THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 66:178–182, 1976

Vol. 66, No. 3
Printed in U.S.A.

GROWTH REQUIREMENTS AND LIPID METABOLISM OF PITYROSPORUM ORBICULARE

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The yeast, *Pityrosporum orbiculare*, isolated from lesions of tinea versicolor, grows in vitro only if fatty acids from the C_{12} to C_{24} series are added to the culture medium. Except for elaidinic and nervonic acids, all saturated and unsaturated fatty acids tested support growth. *P. orbiculare* can synthesize various lipid fractions containing both saturated and unsaturated fatty acids from a single fatty acid. Glucose and asparagine stimulate growth but exogenous vitamins do not.

The yeast-like organisms, *Pityrosporum ovale* and *Pityrosporum orbiculare*, are both lipophilic and lipid dependent [1–3]. *P. ovale* has been considered a saprophytic organism which colonizes human skin and is often found in association with chronic seborrhea or dandruff. *P. orbiculare*, which has been morphologically and physiologically differentiated from *P. ovale* [3] is regarded as the pathogenic agent in tinea versicolor.

Earlier investigations [5–7] have demonstrated the specific fatty acid requirements and have described the lipid metabolism of *P. ovale*. As a sequel to our previous studies [8–10], in this paper we shall report our investigations on the lipid metabolism of *P. orbiculare* isolated from scales of tinea versicolor.

MATERIALS AND METHODS

Organism. In these experiments, we used mainly strain 4709 and occasionally strain 6311 from our collection of 130 strains of *P. orbiculare*.

Media. Bacto-yeast nitrogen base (Difco) was used as the basal medium with and without glucose and/or asparagine. Different concentrations of fatty acids were added either as free fatty acids, free acids emulsified in 0.2% Triton X-100, or as Tween derivatives. The cultures were grown in 500-ml Erlenmeyer flasks on a gyrorotary shaker at 30°C. The initial cell density of all cultures was adjusted at 5 to 10 μg dry weight of cells/ml. Vitamin requirements were studied in a Bacto-vitamin-free yeast base (Difco) supplemented with Tween 80 as the lipid source. The pH of all culture media was about 5.4 before autoclaving.

Estimation of cell yield. Cell growth was estimated by directly weighing the cells after drying at 80°C for 24 hr. The cells had been previously washed 3 times in physiologic saline, once in distilled water, and then centrifuged at 2500 rpm for 10 min. Cell growth was also determined turbidometrically by measuring the optical density at 640 nm of a suitably diluted culture. A calibration curve was prepared by plotting the optical density at 640 nm of a cell suspension whose concentration had been determined by direct weighing. This method was generally used in growth studies but, when accurate cell densities were necessary, direct weighing was utilized.

Extraction of lipids. Whole cells, mixed with quartz sand, were finely disrupted in a mortar and extracted with 50 volumes of chloroform: methanol (2:1 v/v) for 3 hr at room temperature and then twice with 20 volumes of chloroform:methanol (2:1 v/v). Pooled extracts were dried over anhydrous Na_2SO_4 and the lipids recovered by evaporation of solvents under reduced pressure on a rotatory evaporator below 30°C.

Fractionation of the lipid extracts. The total lipid extracts were separated into their various components by thin-layer chromatography (TLC) according to the method of Boniforti et al [11]. Phospholipids, which remained in the original plate with this method, were reextracted with chloroform:methanol (2:1 v/v) and separated by TLC according to the method of Skipski et al [12]. The lipids on the developed chromatograms were detected by two methods: (1) charring by heating to 150°C with 50% v/v H_2SO_4 for photodensitometry; (2) staining with bromocresol green solution for gas–liquid chromatography.

Photo densitometry. A Joyce-Loebl Chromoscan integrating and recording reflectance photometer was used to scan each line and to quantify the charred chromatograms. The peak areas in the recorder output were calculated by a DuPont curve resolver and adjusted by factors determined by analyzing reference mixtures of known composition [13].

Esterification procedure and gas–liquid chromatography. The esterification procedure and determination of the fatty acid composition of total lipids and phospholipids were performed according to the method of Boniforti et al [11].

