and in the initial cell inoculum after 25 days of culture. To further explore the synthetic capability of meront stage of this parasite, the present study employed stable-isotope-labeled precursors (1,2 ^{13}C-acetate and palmitic-d_{31} acid) to test the activity of de novo synthesis and desaturation of fatty acids. Stable-isotope-labeled precursors have been used previously and successfully in studies investigating synthesis and desaturation/elongation of fatty acid and lipogenesis and cholesterol synthesis employing gas chromatographic/mass spectrometric techniques [26–28].

2. Materials and methods

2.1. Axenic *P. marinus* meront cultures

P. marinus meronts were grown in a medium prepared as described by La Peyre et al. [23]. The lipid concentration of this medium was estimated to be 14.5 μg lipid ml^{-1}, based on the amount of lipid added to the medium, which contains 1% (v/v) of lipid concentrate (100X GIBCO) including 450 μg ml^{-1} of cholesterol, 1 mg ml^{-1} of cod liver oil, and 200 μg ml^{-1} α-tocopherol-acetate. The fatty acid composition of this medium is dominated by 16:0, 16:1(*n*-7), 18:1(*n*-9), 18:1(*n*-7), 20:5(*n*-3), and 22:6(*n*-3), but is deficient in (*n*-6) C20–22 fatty acids [25]. This medium contained amino acids, nucleotides, carbohydrates, and vitamins, but no fetal bovine serum.

Meronts were inoculated (about 1×10^6 ml^{-1}) and cultivated in 10 ml aliquots of medium in T-10 tissue culture flasks at 28 °C. Meronts at exponential growth phase (7 or 9 days old) were harvested and used for all stable isotope precursor incubation or growth experiments.

2.2. Preparation of media containing palmitic-d_{31} acid and 1,2 ^{13}C-acetate

Medium [23] containing palmitic-d_{31} acid (Cambridge Isotope Laboratories Inc., Andover, MA) was prepared according to the method of Ghioni et al. [29]. Briefly, 2 mg of palmitic-d_{31} acid was added to a 250 ml Erlenmeyer flask and dissolved in 5 ml of chloroform–methanol (1:1). The solvent was then evaporated completely under a stream of nitrogen to allow palmitic-d_{31} acid to form a thin film on the bottom of the flask. A 100 μl aliquot of 0.1 M KOH was added to the flask and stirred vigorously for 10 min at 30 °C. Forty milliliters of medium containing 2 g (50 mg ml^{-1}) of fatty acid free-bovine serum albumin (FFA-free BSA) was then added to the flask containing palmitic-d_{31} acid and KOH solution and stirred vigorously at 30 °C for 2 h to facilitate binding of palmitic acid to the BSA. The medium was then filter sterilized (0.22 μm), diluted by the addition of 40 ml media without BSA or palmitic-d_{31} acid, and stored at 4 °C until use. The incorporation of palmitic-d_{31} acid into the medium was determined by lipid extraction of 5 ml of media and subsequent GC analysis of fatty acid methyl esters (FAMEs) derivatized from the extracted

lipids. The incorporation efficiency of palmitic-d_{31} acid into the media was in excess of 95%. The concentration of palmitic-d_{31} acid in the media was determined to be 875 μM (25 μg ml^{-1}). Media containing ^{13}C-acetate was prepared by adding Na-1,2 ^{13}C-acetate (Cambridge Isotope Laboratories Inc., Andover, MA) into the medium to a desired concentration of 6 mM (0.5 mg ml^{-1}).

2.3. Lipid and fatty acid analyses

Total lipids were extracted from meronts according to the procedure described by Bligh and Dyer [30]. Fatty acid composition and contents of meronts were analyzed using GC/FID. Total lipids were transesterified in 8 ml Wheaton vials containing 20 μg internal standard (23:0), with 10% BF_3 (w/w) in methanol for 15 min at 95–100 °C [31]. After cooling, the FAMEs were extracted with carbon disulfide [32]. The organic phase was evaporated, and redissolved in hexane. Separation of FAMEs was carried out on a GC (Varian 3300; Varian Analytical Instruments, Sunnydale, CA) equipped with a flame ionization detector, using a DB-WAX capillary column (25 m × 0.32 mm; 0.2 μm film thickness; J&W Scientific, Folsom, CA). The column was temperature programmed from 60 to 150 °C at 30 °C min^{-1} and 150 to 220 °C at 2 °C min^{-1}, injector and detector temperatures were 230 and 250 °C, respectively; the flow rates of air and hydrogen were 300 and 30 ml min^{-1}. Helium was used as the carrier gas (1.5 ml min^{-1}). Identification of FAMEs was based on the comparison of their retention times with those of authentic standards and confirmed by gas chromatograph-mass spectrometry (GC/MS). The quantity of each component was calculated based on the internal standard.

To detect incorporation of stable isotope precursors, FAMEs were further analyzed qualitatively and quantitatively by mass spectroscopy with a Varian 3400 gas chromatograph equipped with a Varian Saturn 4D GC/MS/MS detector. Methane was used as the reagent gas for positive chemical ionization (CI). The same column used for GC/FID analysis of the FAME samples (J&W DB-WAX, 25 m × 0.32 mm; 0.2 μm film thickness) was used for GC/MS analysis. Carrier gas (helium) flow rate was 1 ml min^{-1}. Injection port temperature was 230 °C and the interface was 250 °C. The column was temperature programmed from an initial temperature of 60 °C for a 4 min hold, followed by a 30 °C min^{-1} increase to 150 °C and 2 °C min^{-1} to 220 °C. Data were collected and processed using Varian Saturn GC/MS software version 5.2. FAMEs were identified by retention time relative to known standards, fragmentation pattern and mass of the molecular ion. FAMEs containing ^{13}C derived from acetate were quantified using standard curves constructed for each FAME standard and ratio with internal standard (23:0). Briefly, the proportion of each FAME that contained one or more molecules of ^{13}C was determined using standard curves for each FAME. The standard curves were created using four concentrations of each FAME comparable to the concentration of the sample, along with the C23:0 internal standard. The molecular ions in spectra of each FAME were used to quantify masses containing exogenous ^{13}C relative to the native molecule. FAMEs derived from palmitic-d_{31} acid were identified by GC/CI/MS and subsequently quantified by GC/FID using the same column and a C23:0 internal standard. Data (FAMEs containing ^{13}C derived from acetate and FAMEs derived from palmitic-d_{31} acid) are expressed as weight percent of fatty acids containing stable isotope and micrograms of fatty acids containing stable isotope.

