

Difference in the late ergosterol biosynthesis between yeast spheroplasts and intact cells

Terenzio Ferrante, Franca Viola, Gianni Balliano and Simonetta Oliaro-Bosso[✉]

University of Torino, Department of Drug Science and Technology, Torino, Italy

A comparative study on post-squalene sterol synthesis in intact yeast cells and spheroplasts was carried out with strains from three genera (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*) as well as with engineered *S. cerevisiae* cells altered in regard to the late ergosterol synthesis pathway. A common outcome of incubation experiments with radioactive acetate was that in intact cells the metabolic pathway flows till its specific end product (ergosterol and its precursor, depending on the enzyme deficiency), whereas in spheroplasts the pathway was stalled some step upstream. For example, in spheroplasts from wt strains, non-cyclic triterpenes squalene and oxidosqualene accumulated as though the metabolic path was kept from producing steroid-shaped molecules different from the end product. Accumulation of non-cyclic triterpenes was observed also in spheroplasts from *S. cerevisiae* cells lacking 3-ketosteroid reductase activity, an enzyme belonging to the C4-demethylase complex. When production of cyclic triterpenes was compromised by loss or poor functionality of oxidosqualene cyclase (EC 5.4.99.7), the difference between intact cells and spheroplasts was still remarkable, yet limited to the different oxido/dioxidosqualene ratio. The characteristics of spheroplasts as non-proliferating cells may partially explain the observed differences in post-squalene pathway from intact cells. We cannot say if the difference in metabolic pathways in spheroplasts and intact cells is a rule. We think, however, that it is worthwhile to search for an answer, as a wider picture of the points where the metabolic pathways are stalled in spheroplasts could provide original ideas about the metabolic network in yeast.

Key words: yeast cell wall, spheroplasts, late sterol biosynthesis, engineered yeast strains, Erg27p, Erg7p

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INTRODUCTION

Yeast spheroplasts created from intact cells by lytic digestion of the cell wall (Daum *et al.*, 1982) provide a useful model to study in eukaryotes the effect of compounds that are blocked by the presence of the cell wall (Becker & Schmitt, 2011). For instance, if the molecules are designed as inhibitors of metabolic pathways occurring in mammalian cells, preliminary studies can be carried out with the yeast spheroplasts, which mimic intact mammalian cells in being separated from the medium only by the plasma membrane. Spheroplasts may prove to be useful also in studies aimed at designing anti-parasitic/anti-protzoarian molecules, where the ability of

the designed molecule to enter the intact cell and reach the target enzyme has to be assayed (Oliaro-Bosso *et al.*, 2004). In this case, the yeast spheroplasts prepared from engineered yeast cells expressing the parasite/protzoan target enzyme roughly mimic the single cells of the unicellular organism, although in a non-proliferating state. In a study aimed at designing new anti-Trypanosoma molecules, we used yeast (*Saccharomyces cerevisiae*) cells expressing Trypanosoma oxidosqualene cyclase (OSC, EC 5.4.99.7), the target enzyme, as a preliminary *in vitro* model (Balliano *et al.*, 2009). As we knew from previous experiments, that Trypanosoma OSC perfectly complements yeast OSC in sterol biosynthesis (Milla *et al.*, 2002a; Oliaro-Bosso *et al.*, 2004), we expected to observe in control (non-treated) spheroplasts incubated with radiolabelled acetate approximately the same distribution of radioactive post-squalene lipids as detected in intact cells,

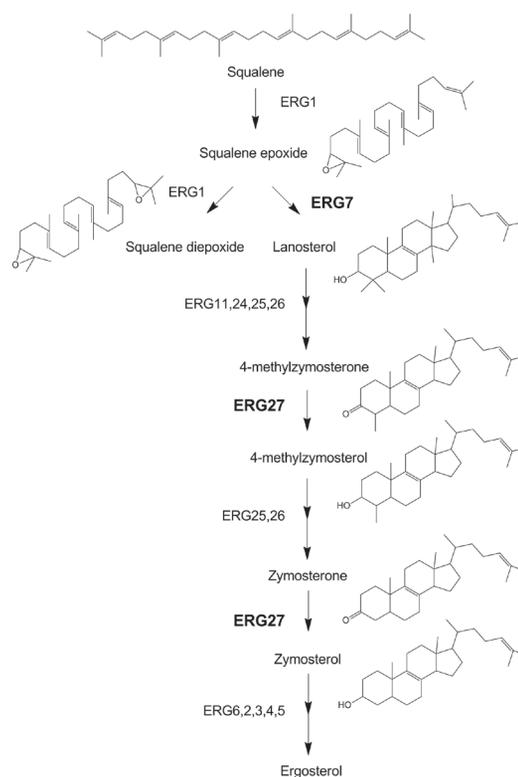


Figure 1. Post-squalene pathway in *S. cerevisiae*. In bold, the mutant steps in the strains used in this work.