Determination of glucose consumption and Tween 80. Glucose was determined by the hexokinase reaction described by Schmidt [14]. Tween 80 was determined as total lipids according to the method of Zöllner and Kirsch [15]. Reference standards for thin-layer chromatography and gas–liquid chromatography was obtained from Analabs (Analabs Inc. North Avon, Connecticut).

RESULTS

Figure 1 shows the growth curves of *P. orbiculare*, strain 4709, grown at 30°C in basal

Manuscript received March 24, 1975; in revised form October 24, 1975; accepted for publication November 4, 1975.

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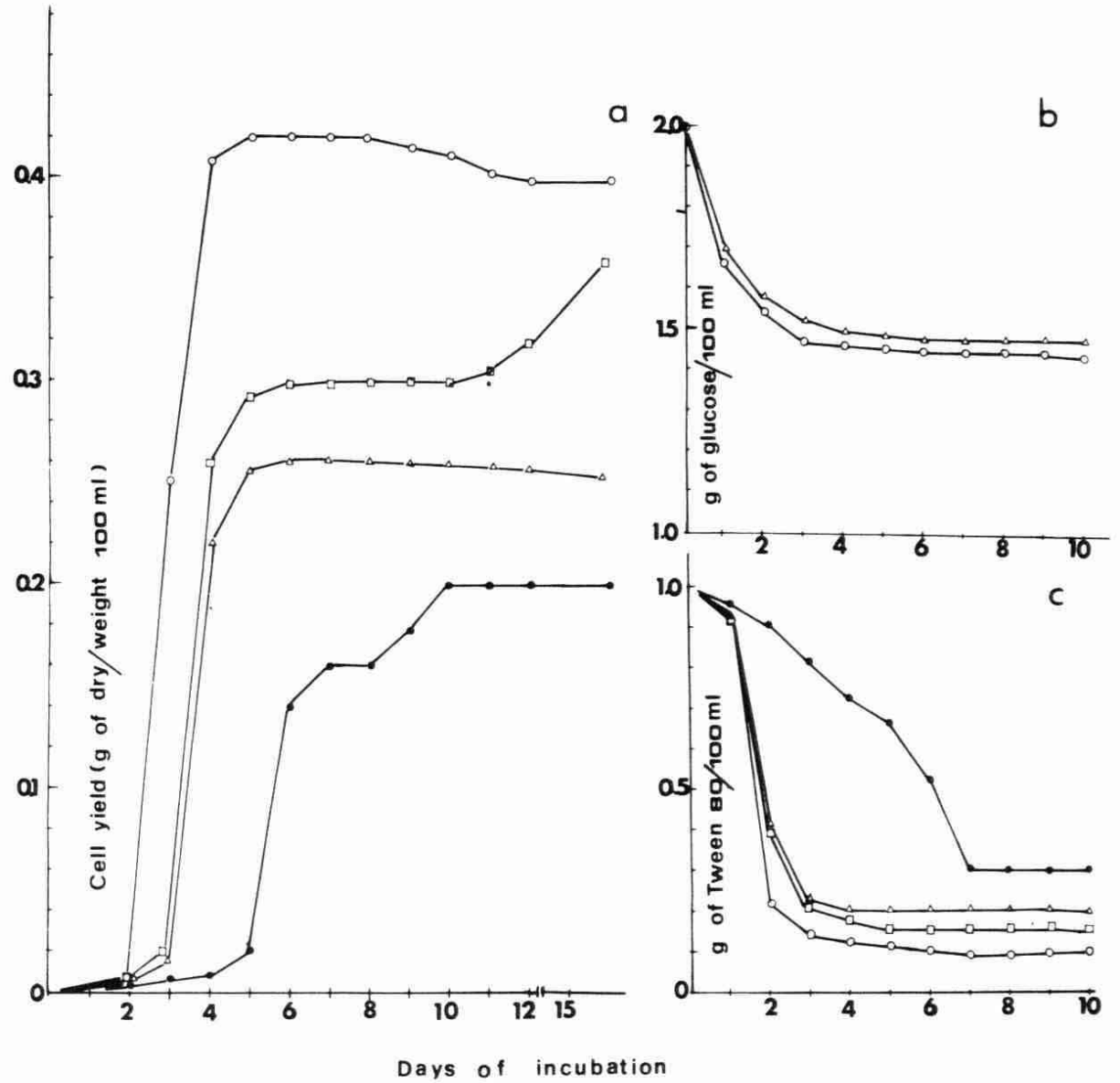