2.4. Experiments

A series of experiments were conducted to examine the capability of in vitro cultured *P. marinus* meronts to incorporate and use 1,2 ^{13}C-acetate or palmitic-d_{31} acid as substrates to synthesize fatty acids.

2.4.1. Incorporation of 1,2 ^{13}C-acetate into fatty acids of *P. marinus* meronts after 7, 14, and 21 days incubation

This experiment tested the qualitative and kinetic incorporation of ^{13}C-acetate into *P. marinus* meronts at different growth phases (exponential to stationary). Nine-day-old meronts were inoculated at a concentration of about 10^6 cells ml^{-1} into three T-10 tissue culture flasks containing Na-^{13}C-acetate in 10 ml of media (0.5 mg ml^{-1}) and cultivated at 28 °C for 21 days. Seven days post-inoculation, cell counts were performed on each flask and cells were pelleted by centrifugation. The cell pellet from one flask was washed with 0.22 μm-filtered/sterilized York River water adjusted to 28 ppt, a salinity similar to the medium, and freeze-dried for subsequent lipid analysis. The cell pellets from the remaining two flasks were combined, resuspended in 30 ml of fresh media containing stable isotope precursor (Na-^{13}C-acetate) and divided equally among three new flasks. The latter procedure was repeated at 7 and 14 days post-inoculation.

2.4.2. Incorporation of 1,2 ^{13}C-acetate into fatty acids of *P. marinus* meronts after 1, 2, 3, and 7 days incubation

This experiment tested the qualitative and quantitative incorporation of ^{13}C-acetate into *P. marinus* meronts. Seven-day-old meront cells were used for

culture inoculation. Experimental condition was similar to the first experiment described above and the culture medium containing the same concentration of ^{13}C-acetate (0.5 mg ml^{-1}). However, since results from the first experiment indicated that there was no significant change in qualitative incorporation of ^{13}C-acetate in *P. marinus* meronts between, 7, 14, and 21 days, cultures were maintained for only 7 days. Meronts were sampled 1, 2, 3 and 7 days post-inoculation ($n = 3$ for each sampling date) and analyzed for qualitative and quantitative ^{13}C-acetate incorporation.

2.4.3. Incorporation of palmitic-d_{31} acid into fatty acids of *P. marinus* meronts after 7, 14, and 22 days incubation

This experiment examined the incorporation of palmitic-d_{31} acid into *P. marinus* meronts. Experimental condition and sampling protocol were similar to the first experiment described above. But this experiment differed from the first experiment in that the media contained palmitic-d_{31} acid (875 μM; 25 μg ml^{-1}), but not ^{13}C-acetate.

2.5. Statistical analysis

Results from the incubations of *P. marinus* meronts with ^{13}C-acetate for up to 7 days were subjected to one-way analysis of variance (ANOVA). When appropriate, differences between sampling dates were determined by Tukey's test. Values were considered to be significantly different at the $P < 0.05$ level.

3. Results

3.1. Fatty acid composition and GC profiles

The major fatty acids in cultivated meronts are 16:0, 18:0, 22:0, 24:0, 18:1(*n*-9), 20:1(*n*-9), 18:2(*n*-6), 20:2(*n*-6), and 20:4(*n*-6) (Fig. 1) with a weight percentage of 13.3 ± 0.3, 3.9 ± 0.9, 2.5 ± 0.2, 4.0 ± 0.1, 27.0 ± 0.4, 4.1 ± 0.6, 3.1 ± 0.1, 2.6 ± 0.1, and 24.8 ± 1.6, respectively ($n = 3$). The long chain PUFAs, 20:5(*n*-3) and 22:6(*n*-3) account for only 2.0 ± 0.0 and 0.4 ± 0.0% of the total fatty acids, respectively. The most prevalent polyunsaturated fatty acid (PUFA) is 20:4(*n*-6). This fatty acid accounts for 24.5% of the total fatty acids and 85% of the total PUFAs.

3.2. Qualitative incorporation of 1,2 ^{13}C-acetate in meront fatty acids after 7, 14, and 21 days incubation

Each FAME containing ^{13}C derived from acetate eluted as part of a larger peak comprised of a single FAME with a range of molecular weights (Figs. 2 and 3). Fatty acids containing levels of ^{13}C label above the calculated natural abundance per molecule were identified, indicating that ^{13}C-acetate incorporated into a range of saturated and unsaturated fatty acids. The saturated fatty acids 14:0, 16:0, 18:0, 20:0, 22:0, 24:0 and the unsaturated fatty acids, 16:1(*n*-7), 16:1(*n*-9), 18:1(*n*-9), 18:2(*n*-6), 20:1(*n*-9), 20:2(*n*-6), 20:2(*n*-9), 20:3 (*n*-6), 20:4(*n*-6) were found to contain ^{13}C, after 7, 14, and 21 days incubation (Table 1). Most of the above

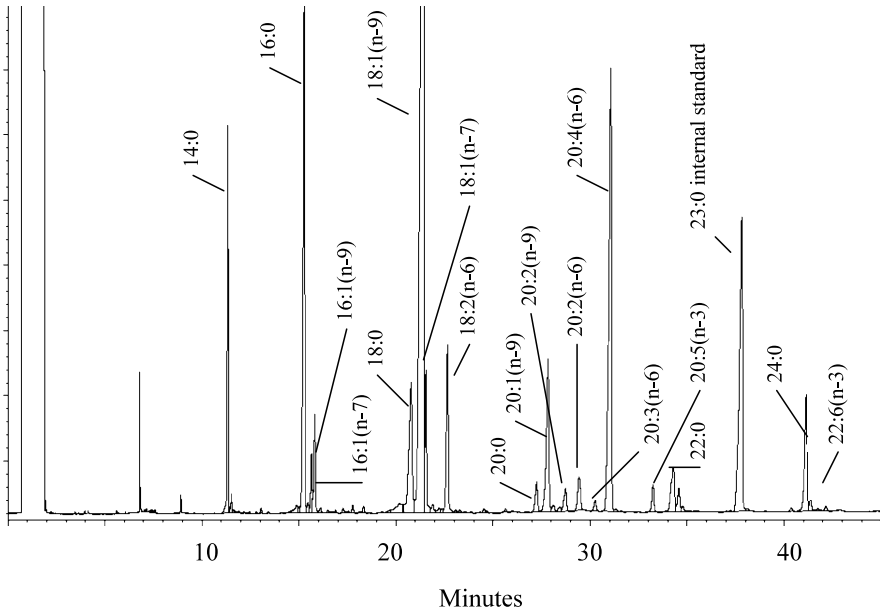

Fig. 1. Portion of a gas chromatography/flame ionization detection (GC/FID) chromatogram of FAMEs derived from *P. marinus* cells grown in media containing 6 mM sodium acetate 1,2 $^{13}C_2$ for 7 days. The same column (J&W DB-WAX, 25 m × 0.32 mm; 0.2 μm film thickness, polyethylene glycol stationary phase, J&W Scientific, Folsom, CA) and temperature program were used for gas chromatography/mass spectroscopy analysis. Peaks were identified by comparison to known standards and confirmed by GC/MS.