[✉] e-mail: simona.oliaro@unito.it

Abbreviations: OSC, oxidosqualene cyclase; Erg7p, oxidosqualene cyclase; Erg27p, 3-ketosteroid reductase; DOS, dioxidosqualene

Table 1. Yeast strains used in this study

Strains	Genotype	Source
<i>S. cerevisiae</i>		
SCY876 (wt)	<i>MATa, upc2-1, hap1Ty, ura3, his3, leu2, trp1</i>	(Taramino <i>et al.</i> , 2010a)
STY2 (<i>erg7Δ</i>)	<i>MATa, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg7Δ::HIS3</i>	(Taramino <i>et al.</i> , 2010a)
BTY6-5-3 (<i>erg27Δ</i>)	<i>MATa, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg27Δ::LEU2</i>	(Taramino <i>et al.</i> , 2010b)
BTY6-5-3 (K206A-Erg27p)	<i>MATa, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg27-K206A</i>	(Teske <i>et al.</i> , 2008)
BTY6-5-3 (R40A-Erg27p)	<i>MATa, upc2-1, hap1Ty, ura3, his3, leu2, trp1, erg27-R40A</i>	(Teske <i>et al.</i> , 2008)
<i>S. pombe</i> KGY425 (wt)	<i>h-, his3-D1, leu1-32, ura4-D18, ade6-M210</i>	Prof. P. Espenshade (Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, United States)
<i>P. pastoris</i> GS115 (wt)	<i>his4</i>	Life Technologies

namely most radioactivity accumulated into ergosterol, the end product of the pathway. Surprisingly, the lipid composition in spheroplasts was dramatically different, being ergosterol a minor fraction, while most radioactivity accumulated into squalene, a non-cyclic triterpene of the sterol biosynthetic pathway. Results suggested carrying out a careful comparative study of the metabolic fate of post-squalene pathway (Fig. 1) in yeast intact cells and spheroplasts, and to extend the study to other unicellular cell wall-equipped eukaryotes such as *Pichia pastoris* and *Schizosaccharomyces pombe*. The study was completed by using engineered yeast strains altered in regard to the post squalene section of ergosterol biosynthesis. Tracer experiments with radioactive acetate revealed that the main difference between intact cells and spheroplasts, irrespective of the organism used, was the accumulation of non-cyclic precursor of ergosterol, squalene and oxisqualene. The differences observed suggest that a deep metabolic rearrangement might occur within other pathways of the lipid metabolism when cells are deprived of their cell wall.

MATERIALS AND METHODS

Chemicals. Solvents, buffers, culture media and ergosterol were purchased from Sigma-Aldrich (Italy). [2-¹⁴C]acetic acid (2.04 GBq/mmol) was purchased from Perkin Elmer Italia SpA (Italy).

Yeast strains and culture conditions. The strains used in this study are listed in Table 1.

Both *P. pastoris* GS115 and *S. cerevisiae* SCY876 wild-type strains were grown to early stationary phase at 30°C in YPD medium (1%, w/v, yeast extract, 2%, w/v, peptone, 2%, w/v, glucose). The *S. pombe* KGY425 wild-type strain was grown to early stationary phase at 30°C in YD medium (0.5%, w/v, yeast extract, 3%, w/v, glucose) supplemented with 0.225 mg/mL each of histidine, leucine, adenine, lysine and uracil.

S. cerevisiae deletant mutant strains for either *ERG7* (STY2) or *ERG27* (BTY6-5-3) were grown to early stationary phase at 30°C in YPD medium, supplemented with ergosterol (0.02 mg/mL). *S. cerevisiae* K206A-Erg27p and R40A-Erg27p mutant strains were grown at 30°C in synthetic complete medium without uracil SC-UraD (0.17%, w/v, yeast nitrogen base, 0.2%, w/v, amino acids, 0.5%, w/v, ammonium sulphate, 2%, w/v, glucose), supplemented with ergosterol (0.02 mg/mL). STY2,

BTY6-5-3, K206A-Erg27p and R40A-Erg27p strains were also able to grow under aerobic conditions with sterol supplementation because they contain the allele *upc2-1*, that allows aerobic sterol uptake (Wilcox *et al.*, 2002).