FIG. 1. Growth of *Pityrosporum orbiculare* (*a*), consumption of glucose (*b*), and Tween 80 (*c*). Each result represents the average of two experiments. Original inoculum was adjusted at 5 to 10 μg dry weight cells/ml per flask. Fatty acid supplements were added in the form of Tween 80 at a concentration of 1 gm/100 ml. ●——●, Basal medium; △——△, basal medium + glucose (2 gm/100 ml); □——□ basal medium + asparagine (0.15 gm/100 ml); ○——○, basal medium + glucose (2 gm/100 ml) + asparagine (0.15 gm/100 ml).

medium with and without glucose and/or asparagine with Tween 80 added at a concentration of 1 mg/ml. The diauxic growth probably results from the utilization of the sorbitol moiety of the Tween. The same figure shows the consumption of glucose and Tween 80 during incubation.

Figures 2 and 3 show the effect of varying concentrations of Tween 80 on the growth pattern on the 4th and 10th days. Tween 80 was used because of its solubility in the medium. Note that although *P. orbiculare* utilized Tween 80 as the sole source of energy, high concentrations of Tween inhibit growth when glucose and/or asparagine are omitted.

Figure 4 indicates the stimulating effect of increasing amounts of asparagine on the growth of *P. orbiculare*.

The effect on growth of adding different, straight-chain fatty acids, both saturated and unsaturated, to the basal medium + asparagine + glucose is summarized in Table I. The experiments were performed both with and without the addition of the detergent, Triton X-100.

To determine whether *P. orbiculare* requires the addition of exogenous vitamins, the yeast was grown in basal medium containing glucose, asparagine, and Tween 80, but lacking vitamins. In the vitamin-free medium the rate of growth was identical to that observed in the basal medium.

Photodensitometric and gas chromatographic

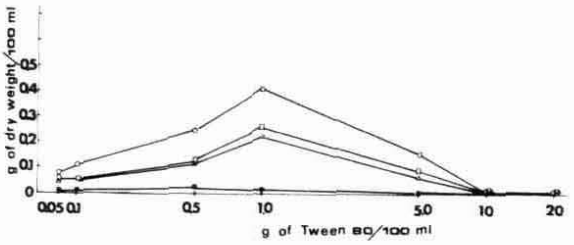

FIG. 2. Growth of *Pityrosporum orbiculare* at the 4th day with different supplements of Tween 80. Each plot represents the average of two experiments. Original inoculum was adjusted at 5 to 10 μg dry weight cells/ml per flask. ●——●, Basal medium; Δ——Δ basal medium + glucose (2 gm/100 ml); □——□ basal medium + asparagine (0.15 gm/100 ml); ○——○, basal medium + glucose (2 gm/100 ml) + asparagine (0.15 gm/100 ml).

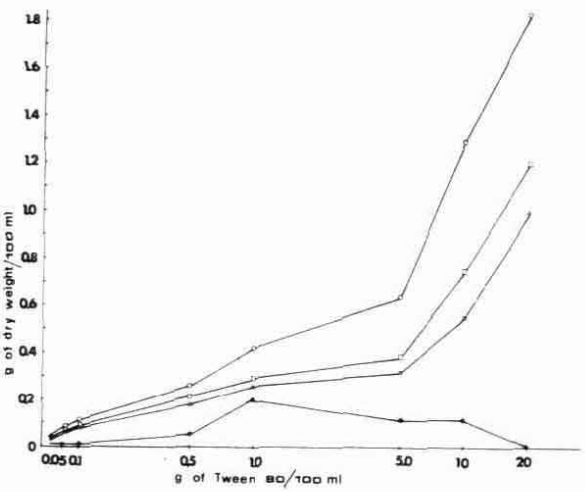

FIG. 3. Growth of *Pityrosporum orbiculare* at 10th day with different supplements of Tween 80. Each result represents the average of two experiments. Original inoculum was adjusted at 5 to 10 μg dry weight cells/ml per flask. ●——●, Basal medium; Δ——Δ basal medium + glucose (2 gm/100 ml); □——□ basal medium + asparagine (0.15 gm/100 ml); ○——○, basal medium + glucose (2 gm/100 ml) + asparagine (0.15 gm/100 ml).

analyses of cell lipids are reported in Tables II and III.

