Osmotic Lysis of Sphaeroplasts from *Saccharomyces cerevisiae* Grown Anaerobically in Media Containing Different Unsaturated Fatty Acids

By F. ALTERTHUM* AND A. H. ROSE

*Microbiology Laboratories, School of Biological Sciences,
Bath University, Bath*

(Received 5 March 1973)

SUMMARY

Saccharomyces cerevisiae NCYC 366 was grown anaerobically under conditions that induce a requirement for a sterol and an unsaturated fatty acid. In media containing ergosterol and either oleic acid, linoleic or γ -linolenic acid, organisms grew at about the same rate, although the duration of the lag phase of growth was extended as the degree of unsaturation in the exogenous fatty acid was increased. Organisms grown in each of the three media did not differ in their contents of total lipids or total phospholipids. Between 54 and 65% of the fatty-acid residues in lipids extracted from organisms were the same as the fatty acid supplied in the medium. Organisms grown in linoleic acid-containing medium were less susceptible to sphaeroplast formation, by digestion of the wall with a basidiomycete glucanase, than were organisms grown in the presence of oleic acid. Sphaeroplasts could be obtained from organisms grown in the presence of linolenic acid if spermine was included in the glucanase digest. Sphaeroplasts formed in the presence of spermine from organisms grown in oleic acid-containing medium were less susceptible to osmotic lysis than sphaeroplasts formed from these organisms in the absence of spermine. The effect of spermine was less pronounced with organisms grown in media containing linoleic acid. The inclusion of spermine in the hypotonic solutions of sorbitol did not affect the kinetics of lysis of sphaeroplasts from organisms grown in medium containing oleic acid or linoleic acid.

INTRODUCTION

Much useful information on relationships between lipid composition and physiological activity in biological membranes has come from studies on fatty acid-requiring micro-organisms, the membrane composition of which can be modified by altering the nature of the exogenously provided fatty acid. A nutritional requirement for a fatty acid is not common among micro-organisms. The majority of studies have been done with mycoplasmas (reviewed by Razin, 1973), and these have furnished information on the role of unsaturation in fatty-acid residues in membrane fragility (Razin, Tourtellotte, McElhaney & Pollack, 1966). Fatty acid-requiring mutants of several other microbial species have been isolated and, since many of these species are more amenable to biochemical and genetical manipulation than are mycoplasmas, these mutants will in future probably be more widely used. Considerable interest has been shown in fatty acid-requiring mutants of haploid strains of *Saccharomyces cerevisiae*. Two classes of mutant have been reported. Representatives of the first class were isolated by Resnick & Mortimer (1966). These are Δ^9 desaturase mutants,

* Present address: Instituto de Química da Universidade de São Paulo, Departamento de Bioquímica, Caixa Postal 20.780, São Paulo, S.P. Brazil.

Fig. 1. Culture apparatus used for anaerobic cultivation of *Saccharomyces cerevisiae* NCYC 366. See text for details.

and require an unsaturated fatty acid for growth; the specificity of the fatty-acid requirement depends on the strain (Wisniewski & Kiyomoto, 1972). A second series of fatty acid-requiring mutants of *Sacch. cerevisiae* have been described by Schweizer & Bolling (1970), Schweizer, Kühn & Castorph (1971) and Henry & Keith (1971). These are chain-elongation mutants, and have a requirement for a saturated fatty acid. Both types of mutant have been used to study the effect of fatty-acid composition on membrane behaviour (Eletr & Keith, 1972).