Incorporation of [2-¹⁴C]acetate into the non-saponifiable lipids of whole yeast cells. The incorporation of [2-¹⁴C]acetate in whole yeast cells was performed as described previously (Balliano *et al.*, 1988). Briefly, washed cells (1 × 10⁸ cells) were resuspended in 1 mL of fresh media and incubated with 0.1 μCi of [2-¹⁴C]acetate in presence of Tween-80 (0.2 mg/mL). Cells were shaken for 3 h at 30°C, then saponified in 1 mL of methanolic KOH (15%, w/v) for 30 min at 80°C. Non-saponifiable lipids were extracted twice with 1.5 mL of petroleum ether and separated on silica gel TLC plates (20 × 20 cm × 0.25 mm) (Macherey-Nagel, Germany), using cyclohexane/ethyl acetate (85:15, v/v). Squalene, oxidosqualene, dioxidosqualene, lanosterol and ergosterol were used for reference. Radioactivity in separated bands was determined using a System 200 Imaging Scanner (Hewlett-Packard, Palo Alto, CA, USA).

Chemical reduction of radioactive non-saponifiable lipids of the *S. cerevisiae* K206A-Erg27p mutant strain with NaBH₄. The ketonic nature of the compounds was established by treatment with NaBH₄ (Sigma-Aldrich, Italy) (Taramino *et al.*, 2010a). An aliquot of petroleum extract was then incubated with 5 mg of NaBH₄ in 1 mL of ethanol for 30 min with magnetic stirring. The reaction was stopped by adding 1 mL of water, and the reaction mixture was extracted twice with 1 mL of petroleum ether and separated on silica gel TLC plates (20 × 20 cm × 0.25 mm) (Macherey-Nagel, Germany), using cyclohexane/ethyl acetate (85:15, v/v).

Incorporation of [2-¹⁴C]acetate into the non-saponifiable lipids of spheroplast yeast cells. The incorporation of [2-¹⁴C]acetate in spheroplasts was performed as described previously (Oliaro-Bosso *et al.*, 2004). Briefly, the spheroplasts (3 × 10⁸ cells), obtained by lysing the cell walls with lyticase (Sigma-Aldrich, Italy) (Daum *et al.*, 1982), were resuspended in 2.5 mL of 20 mM phosphate buffer, pH 7.4, containing 1.2 M sorbitol, 1% (w/v) glucose, and incubated, in presence of Tween-80 (0.2 mg/mL), with 0.25 μCi of [2-¹⁴C]acetate. Spheroplasts were shaken for 2 h at 30°C, then saponified in 1 mL of methanolic KOH (15%, w/v) for 30 min at 80°C. The extraction and analysis of non-saponifiable lipids were performed as previously reported.

Table 2. [^{14}C]acetate incorporation into the non-saponifiable lipid fraction of yeast wild-type strains, *S. cerevisiae* *ERG7* and *ERG27* deletant strains, *S. cerevisiae* *ERG27* mutant strains.