DISCUSSION

As a result of these experiments we can delineate several features of the growth requirements and lipid metabolism of *P. orbiculare*.

1. *P. orbiculare* requires a lipid source for growth. Although lipids can be the only carbon source, growth is accelerated by the addition of glucose or asparagine. These nutrients must affect different metabolic pathways because optimal growth is attained only when both are present at the same time (Figs. 1-3). Growth also occurs with small amount of lipids (10–20 mg of Tween 80/100 ml of medium) in the presence of glucose and/or asparagine, indicating that lipids are not essential as an energy source. A probable explanation is that *P. orbiculare* is incapable of synthesizing lipids and requires an exogenous source of fatty acids for membrane synthesis. However, at high concentrations, lipids retard growth; growth is also completely inhibited in media without asparagine and/or glucose. When glucose and/or asparagine are present, growth is temporarily retarded (Fig. 2), but on the 10th day growth is maximally stimulated (Fig. 3). The probable explanation is that high concentrations of lipids increase the intracellular level of NADH and ATP which would lower the levels of NAD^+ and ADP. The high $NADH/NAD^+$ ratio would in turn inhibit the Krebs and glyoxilic cycles, particularly the conversion of malate to oxalacetate which occurs only at high $NAD^+/NADH$ ratios [16]. Moreover, a high ATP/ADP ratio inhibits allosterically the Krebs cycle enzyme, isocitric dehydrogenase [16]. Fur-

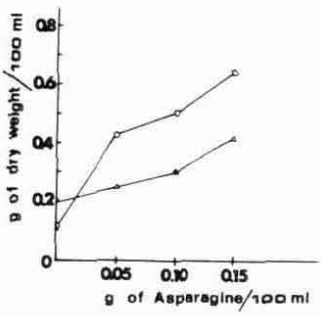

FIG. 4. Effect of asparagine on the growth of *Pityrosporum orbiculare* at the 10th day. Each result represents the average of two experiments. Δ——Δ Tween 80 at the concentration of 1 gm/100 ml; ○——○, Tween 80 at the concentration of 5 gm/100 ml.

TABLE I. *Effect of varying fatty acids on growth of Pityrosporum orbiculare at 10 days*

Fatty acid supplements (*2 mg/ml*)	Basal medium (*mg dry weight/100 ml*)	Basal medium + 0.2% Triton X-100
None	0	0
n-Octanoic	0	0
n-Decanoic	0	0
n-Dodecanoic	8.5	24.4
n-Tetradecanoic	17.2	132.0
n-Hexadecanoic	181.0	318.0
n-Octadecanoic	114.0	281.5
n-Eicosanoic	173.5	186.4
n-Docosanoic	45.5	52.5
n-Tetracosanoic	15.7	22.4
9-Tetradecenoic	29.0	11.4
9-Hexadecenoic	114.0	48.5
9-Octadecenoic	360.0	80.5
trans-9-Octadecenoic	0	0
12 OH-9-Octadecenoic	268.0	31.2
cis-13-Docosenoic	197.3	8.2
cis-15-Tetracosenoic	6.1	2.0
Vit F (linoleic, linolenic, and arachidonic)	214.0	67.4
Tween 40	195.0	Not done
Tween 60	147.4	Not done
Tween 80	178.5	Not done

TABLE II. *Effect of addition of varying fatty acids on lipid composition of Pityrosporum orbiculare after 10 days of growth*

Total lipid content of cells varied from 22 to 26 gm/100 dry weight cells. The results are the average of two determinations.