In this paper, we describe the use of another method for effecting specific alterations in the fatty-acid composition of membranes in *Saccharomyces cerevisiae*. When this organism is grown anaerobically, a nutritional requirement is induced for an unsaturated fatty acid and for a sterol (Andreasen & Stier, 1954). The anaerobically induced requirements for both the unsaturated fatty acid (Light, Lennarz & Bloch, 1962) and the sterol (Proudlock, Wheeldon, Jollow & Linnane, 1968) are relatively non-specific and, providing that the incorporated fatty acid or sterol is not modified metabolically, the method provides potentially a valuable technique for studying composition-function relationships in yeast membranes. The anaerobic technique has an advantage over the use of fatty acid-requiring mutants of yeast in that the fatty-acid and sterol composition of yeast membranes can be varied simultaneously. In this paper, we report on the incorporation of oleic, linoleic or γ -linolenic acid by anaerobically grown *Sacch. cerevisiae* NCYC 366, and the effect of these fatty-acid substitutions on the susceptibility of sphaeroplasts to osmotic lysis. Certain of these data were communicated at the 3rd International Symposium on Yeast Protoplasts held in October 1972 in Salamanca, Spain.

METHODS

Organism. The organism used was *Saccharomyces cerevisiae* NCYC 366, a strain which can readily be converted into sphaeroplasts (Eddy & Rudin, 1958). The yeast was maintained on slopes of glucose-salts-vitamins medium (Diamond & Rose, 1970).

Experimental cultures. The yeast was grown anaerobically under conditions which induce a nutritional requirement for a sterol and an unsaturated fatty acid (Andreasen & Stier, 1954). The medium contained per l: glucose, 100 g; $(\text{NH}_4)_2\text{SO}_4$, 3.0 g; KH_2PO_4 , 3.0 g; yeast extract (Oxoid), 2.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 25.0 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 25.0 mg; ergosterol, 5.0 mg; and 30.0 mg of the unsaturated fatty acid indicated. The anaerobically induced nutrients were added as solutions in 95% (v/v) ethanol (2.5 ml each of 2.0 mg ergosterol/ml and 30 mg fatty acid/ml). One-litre portions of medium were dispensed into 2 l round, flat-bottomed Pyrex flasks each fitted with a fermentation lock containing water, and with a latex-rubber port on the side (Fig. 1). Medium was sterilized momentarily at 110 °C. Whilst still warm, the flask was placed in an incubator water-bath as described by Patching & Rose (1969) and the medium sparged with white-spot nitrogen gas (Air Products, Ltd) that had been freed from traces of oxygen with a Nilox scrubber (Jencons Ltd, Hemel Hempstead, Hertfordshire). Each anaerobic culture or set of cultures was incubated together with a control culture the medium for which was not supplemented with an unsaturated fatty acid. After at least 2 h sparging with nitrogen gas, each batch of medium was inoculated through the port with a portion of inoculum culture containing 1.0 mg dry wt equiv. Inocula were taken from cultures prepared by inoculating 100 ml portions of sterol- and fatty acid-free medium in a 250 ml conical flask with organisms from a slope culture. These cultures were incubated statically overnight at 30 °C, after which time the density was about 1.0 mg dry wt equiv./ml. Anaerobic cultures were incubated at 30 °C and sparged with nitrogen gas at the rate of 100 ml/min. Growth was followed by removing portions of the culture with a hypodermic syringe through the port, and measuring the absorbance of the culture in a Hilger Spekker absorptiometer with neutral green-grey (H 508) filters and a medium blank. Absorptiometer readings were related to dry weight. Cultures were harvested at a density of 0.22 to 0.24 mg dry wt equiv./ml. If the density of the control cultures exceeded 0.04 mg dry wt equiv./ml, all cultures were rejected because this indicated possible contamination of the anaerobic culture with oxygen. When cultures reached the required density, 2 ml of a solution containing chloramphenicol (10 mg) and Actidione (10 mg) were added through the port. The culture was then incubated and sparged with nitrogen gas for 15 min when the organisms were harvested by centrifuging for 5 min at 12250g at 4 °C. Organisms that were to be analysed for lipid content or composition were washed twice with water at 4 °C, freeze dried, and stored at -20 °C over silica gel.