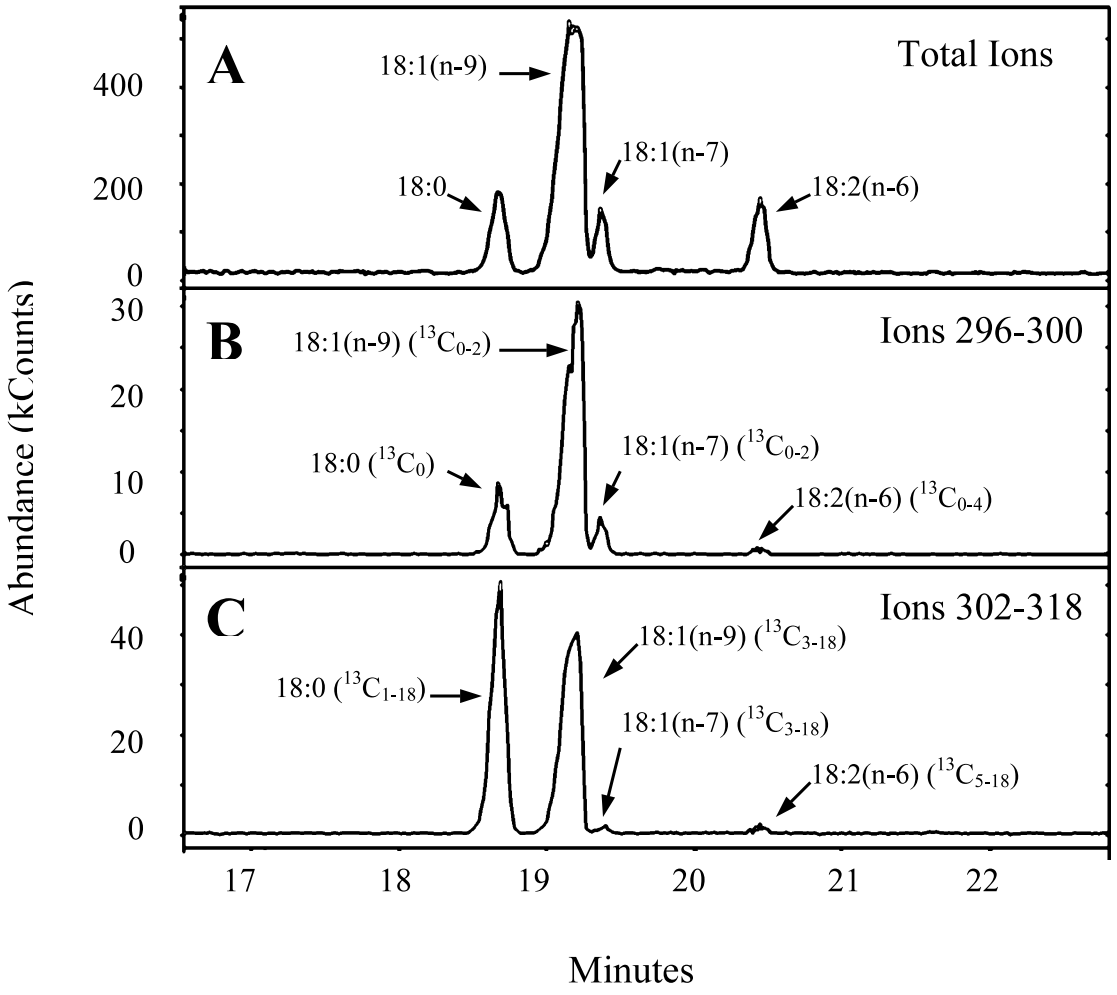

Fig. 2. Portion of a gas chromatography/mass spectroscopy chromatogram of FAME derived from *P. marinus* cells grown in media containing 6 mM sodium acetate 1,2 $^{13}C_2$ for 7 days. Chromatograms show total ion (A) and selective ion monitoring at molecular weights 296–300 corresponding to 18:0 ($^{13}C_0$), 18:1(*n*-9) ($^{13}C_{0-2}$), 18:1(*n*-7) ($^{13}C_{0-2}$) and 18:2(*n*-6) ($^{13}C_{0-4}$) (B) and molecular weights 302–318 corresponding to 18:0 ($^{13}C_{1-18}$), 18:1(*n*-9) ($^{13}C_{3-18}$), 18:1(*n*-7) ($^{13}C_{3-18}$) and 18:2(*n*-6) ($^{13}C_{5-18}$) (C). The molecular weights of native methyl esters of 18:0, 18:1(*n*-9), 18:1(*n*-7) and 18:2(*n*-6) are 300, 298, 298 and 296, respectively.

fatty acids including 20:4(*n*-6) contained up to 9 or more atoms of ^{13}C. The percent of fatty acids containing ^{13}C derived from acetate ranged from 77.7 to 95.9% in saturated fatty acids and from 53 to 86.8% in unsaturated fatty acids and varied slightly between sample dates. The percentage of 16:1(*n*-9), 18:1(*n*-9), 18:2(*n*-6) and 20:4(*n*-6) that contained ^{13}C increased slightly from 7 to 21 days post-inoculation. Meront cells number in culture increased from day 1 to day 21, from 1.0×10^6 to about 12×10^6 cells ml^{-1}.