Fatty acid supplements (2 mg/ml)	All lipid fractions (%)							Phospholipids only (%)				
	PL	MG	ST	DG	FFA	TG	SE	DPG	PC	PE	PI	PS
n-Hexadecanoic	10.5	1.0	1.0	3.2	44.8	30.0	9.5	11.5	40.0	22.5	24.5	1.5
n-Hexadecanoic + Triton	12.1	0.5	1.2	2.8	43.7	29.5	10.2	11.0	42.0	20.5	25.0	1.5
n-Octadecanoic	11.0	1.2	0.9	3.7	46.0	28.0	9.2	10.5	44.5	18.5	25.3	1.2
Tween 60	12.2	0.5	1.1	3.0	45.3	28.5	9.4	11.5	43.5	21.0	22.5	1.5
9-Octadecenoic	11.5	1.2	1.4	3.5	48.2	23.2	11.0	10.0	42.5	20.5	25.0	2.0
Tween 80	13.0	0.6	0.6	3.2	43.0	29.0	10.6	11.0	43.0	21.0	23.2	1.8
12 OH-9-Octadecenoic	11.6	0.5	0.8	2.5	49.4	25.4	9.8	13.5	41.5	23.0	19.5	2.5
n-Eicosanoic	12.5	0.2	1.2	1.8	55.2	20.6	8.5	12.2	40.5	19.5	25.8	2.0
cis-13-Docosenoic	12.2	0.2	1.5	1.5	60.1	14.6	9.9	11.5	42.5	20.5	24.0	1.5

Abbreviations: PL, phospholipids; MG, monoglycerides; ST, sterols; DG, diglycerides; FFA, free fatty acids; TG, triglycerides; SE, sterol esters; DPG, diphosphatidyl glicerol (cardiolipin); PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine

TABLE III. *Effect of varying fatty acid composition of total lipids and phospholipid fraction of Pityrosporum orbiculare after 10 days of growth*

All fatty acids added at a concentration of 2 mg/ml. The results are the average of two determinations.

Fatty acids	Fatty acid supplements																	
	n-Hexadecanoic		*n*-Hexadecanoic + Triton X-100		*n*-Octadecanoic		Tween 60		*n*-Octadecenoic		Tween 80		12 OH-9-Octadecenoic		*n*-Eicosanoic		*cis*-13-Docosenoic	
	TL[a]	PL[a]	TL[a]	PL[a]	TL[a]	PL[a]	TL[a]	PL[a]	TL[a]	PL[a]	TL[a]	PL[a]	TL[a]	PL[a]	TL[a]	PL[a]	TL[a]	PL[a]
Tetradecanoic (C_{14})	0.5	1.2	0.6	1.0	0.5	1.5	0.6	1.3	0.3	1.0	0.4	1.2	0.1	1.0	0.8	0.8	0.6	0.8
Tetradecenoic (C_{14}=)	0.9	1.0	0.8	1.0	0.5	1.0	0.3	0.9	0.4	1.2	0.2	0.8	0.1	0.6	0.2	0.6	0.1	0.6
Pentadecanoic (C_{15})	0.3	0.1	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.3	0.2
Pentadecenoic (C_{15}=)	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.3	0.2	0.1	0.2	0.2	1.2	0.1	0.1	0.1	0.8	0.2
Hexadecanoic (C_{16})	52.6	25.1	48.5	25.4	30.5	24.7	36.5	28.6	18.6	16.3	13.0	14.0	11.4	14.8	7.5	24.5	2.4	16.1
Hexadecenoic (C_{16}=)	1.9	3.0	2.2	3.5	1.8	3.8	0.8	2.8	4.4	3.5	5.0	2.5	3.1	6.1	0.8	3.6	1.6	6.5
Heptadecanoic (C_{17})	2.6	0.3	2.4	0.2	2.5	0.4	0.8	0.3	0.5	0.2	0.5	0.2	0.2	0.3	0.1	0.4	0.8	0.1
Heptadecenoic (C_{17}=)	0.7	0.3	0.6	0.2	0.5	0.3	0.2	0.4	0.8	0.2	0.6	0.3	3.4	0.4	0.1	0.4	1.0	0.4
Octadecanoic (C_{18})	16.1	15.4	14.5	14.8	46.8	18.2	30.8	16.4	8.6	10.6	17.5	12.0	0.5	10.5	4.3	18.3	2.1	9.6
Octadecenoic (C_{18}=)	17.1	33.4	21.2	34.7	10.6	28.9	24.2	34.0	58.1	47.6	55.5	54.5	42.7	41.5	30.5	32.8	22.1	45.8
Octadecadienoic ($C_{18}2$=)	4.6	16.0	5.1	15.5	3.0	17.5	2.6	12.9	5.3	14.4	2.2	10.5	8.1	18.3	8.5	14.5	3.5	16.5
Others	2.6	4.0	3.8	3.5	3.1	3.5	2.9	2.0	2.7	4.8	4.8	3.7	29.0[b]	6.3	47.0[c]	3.9	64.7[d]	3.2