Strain	% total radioactivity incorporated									
	S	OS	4-MMS	DOS	4,4-DMS	X	L	4-MM Sterols	E	OS/ DOS
<i>S. cerevisiae</i> SCY876	18.59	*	–	1.31	–	–	9.93	4.01	*	10.21
whole cell	21.96	13.38	–	0.28	–	–	8.48	2.79	52.78	193.03
spheroplast		54.05							12.44	
<i>S. pombe</i> KGY425	22.42	*	–	1.09	–	–	13.75	2.77	*	11.64
whole cell	17.33	12.69	–	0.75	–	–	10.23	3.05	47.28	81.89
spheroplast		61.42							7.22	
<i>P. pastoris</i> GS115	2.92	*	–	0.71	–	–	2.01	4.35	*	5.80
whole cell	4.01	4.12	–	0.69	–	–	3.42	2.40	85.89	88.92
spheroplast		61.38							28.10	
STY2 (<i>erg7Δ</i>)	*	*	–	*	–	–	0.36	–	1.17	0.26
whole cell	0.67	20.12	–	77.68	–	–	0.40	–	1.83	3.77
spheroplast	5.22	73.15	–	19.40	–	–				
BTY6-5-3 (<i>erg27Δ</i>)	*	*	–	*	–	–	0.64	–	1.13	0.25
whole cell	2.02	19.03	–	77.18	–	–	0.91	–	2.23	9.39
spheroplast	8.21	80.12	–	8.53	–	–				
R40A-Erg27p	*	*	40.97	1.01*	22.95	9.71	4.56	4.24	6.96	5.21
whole cell	4.33	5.27	27.54	0.42*	4.39	5.58	5.15	1.91	4.24	27.24
spheroplast	39.33	11.44								
K206A-Erg27p	*	*	30.06	2.22	27.24	11.30	5.65	5.76	7.57	2.78
whole cell	4.02	6.18	34.92	1.13	9.45	7.56	6.12	2.21	4.87	11.69
spheroplast	20.53	13.21								

S: squalene; OS: oxidosqualene; 4-MMS: 4-monomethylsterones (compounds chromatographically corresponding to 4-methylzymosterone); DOS: dioxidosqualene; 4,4-DMS: 4,4-desmethylsterones (compounds chromatographically corresponding to zymosterone); L: lanosterol; 4-MM Sterols: 4-monomethylsterols (TLC bands with Rf values ranging from lanosterol to ergosterol); E: ergosterol; X: compound not identified. *An asterisk indicates a significant difference ($P < 0.05$) between the proportion of radioactivity incorporated into whole cells and spheroplasts (two tailed *t*-test). Whole and spheroplast yeast cells were incubated with [^{14}C]acetate in presence of Tween-80 for 3 and 2 h at 30°C, respectively, then saponified for 30 min at 80°C. Non-saponifiable lipids were extracted with petroleum ether and separated on silica gel TLC plates.

Statistical analysis. Results are the means of at least three experiments, each with duplicate incubations.

Prior to statistical analysis, percentages (%) of incorporation in each product or group of products have been transformed to the arcsin ($^\circ$)^{0.5} in order to stabilize the variance. Transformed data were compared by the one-tailed *t*-test with unequal variances.

RESULTS

To compare sterol biosynthesis in yeast cells and spheroplasts, we traced the distal section of ergosterol biosynthesis by incubating either intact cells or spheroplasts of *S. cerevisiae*, *S. pombe* and *P. pastoris* with radiolabelled acetate. Spheroplasts from *P. pastoris* and *S. pombe* were prepared through a procedure developed for *S. cerevisiae* (Daum *et al.*, 1982). After incubation, cultures containing either cells or spheroplasts were directly saponified, and non-saponifiable lipids were separated by TLC. The distribution of radioactivity among the non-saponifiable lipids was then detected by radioscanning the TLC plates.

Intact cells and spheroplasts from wt strains of *S. cerevisiae*, *S. pombe* and *P. pastoris*

A notable difference between intact cells and spheroplasts, shared by all three organisms, was that in intact cells most radioactivity accumulated into ergosterol, the end product of the pathway, whereas in spheroplasts the non-cyclic squalene-type intermediates kept the bulk (>60%) of the radioactivity of the non-saponifiable fraction. In the spheroplasts, however, the metabolic flow

toward the end product was not completely blocked at the level of non-cyclized precursors, as some radioactive lanosterol and ergosterol were detectable on the TLC plates (Table 2). In spheroplasts from all three organisms, the squalene dioxide was completely absent, a result that deserves attention, being the squalene dioxide the usual partner of the squalene monoxide when the latter accumulates in intact cells (Milla *et al.*, 2002b; Mo *et al.*, 2003).