3.3. Cultivation of meronts supplemented with 1,2 ^{13}C-acetate for 1, 2, 3 and 7 days

Generally, synthesis of fatty acids using ^{13}C-acetate increased with time and cell number (Table 2). All fatty acids with quantifiable amounts of ^{13}C incorporation contained significantly more ^{13}C at day 7 than at 24 h. The amount (μg) of total ^{13}C-labeled fatty acids increased significantly from 24 h to 7 days (Table 2). Meronts rapidly incorporated ^{13}C-acetate and used it as a substrate to make saturated fatty acids, 14:0, 16:0, 18:0, 20:0, 22:0, 24:0 and the unsaturated fatty acid, 20:4(*n*-6) after 24 h incubation. Trace amounts of ^{13}C-labeled 16:1(*n*-9), 16:1(*n*-7), 18:1(*n*-9), 18:1(*n*-7), 18:2(*n*-6), 20:1(*n*-9), 20:2(*n*-6) and 20:3(*n*-6) were detected after 24 h. Incorporation of ^{13}C into 18:1(*n*-9) and 20:1(*n*-9) was detected at 48 h and continued to rise significantly up to day 7 (Fig. 4). Arachidonic acid, 20:4(*n*-6), the first detected long chain PUFA, increased significantly from 24 to 72 h and reached a plateau thereafter. The fatty acid, 20:2(*n*-6), contained detectable ^{13}C incorporation only at 2 and 3 days post-inoculation.

3.4. Incubation with palmitic-d_{31} acid (16:0-d_{31}) for 7, 14, and 22 days

In vitro cultured meronts usually proliferate up to

9–11 days and remain healthy and viable for up to 1 month. Unexpectedly, meront cell number decreased with incubation time (1.1×10^6, 1.4×10^6, 0.8×10^6, and 0.4×10^6 cells ml^{-1} in day 1, 7, 14 and 22 post-inoculation) and dead cells were noted after 14 and 22 days post-inoculation. It is not known whether the amounts of BSA and/or palmitic acid present in the incubation media were the cause of the impaired growth and mortality. Low numbers of live meront cells and high concentration of debris from dead cells prevented the analysis of 22-day-old culture. Thus, lipid analysis was conducted only on 7 and 14 day meront cells. GC/MS analysis showed that FAMEs derived from palmitic-d_{31} acid eluted as discrete peaks (Fig. 5). The major metabolites of 16:0-d_{31} in meronts formed by chain elongation. 16:0-d_{31} elongated to 18:0-, 20:0-, 22:0- and 24:0-d_{31} after 7 and 14 days incubation (Table 3). Most of the incorporated 16:0-d_{31} remained as 16:0-d_{31}. The rest was elongated to 18:0, 20:0, 22:0, and 24:0. About 18.7–19.5% of the incorporated 16:0-d_{31} elongated to 18:0. Small amounts of 18:1-d_{29} were detected (1.1 and 0.1 μg at 7 and 14 days post-incubation, respectively). Decarboxylation (β-oxidation) also occurred. Approximately 19% of 14:0 was 14:0-d_{27}. While the amount of fatty acids derived from elongation and β-oxidation of 16:0-d_{31} decreased with meront cell number and time, the percentage of incorporation in these fatty acids did not change (Table 3).

4. Discussion

Unlike the typical marine fatty acid profile which is rich in (*n*-3) polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [33,34], the fatty acid composition of *P. marinus* meronts exhibits a characteristic 'terrestrial'

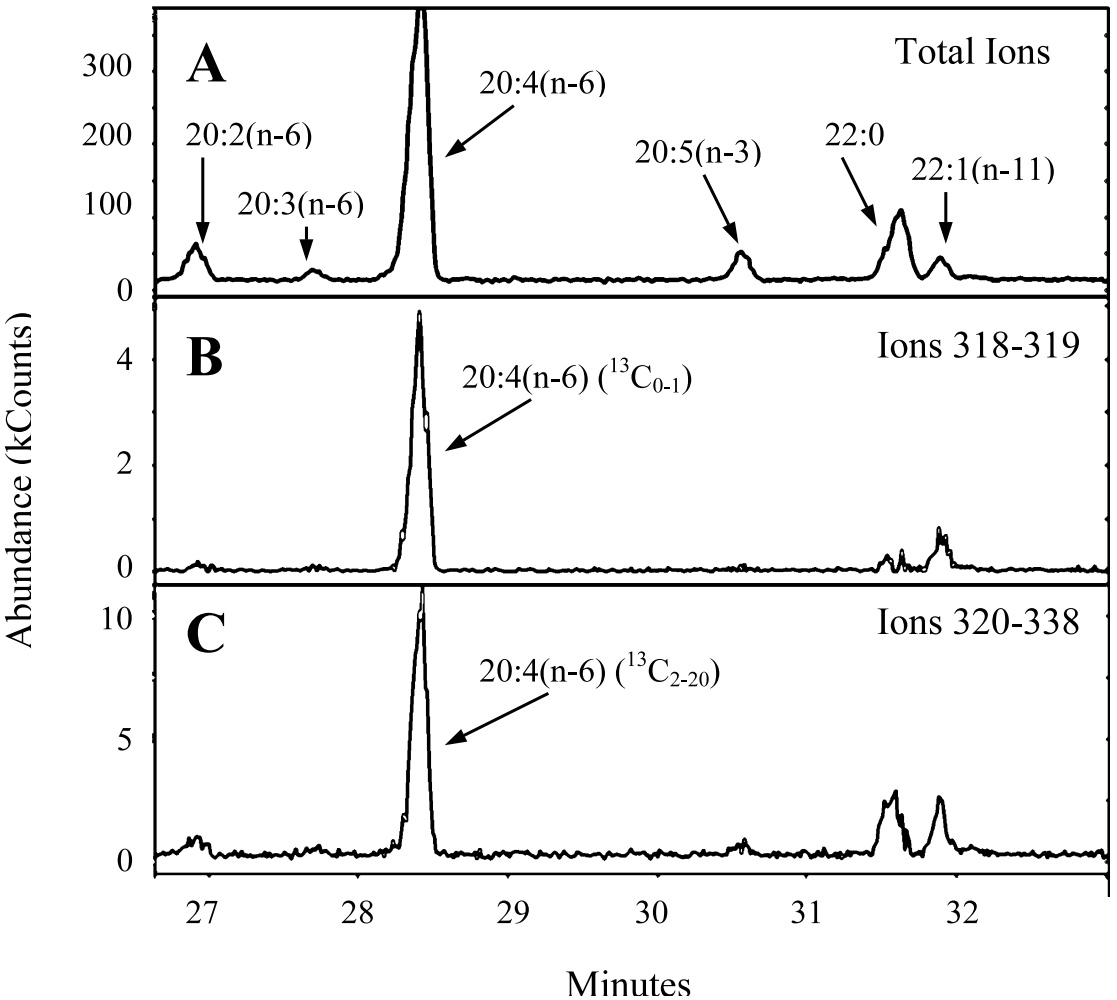

Fig. 3. Portion of a gas chromatography/mass spectroscopy chromatogram of FAME derived from *P. marinus* cells grown in media containing 6 mM sodium acetate 1,2 $^{13}C_2$ for 7 days. Chromatograms show total ion (A) and selective ion monitoring at molecular weights 318–319 corresponding to 20:4(*n*-6) ($^{13}C_{0-1}$) (B) and molecular weights 320–338 corresponding to 20:4(*n*-6) ($^{13}C_{2-20}$) (C). The molecular weight of native 20:4(*n*-6) methyl ester is 318.