Abbreviations: TL, total lipids; PL, phospholipids

[a] Data represent percent of specific fatty acid in fraction

[b] C_{18}=(OH):22.5; $C_{18}3$=:0.8; C_{20}=:4.7; C_{22}:0.5; C_{22}=:0.5

[c] $C_{18}3$=:1.0; C_{20}:46.0

[d] $C_{18}3$=:3.0; C_{20}:4.5; C_{20}=:18.5; C_{22}:2.2; C_{22}=:36.5

ther data are needed for confirmation but our data tend to support this possibility.

The addition of all fatty acids with the exception of *trans*-9-octadecenoic (elaidinic) acid and probably *cis*-15-tetracosenoic (nervonic) acid, stimulate growth (Tab. I). When Triton X-100 is added, however, the unsaturated fatty acids achieve toxic levels probably because Triton, by solubilizing fatty acids, increases their concentration in the medium. Since unsaturated fatty acids are probably toxic at high concentrations, this may explain why Shifrine and Marr [5] found that pure oleic acid does not stimulate the growth of *P. ovale*.

The inability of *P. orbiculare* to synthesize longer-chain fatty acids without an external source of fatty acids of chain lengths C_{12} or greater (Tab. I) suggests that "de novo" synthesis of lipids probably does not occur. Rather it appears likely that chain length elongation occurs via mitochondrial synthetic pathways [17]. Since saturated and

unsaturated, odd-chained, fatty acids (C_{13} to C_{17}) are found in the cell lipids of *P. orbiculare* (Tab. III) this suggests the presence of an α oxidation in which carboxyl carbon is lost as CO_2 and the α carbon is first oxidized to fatty aldehyde and then to a corresponding carboxylic acid.

2. *P. orbiculare* appears to have the ability to synthesize from a single fatty acid a variety of lipids containing both saturated and unsaturated fatty acids (Tabs. II, III). However, this ability is probably somewhat limited since the fatty acid composition of the phospholipids partially reflects the composition of the fatty acids added to the medium (Tab. III). *P. orbiculare* also seems to possess the ability to interconvert saturated and unsaturated fatty acids. The dependence on the specific fatty acid supplement becomes ever more evident as demonstrated by the determination of the fatty acid composition of the total cell lipids, particularly when 12OH,9-octadecenoic (ricinoleic) acid, *n*-eicosanoic (arachidic) acid, and *cis*-13-docosaenoic (erucic) acid are used as fatty acid supplements (Tab. III).

3. *P. orbiculare* continues to proliferate after continuous subculturing in a vitamin-free medium supplemented with Tween 80, which indicates that this proliferation does not require an external source of vitamins. This conclusion agrees with the results of Weary [7] who showed that exogenous vitamins do not contribute substantially to the growth of *P. ovale*.

4. Asparagine also supports growth (Fig. 4), probably because this amino acid can be readily converted to oxalacetate. This suggestion is supported by Benham's [18] demonstration that in *P. ovale* ethyloxalacetate can substitute for asparagine as a growth factor. Evidently, if lipids constitute the sole carbon source, insufficient oxalacetate is present to provide maximum efficacy to the Krebs cycle. The addition of exogenous glucose probably increases growth by providing an additional source of oxalacetate which is being converted to phosphoenolpyruvate [16].

5. Our results, when compared with those obtained with *P. ovale* [5–7], indicated that the growth requirements and lipid metabolism of the two species are similar and probably identical. Since both *P. orbiculare* and *P. ovale* are able to utilize oleic acid, the distinction between these two species should not be based (as it usually is) on differences in their ability to utilize unsaturated fatty acids in vitro. Whether they constitute different species on the basis of morphologic differences cannot be resolved at the present time.

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