Intact cells and spheroplasts from *ERG7* and *ERG27* deletant yeast (*S. cerevisiae*) strains

ERG7 and *ERG27* deletant yeast strains share the absence of oxidosqualene cyclase activity, as (i) the *ERG7* deletant strain lacks the enzymatic protein that cyclizes the oxidosqualene, whereas (ii) the *ERG27* deletant strain lacks the steroid-3-ketoreductase (EC 1.1.1.270), a protein belonging to the C4 demethylase complex of sterol biosynthesis that acts as an absolutely-required chaperonine-like protein toward the oxidosqualene cyclase (Mo *et al.*, 2003; Teske *et al.*, 2008). These two strains were included in the study due to their intrinsic propensity to accumulate epoxide derivatives of squalene, a trait similar to that observed in the spheroplasts from wild-type cells. Indeed, intact cells of both strains, incubated with radiolabelled acetate, accumulated radioactive epoxysqualenes, mainly dioxidosqualene (DOS), as expected when oxidosqualene cyclase activity is abolished (Table 2). No significant difference between the two gene-deletant strains was observed. When spheroplasts from both strains were incubated with the radioactive precursor, radioactivity accumulated also into the epox-

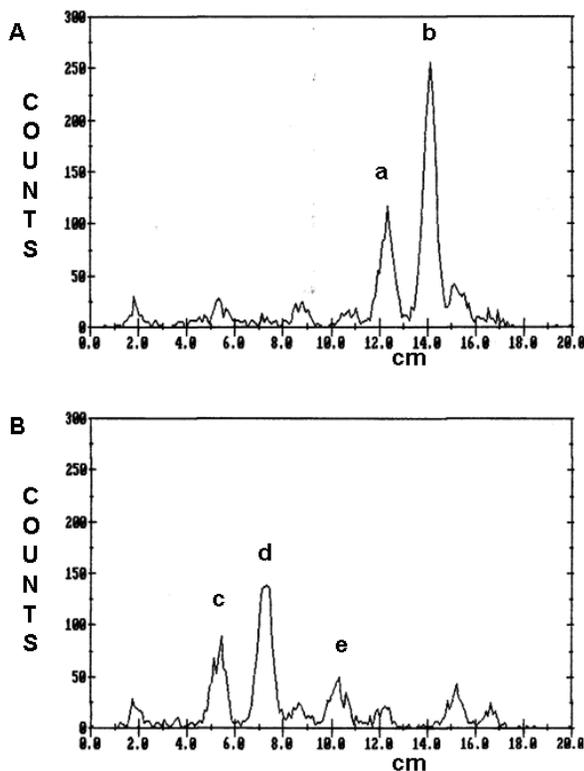


Figure 2. Chemical reduction of non-saponifiable lipid extracted from whole cell of K206A-Erg27p mutant strain. Radiochromatograms A) before reduction, and B) after reduction with NaBH_4 . Peak a: 4,4-desmethylsterone, peak b: 4-monomethylsterone, peak c: β -hydroxy derivative of 4,4-desmethylsterone, peak d: β -hydroxy derivative of 4-monomethylsterone and the α -hydroxy derivative of 4,4-desmethylsterone coeluted, peak e: α -hydroxy derivative of 4-monomethylsterone (Taramino *et al.*, 2010a).

ysqualene fraction, but the mono-/diepoxide ratio appeared dramatically different from intact cells, with the monoepoxide completely predominant over dioxide in spheroplasts.

Intact cells and spheroplasts from R40A-Erg27p and K206A-Erg27p yeast (*S. cerevisiae*) strains

The R40A-Erg27p and K206A-Erg27p belong to a group of engineered yeast strains constructed with the aim of separating the catalytic function of Erg27p from its Erg7p chaperone ability (Teske *et al.*, 2008). Unlike *ERG27* knockout strains, in these point mutation strains, the chaperone ability of Erg27p toward Erg7p is maintained, thus OSC (Erg7p) does work properly, whereas the keto-reductase activity is substantially reduced. In tracer experiments with intact cells of R40A-Erg27p and K206A-Erg27p grown in the presence of radiolabelled acetate, most radioactivity accumulated into two compounds, chromatographically corresponding to 4-methyl zymosterone and zymosterone on TLC plates (Table 2). The 3-keto nature of the compounds was unambiguous, as seen by treating part of the non-saponifiable extract of K206A-Erg27p yeast strain with NaBH_4 before TLC separation; as reported in Fig. 2, the strong reducing agent effectively transformed the original compound into two more polar compounds, presumably the α - and β -hydroxy derivatives (Taramino *et al.*, 2010a). Negligible amounts of other radioactive non-saponifiable lipids,

such as squalene and squalene monoxide were detectable on the TLC plates. In spheroplasts, the distribution of radioactivity among the components of the lipid extract changed abruptly with a dramatic increase of squalene. However, a fair amount of a compound chromatographically corresponding to 4-methyl zymosterone was still present (Table 2).