Table 1
Percent of fatty acids in *P. marinus* cells containing ^{13}C derived from ^{13}C-acetate

FAME	Day 7	Day 14	Day 21
14:0	90.8	92.8	90.0
16:0	77.7	84.1	78.6
18:0	90.7	92.1	89.0
20:0	95.1	95.9	96.7
22:0	90.7	92.6	89.4
24:0	88.4	95.3	95.7
16:1(*n*-9)	53.0	72.5	73.4
16:1(*n*-7)	64.1	67.2	62.5
18:1(*n*-9)	78.3	84.4	82.0
20:1(*n*-9)	80.2	80.5	78.3
18:2(*n*-6)	52.6	61.8	64.3
20:4(*n*-6)	76.9	83.5	86.8

The percentage of each FAME containing ^{13}C derived from ^{13}C-acetate was determined by GC/MS using a FAME-specific standard curve. Selected ions within the spectra were used to quantify both native fatty acids containing only naturally occurring stable isotopes and fatty acids containing an additional isotope derived from ^{13}C-acetate. The methyl esters of 20:2(*n*-9), 20:2(*n*-6) and 20:3(*n*-6) all contained ^{13}C, but were present in quantities too low (<1.5% total fatty acids) to determine levels of stable isotope incorporation.

profile with the PUFAs are mainly of the *n*-6 series fatty acids (Fig. 1). This profile differed from the culture medium and the host [25]. The host oyster has high weight percentages of (*n*-3) PUFAs, particularly EPA ($\approx$17.5%) and DHA ($\approx$9%), a characteristic of marine organisms [25]. This finding is consistent with our previous result [25]. While the finding of a terrestrial type of fatty acid profile in a marine parasitic protozoan is interesting, at this stage, the reason for its presence in *P. marinus* is not known.

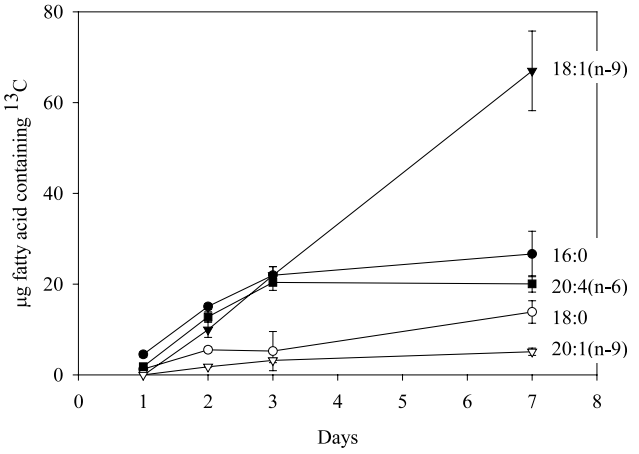

Fig. 4. Incorporation of ^{13}C-acetate into selected fatty acids of *P. marinus* ($n = 3$).

Most of the parasitic protozoans that have been studied showed limited ability for chain elongation and desaturation and cannot synthesize fatty acids from acetate [3,6–9,35–38]. Only three parasitic protozoans, *T. cruzi*, *P. falciparum* and *L. donovani* were able to synthesize fatty acids from acetate [10–12]. *T. cruzi* incorporated ^{14}C-labeled acetate into 16:0, 18:0, 18:1 and 18:2 [10] and *P. falciparum* synthesized 10:0, 12:0, and 14:0 from ^{14}C-labeled acetate [12]. Results of the present study reveal that in vitro cultured *P. marinus* meronts' synthetic capability was far beyond those described for *T. cruzi* and *P. falciparum*. *P. marinus* meronts synthesized a wide range of saturated and unsaturated fatty acids including the essential fatty acid, arachidonic acid (AA), utilizing acetate. These

Table 2
Percentages and micrograms of individual fatty acids containing ^{13}C derived from sodium-^{13}C-acetate in *P. marinus* meronts

FAME	24 h, $1.8 \pm 0.1 \times 10^7$ cells		48 h, $2.4 \pm 0.3 \times 10^7$ cells		72 h, $12.3 \pm 1.3 \times 10^7$ cells		7 days, $20.48 \pm 4.1 \times 10^7$ cells	
	Percent	μg	Percent	μg	Percent	μg	Percent	μg
14:0	42.0 ± 1.2	0.9 ± 0.8^{a}	56.7 ± 0.7	3.9 ± 0.5^{ab}	61.9 ± 0.6	6.5 ± 2.6^{ab}	77.8 ± 1.6	8.0 ± 4.2^{b}
16:0	55.8 ± 1.9	4.5 ± 0.6^{a}	69.7 ± 1.7	15.1 ± 0.7^{b}	72.9 ± 1.5	22.0 ± 1.9^{bc}	76.5 ± 0.8	26.6 ± 5.0^{c}
18:0	48.9 ± 1.0	1.3 ± 0.1^{a}	63.2 ± 2.9	5.5 ± 0.6^{a}	65.8 ± 2.6	5.3 ± 4.3^{a}	74.7 ± 0.4	13.9 ± 2.5^{b}
20:0	49.7 ± 1.0	0.2 ± 0.1^{a}	58.2 ± 0.3	1.0 ± 0.1^{b}	64.0 ± 2.3	1.1 ± 0.1^{b}	70.6 ± 1.9	2.3 ± 0.4^{c}
22:0	32.1 ± 27.8	0.2 ± 0.2^{a}	62.7 ± 0.6	0.4 ± 0.1^{a}	67.8 ± 1.5	2.0 ± 1.3^{a}	73.2 ± 0.5	5.4 ± 0.9^{b}
24:0	27.5 ± 1.9	0.4 ± 0.1^{a}	52.4 ± 1.5	2.5 ± 0.4^{b}	59.6 ± 0.9	5.8 ± 0.5^{c}	66.3 ± 0.3	8.5 ± 0.9^{d}
16:1(*n*-9)	Trace	Trace^{a}	Trace	Trace^{a}	Trace	Trace^{a}	12.0 ± 5.0	0.4 ± 0.2^{b}
18:1(*n*-9)	Trace	Trace^{a}	20.4 ± 3.2	10.0 ± 1.7^{b}	29.5 ± 2.7	21.9 ± 1.9^{c}	53.1 ± 2.4	67.0 ± 8.8^{d}
20:1(*n*-9)	Trace	Trace^{a}	18.8 ± 1.0	1.8 ± 0.1^{b}	20.9 ± 2.0	3.2 ± 0.5^{c}	30.3 ± 2.0	5.1 ± 0.8^{d}
18:2(*n*-6)	Trace	Trace^{a}	Trace	Trace^{ab}	Trace	Trace^{ab}	6.6 ± 1.8	0.8 ± 0.3^{b}
20:2(*n*-6)	Trace	Trace	19.1 ± 4.8	0.7 ± 0.2	22.7 ± 6.1	1.2 ± 0.2	Trace	0.8 ± 1.1
20:4(*n*-6)	17.5 ± 4.4	1.8 ± 0.4^{a}	34.6 ± 1.0	12.8 ± 1.1^{b}	39.2 ± 1.1	20.4 ± 1.8^{c}	40.6 ± 2.1	20.0 ± 1.8^{c}
Total μg		8.3 ± 2.0^{a}		53.4 ± 3.7^{b}		89.3 ± 6.1^{c}		159.0 ± 24.5^{d}

All values are mean $\pm$ standard deviation of three replicates of 10 ml cultures. The mass and percentage of each FAME containing ^{13}C derived from ^{13}C-acetate was determined by GC/MS using a FAME-specific standard curve. Selected ions within the spectra were used to quantify both native fatty acids containing only naturally occurring stable isotopes and fatty acids containing additional isotope derived from ^{13}C-acetate. The fatty acids 16:1(*n*-7), 18:1(*n*-7), 20:2(*n*-9) and 20:3(*n*-6) contained only trace quantities of ^{13}C (<0.1 μg or SD>mean). Different letters denote that the masses of the fatty acids on those sampling dates are significantly different at the $P<0.05$ level (ANOVA, Tukey's test).

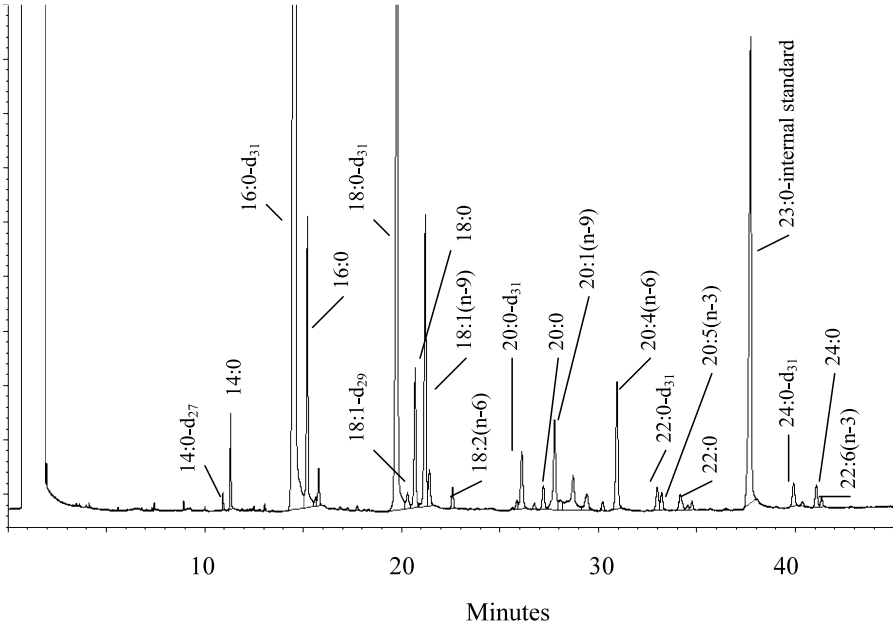

Fig. 5. Portion of a gas chromatography/flame ionization detection (GC/FID) chromatogram of FAME derived from *P. marinus* cells grown in media containing 875 μM palmitic-d_{31} acid for 7 days. FAME derived from palmitic-d_{31} acid was identified by gas chromatography/mass spectroscopy using the same column and temperature program as was used for the GC/FID.

results confirm our previous findings that the increased fatty acids and lipids during meront proliferation were derived from the de novo synthesis [25]. *L. donovani* was reported to incorporate ^{14}C-acetate to various saturated and unsaturated fatty acids [11]. However, whether *L. donovani* synthesize AA from acetate is inconclusive, since in Jacobs et al.'s study [11], fatty acids were separated using a packed GC column and 20:3(*n*-3) eluted together with 20:4(*n*-6) (AA) as one peak.

Since 16:4(*n*-6), 18:3(*n*-6), and 18:4(*n*-6) were not detected in the medium [25], it is very unlikely that the formation of 20:4(*n*-6) is a result of the elongation of 16:4(*n*-6) and 18:4(*n*-6) or the end product of elongation and desaturation of 18:3(*n*-6). The common pathway for synthesis of (*n*-6) 20-carbon polyenoic acids begins with Δ-6 desaturation of 18:2(*n*-6) to 18:3(*n*-6) followed by two-carbon elongation to 20:3(*n*-6) and then further desaturation to 20:4(*n*-6) by Δ-5 desaturase. The alternate Δ-8 pathway begins with an elongation of 18:2(*n*-6) to 20:2(*n*-6) followed by Δ-8 desaturation and a second desaturation at the Δ-5 position. Incorporation of ^{13}C-acetate into 18:3(*n*-6) was not detected, but found in 20:2(*n*-6), 20:3(*n*-6), and 20:4(*n*-6) (Table 2, Fig. 3). The fatty acid 18:3(*n*-6) was also not present in *P. marinus* cultivated in two different media [25]. Thus, it is more likely that *P. marinus* meronts used the alternate Δ-8 pathway rather than Δ-6 pathway to make 20:4(*n*-6). A Δ-8 desaturase has been previously isolated and characterized from the free living protozoan *Euglena gracilis* [39]. In *P. marinus* meronts, it is probable that 18:1(*n*-9) is first desaturated to 18:2(*n*-6) by Δ-12 desaturase, then elongated to 20:2(*n*-6) and ultimately desaturated to 20:3(*n*-6) and 20:4(*n*-6) via the sequential use of Δ-8 and Δ-5 desaturases. The much higher amounts and percentage of ^{13}C-labeled 20:2(*n*-6) and 20:4(*n*-6) than 18:2(*n*-6) and 20:3(*n*-6) accumulated in meronts from 24 to 72 hrs suggest that the steps from 18:2 (*n*-6) to 20:2(*n*-6) and from 20:3(*n*-6) to 20:4(*n*-6) may be coupled. Once 18:2(*n*-6) and 20:3(*n*-6) were formed, they were readily used, respectively, by the elongation and desaturation pathways. Synthesis of 20:4(*n*-6) usually utilizes the Δ-6