DISCUSSION

In this report, we are comparing the metabolic flow through the post-squalene section of ergosterol biosynthesis in intact growing cells and spheroplasts from *S. cerevisiae*, *S. pombe* and *P. pastoris* wild-type strains (group 1), as well as from *S. cerevisiae* strains lacking oxidosqualene cyclase (group 2) or steroid-3-keto reductase activity (group 3).

An overall difference that could be observed among the cell lines was that in intact cells the metabolic pathway flows till the end product, whereas in spheroplasts the pathway is detained some step before. Ergosterol was indeed the main radioactive product formed in intact growing cells of different wt yeast strains cultured in the presence of radioactive acetate, whereas in spheroplasts the acyclic intermediates, squalene and oxidosqualene, retained most of the non-saponifiable lipid radioactivity. Apparently, in spheroplasts the sterol biosynthetic flow was kept from entering the complex distal section of the pathway, crowded with steroid-shaped precursors of the end product. Whether this depends on the spheroplasts being cell wall-free or non-proliferating cells (or both) was difficult to establish: indeed, their requirement of ergosterol for new membranes is poor, thus a mechanism of early damping of the metabolic path before the formation of steroid products (hard to address and dispose) may represent a defense strategy. Surprisingly, dioxidosqualene, which usually matches the accumulation of squalene monoepoxide (Nelson *et al.*, 1981; Wong *et al.*, 2008), was absent in spheroplasts from all three wt yeast strains studied. Moreover, in the strains lacking oxidosqualene cyclase activity (group 2), thus endowed with a high propensity to form dioxidosqualene (Milla *et al.*, 2002b; Mo *et al.*, 2003), this compound accumulated exclusively in growing intact cells (till 80% of the non-saponifiable lipids), being poorly present in spheroplasts. No significant difference was observed between cells lacking oxidosqualene cyclase activity due to the *ERG7* gene deficiency and cells in which the protein oxidosqualene cyclase was present but inactive (Milla *et al.*, 2002b). In this kind of cells, DOS may be regarded either as a side product, being a derivative of an intermediate of ergosterol biosynthesis (squalene monoxide), or as an end product of the pathway, with squalene epoxidase being the last functional step of the shortened pathway. In the intact cells, squalene epoxidase seemed to take care of the monoepoxysqualene accumulated in a consequence of the absence of the OSC activity. The unusual "second" epoxidase activity of squalene epoxidase could be attributed either to simple "mass pressure" exerted by the accumulated monoepoxide offering its second epoxidable end, or perhaps to some regulatory role of the enzyme oriented to generate precursors of signaling molecules such as the oxysterols (Beh *et al.*, 2004; Gill *et al.*, 2008). Willing to hazard a guess, the drop in the second epoxidation of squalene monoepoxide in spheroplasts could result from the fading out of such a regulatory machinery in these abnormal cell wall-free non-proliferating cells. In cells from group 3,

the ergosterol biosynthesis resulted strongly depressed at the level of 3-ketosteroid reductase, one of the enzymes of the C4-demethylation apparatus. Point mutations R40A and K206A caused, each, an almost complete reduction of the catalytic activity of the protein (Teske *et al.*, 2008), thus determining in intact cells an accumulation of 3-ketosteroids, mainly C4-methyl zymosterone, which therefore represented the end product of the altered pathway. As in wild type cells, also in spheroplasts from these mutants incubated with radioactive acetate, the metabolic flow was hampered at the boundary between cyclized and non-cyclized precursors of ergosterol, resulting from the accumulation of squalene along with methyl zymosterone. Notably, this accumulation was not accompanied by the epoxidation of squalene, as though squalene epoxidase were poorly active in a spheroplast environment.

CONCLUSIONS

The comparison of the late steps of ergosterol biosynthesis in intact cells and spheroplasts from different yeast strains revealed sharp differences between the two cellular systems, suggesting the occurrence of a complex control over the post-squalene section of the pathway. To our knowledge, this is the first comparison of a metabolic pathway in intact cells and in spheroplasts, and much additional experimental work should be done to explain the observed differences, mainly to establish whether they depend on the absence of the cell wall or on the non-proliferating status of the spheroplasts. We are confident that the extension of the comparison to other lipid pathways will bring interesting surprises, contributing to the unraveling of the complex lipid metabolism network.

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