Table 3
Incorporation of exogenous palmitic-d_{31} acid into fatty acids of *P. marinus*

FAME	7 day incubation (1.39×10^7 cells)		14 day incubation (8.2×10^6 cells)	
	Percent	μg	Percent	μg
14:0	19.0 ± 0.4	0.8 ± 0.1	19.5 ± 0.4	0.3 ± 0.1
16:0	94.3 ± 0.2	215.2 ± 31.5	95.0 ± 0.2	87.0 ± 12.8
18:0	86.9 ± 0.1	55.3 ± 6.2	86.5 ± 0.1	21.0 ± 1.2
20:0	72.5 ± 1.4	5.0 ± 0.1	72.0 ± 1.7	2.1 ± 0.2
22:0	54.9 ± 1.0	2.2 ± 0.1	55.2 ± 1.1	1.0 ± 0.1
24:0	54.2 ± 0.4	2.4 ± 0.1	54.6 ± 0.5	1.0 ± 0.1
18:1	5.0 ± 1.6	1.1 ± 0.5	3.0 ± 2.0	0.1 ± 0.1
Total μg		281.8 ± 38.0		112.5 ± 13.87

Percent values are for the amount derived from palmitic-d_{31} acid relative to the total amount of each fatty acid. All values are mean ± the standard deviation of three GC/FID analyses of each cell culture.

pathway of desaturation [34]. Usage of the Δ-6 pathway for polyenoic fatty acid synthesis has been described in ciliates, trypanosomatids [6] and some phytoplankton species such as *Porphyridium cruentum* (Rhodophycease), *Ochromonas danica* (Chrysophyceae), and *Spirula platensis* (Cyanophyceae) [40]. Conversely, employing the Δ-8 pathway to synthesize 20:4(*n*-6) has been described in the ciliated protozoan *Tetrahymena pyriformis* [41], in the soil amoebae *Acanthamoeba* spp. [42,43], and in heterotrophic euglenoid species [40]. However, further study is needed to rule out the operation of Δ-6 pathway in *P. marinus* meronts.

It is speculated that 18:1(*n*-9) is consumed immediately for 20:4(*n*-6) synthesis as soon as it is made. Thus, no ^{13}C-labeled 18:1(*n*-9), but labeled 20:4(*n*-6) was found after 24 h incubation. The continuous increase in ^{13}C-labeled 18:1(*n*-9) up to 7 days post-incubation, after synthesis of 20:4(*n*-6) had leveled out, is believed due to its storage as energy and resource for future 20:4(*n*-6) synthesis. The meronts' polar lipid and neutral lipid contained about 26 and 21% of 18:1(*n*-9), respectively [25]. Considerable amount of 20:1(*n*-9) containing ^{13}C was noted and similar to 18:1(*n*-9), the amount and percentage of this fatty acid containing stable isotope label increased from 48 h to 7 days (Fig. 4). While 18:1(*n*-9) is considered the precursor of 20:4(*n*-6), 20:1(*n*-9) is probably an energy storage component rather than an intermediate of 20:4(*n*-6) since a much higher level of 20:1(*n*-9) was found in neutral (about 19%) than in polar (about 4%) fractions in meronts [25]. Nevertheless, we cannot completely exclude the possibility that 18:1(*n*-9) was first elongated to 20:1(*n*-9), then further desaturated to 20:2 (*n*-6). Analysis of the distribution of ^{13}C-labeled 18:1(*n*-9) and 20:1(*n*-9) in polar and neutral lipids in future studies may provide further information on the functional roles of these fatty acids in *P. marinus*.

To the best of our knowledge, no parasitic protozoan has been reported to be capable of synthesizing essential fatty acids such as 20:4(*n*-6) (arachidonic acid, AA) de novo. The ability of *P. marinus* to AA from acetate is truly exceptional. This fatty acid may be a critical membrane component for *P. marinus*. The polar lipids of in vitro cultured meronts had a higher proportion of AA than neutral lipids ($>25.0\%$ in polar fraction compared to 9.0% in neutral fraction) [25]. At this point, it is unknown whether 20:4(*n*-6) has other physiological roles in *P. marinus*. C20 PUFAs are important membrane components of many organisms and serve as the precursors for synthesis of eicosanoid metabolic regulators. Investigations of the metabolism of 20:4(*n*-6) by mammalian cells have delineated biochemical pathways yielding a diverse number of biologically active eicosanoid products including prostaglandins (PGs), thromboxanes and leukotrienes [44,45]. Eicosanoids, are believed to play an active role in the free swimming larvae, cercariae, of the blood fluke *Schistosoma mansoni*, in modifying the behaviors that precede penetration (e.g. cessation of swimming) and penetration of the mammalian host skin [1,46,47]. Production and release of eicosanoids by cercariae, schistosomula and adult worms have been reported [1,46,48]. The biological functions as well as the metabolism and potential pathogenic effects of parasite elaborated AA derivatives have been evaluated in a few parasitic protozoans. Catisti et al. [49] reported that AA plays a role in regulating Ca^{++} entry in *Trypanosoma brucei* and *L. donovani*. Infection of mammals by African trypanosomes is characterized by upregulation of PG. *Trypanosoma brucei* was found to produce PGs from AA and its metabolites [50]. The $PGF_{2\alpha}$ synthase purified from *T. brucei* was found to be completely distinct from mammalian PGF synthase. Similarly, cell homogenates of *P. falciparum* produced PGs of D_2, E_2, and F_2 after incubation with 20:4(*n*-6) [51]. Addition of AA to *P. falciparum* cell culture markedly increased the ability of the cell homogenate to produce PGs and of parasitized red blood cells to accumulate PGs in the culture medium. *P. falciparum* produces PGs in a way distinguishable from the mammalian system [51]. These results are evidences that parasitic protozoans can produce substances that may contribute to pathogenesis since parasite-derived PGs are considered to be pyrogenic, somnogenic and harmful to host defenses [51]. Also, studies on AA metabolism by murine peritoneal macrophages infected with *L. donovani* [52] and *Toxoplasma gondii* [53] suggests that the increased amount of AA metabolites in infected macrophages has the potential for influencing cellular immune function and the inflammatory response to infection. However, whether *P. marinus* meronts synthesize PGs from AA and their biological role in relation to its host remains to be investigated.

Meronts efficiently elongated 16:0-d_{31} to 18:0, 20:0, 22:0, and 24:0, but desaturation activity was limited. Only a small quantity of 18:1-d_{29} was detected (Table 3). This suggests that meronts cannot directly convert exogenous palmitic acid or products of its elongation to unsaturated counterparts. It is believed that limited formation of unsaturated fatty acid from palmitic-d_{31} acid is not due to the relative high cultivation temperature (28 °C), since the same temperature was used for the ^{13}C-acetate experiment and routine *P. marinus* meront cultivation. Similar results were obtained in a study by McKeon et al. [54] that tested the elongation and desaturation of deuterium labeled 16:0-d_2 in the fungus, *Neurospora crassa*. They found that deuterated 16:0 is efficiently elongated to 18:0, but the latter is not efficiently desaturated to 18:1(*n*-9). They believed that elongation of the exogenous 16:0 to 18:0 occurs in a metabolic pool separate from that of de novo fatty acid synthesis. Further study is needed to test whether this is

the case in *P. marinus* meronts. The high 16:0-d_{31} incorporation and detection of deuterated 14:0 suggest that meronts scavenge and catabolize deuterium-labeled 16:0 for energy.

There are two pathways for the biosynthesis of monoenoic fatty acids [7]. These two pathways are (1) CoA-dependent direct desaturation of saturated fatty acids to corresponding unsaturated fatty acids (e.g. 16:0 to 16:1(*n*-9), and (2) oxidatively desaturate the acyl carrier protein thioester of saturated fatty acids. The CoA-dependent direct desaturation mechanism permits the direct conversion of exogenous palmitic or stearic acid to their unsaturated counterparts. This is used by some bacteria, actinomycetes, blue-green algae, red algae, heterotrophic euglenids, chrysomonads, yeasts, molds, slime molds, amoebas, ciliates and metazoa [6,7]. Also all the examined kinetoplastid flagellate species readily converted palmitic and stearic acid to monounsaturated fatty acids [7]. Phototrophic euglenids, algae and higher plants which use the acyl carrier protein-dependent (ACPD) direct desaturation mechanism do not utilize exogeneously supplied palmitic or stearic acids to make monounsaturated fatty acids [7]. Because no 16:1-d_{29} was detected and only a very small amount of 18:1-d_{29} was found in meronts compared to the precursor, 16:0-d_{31}, and its product, 18:0-d_{31}, after 7 and 14 days incubation, it is tempting to speculate that *P. marinus* meront uses ACPD rather than CoA-dependent pathway for monounsaturated fatty acid synthesis.

Plastid organelles have been described in ultrastructural studies of several genera of Apicomplexa [55]. Also, a remnant plastid genome has been reported in several apicomplexan parasites including *P. falciparum*, *T. gondii*, and *Eimeria tenella* [55–57]. The *P. falciparum* plastid does not encode any of the fatty acid biosynthesis machinery [58]. But, in a recent study several genes encoding enzymes such as, acyl carrier protein (acpP), β-ketoacyl carrier protein synthase III (fabH) and β-hydroxyacyl-ACP (fabZ) were found in the nuclear genomes of *P. falciparum* and *T. gondii*. These genes are located on the plastid genomes of some algae [59] and plants [55]. Waller et al. [56] hypothesized that a Type II fatty acid biosynthetic pathway is present in the apicoplasts of *P. falciparum* and *T. gondii*. Based upon ultrastructural and morphological analyses, *P. marinus* had been placed in the Phylum Apicomplexa [60,61]. After reexamination of the ultrastructural evidence [62] and because of the recent published SSU rRNA and actin gene sequence data, it has been proposed that the phylogenetic position of *P. marinus* is closer to the dinoflagellates than to apicomplexans [63–66]. However, both phototrophic and heterotrophic dinoflagellates contain little (*n*-6) PUFA ($< 5\%$) and biosynthesize and accumulate high levels of (*n*-3) series PUFA [67]. A non-photosynthetic plastid has been identified in the biflagellated zoospores of this parasite [60]. There are no reports of finding this organelle in meront or prezoosporangium stages and it is uncertain whether a Type II fatty acid synthetic pathway exists in *P. marinus*. The ability to synthesize fatty acids de novo, however, leads us to believe in the existence of plastid organelles and/or plastid genomes in *P. marinus* meronts.

The observed fatty acid biosynthetic capability in *P. marinus* meronts may be related to the mechanism for disease transmission. *P. marinus* cannot rely on its host to transport infective cells to new host populations. Disease transmission for *P. marinus* is via dispersal of infective cells in the water column. The ability to synthesize fatty acids and store them as triacylglycerol may be critical for meronts to remain viable and infective long enough for them to enter a new host and to infect a distant host population. It is not known, however, whether meronts retain the ability to synthesize fatty acids in vivo, within the host. A preliminary analysis of 'wild' meronts isolated from infected oyster tissues showed that the 'wild' meronts have levels of EPA, DHA and AA similar to their host. Whether the meront stage of *P. marinus* has two divergent trophic lipid metabolic pathways, host-associated and free living, remains to be determined. Unlike *Plasmodium* spp. whose mammalian hosts are capable of synthesizing AA from 18:2(*n*-6) and 18:3(*n*-6) supplied via diet, *P. marinus*'s host, the eastern oyster, does not appear to be able to make AA and must acquire it from dietary sources [68,69].

In summary, in vitro cultured *P. marinus* meronts can synthesize a range of saturated and unsaturated fatty acids using acetate. Meronts efficiently elongated exogenous 16:0-d_{31} to 18:0, 20:0, 22:0, 24:0, but desaturation of 18:0 to 18:1 was limited. The finding of de novo synthesis of 20:4(*n*-6) is particularly interesting. However, future study is needed to determine whether the observed in vitro activities reflect the in vivo activities, when meronts are associated with the host, or meronts have two metabolic phases, host-associated and host-disassociated.

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