Preparation of sphaeroplasts. Organisms that were to be converted into sphaeroplasts were washed twice in citrate-phosphate buffer (pH 6.0) containing sorbitol (1 M) and MgCl_2 (10 mM), and resuspended at 20 mg dry wt equiv./ml in buffered sorbitol- MgCl_2 solution supplemented with basidiomycete glucanase at the concentration stated. The suspension (usually 10 ml in a 100 ml conical flask) was incubated at 30 °C in a shaker-water bath at 100 oscillations/min. Sphaeroplast formation was followed by adding 0.1 ml portions of suspension to 2.9 ml water or buffered sorbitol- MgCl_2 solution, gently shaking the diluted suspension, leaving at room temperature (18 to 22 °C) for 10 min, then measuring the absorbance ($E_{1\text{cm}}^{600}$) of the suspension in a Unicam SP 600 spectrophotometer. Sphaeroplast formation was judged to be complete when the absorbance of the diluted suspension was about one-quarter of that at zero time, and remained at this value after a further 10 min incubation of the glucanase-containing digest. Completion of sphaeroplast formation was confirmed by microscopic examination. Sphaeroplasts were removed from the suspension by centrifuging at room temperature at 1500g for 10 min. They were then gently washed with buffered sorbitol- MgCl_2 solution.

Exo- β -(1-3) glucanase was purified from culture filtrates of basidiomycete QM 806 (Reese &

Mandels, 1959) by a method based on that of Huotari, Nelson, Smith & Kirkwood (1958) and Bastide, Bastide, Bonte, Travé & Torralba (1972) modified in this laboratory by Drs G. E. Wheeler and K. Hunter. A culture of the basidiomycete (kindly provided by Dr E. T. Reese) was maintained on slopes of malt-wort agar; cultures on solid medium were grown at 30 °C. The medium for growing the mould contained (per litre of water): starch, 10 g; $(\text{NH}_4)_2\text{SO}_4$, 1.4 g; KH_2PO_4 , 1.9 g; Tween 80, 0.5 g; urea, 0.3 g; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g; FeSO_4 , 10 µg; MnSO_4 , 5 µg; ZnSO_4 , 5 µg; yeast extract (Oxoid), 0.1 g. One-litre batches of medium (pH 5.5) were dispensed into 2-l round, flat-bottomed flasks and sterilized at 115 °C for 10 min. Sterile portions of medium were inoculated with a suspension of basidiomycete mycelium obtained by scraping mycelium off the surface of fully grown slope cultures (10 ml solidified medium in a 25 ml screw-capped bottle) into 0.1 M- KH_2PO_4 (pH 4.5). The suspension was decanted from the slope and shaken to disperse the mycelium. A portion of suspension, containing approximately one half of the quantity of mycelium from one slope culture, was added to each portion of medium. Streptomycin (0.1 ml of a solution containing 200 µg/ml) was added to each culture. Cultures were incubated for 7 to 9 days as described by Patching & Rose (1969). Initially, the cultures appeared milky but, as the starch was utilized by the mould, they assumed a yellowish colour. Glucanase activity in culture filtrates was monitored after 5 days of incubation, laminarin being used as substrate. The reaction mixture (0.9 ml) contained 0.0556 % (w/v) laminarin and 0.27 % (w/v) gelatin (Oxoid) in citrate-phosphate buffer (0.15 M; pH 4.8), and was supplemented with 0.1 ml culture filtrate. Reaction mixtures were incubated at 37 °C for 15 min, and the reaction stopped by placing the tubes in a bath of boiling water for 5 min. After cooling, the reaction mixture was centrifuged and the concentration of glucose in the supernatant liquid determined by the glucose oxidase assay (Boehringer Corporation, London). One unit of activity is equal to that amount of glucanase which liberates 1 µmol glucose/min. Basidiomycete cultures were harvested when the activity of the culture filtrate attained a value of about 35 units/ml. After harvesting, the culture filtrate was freeze dried. The material was then dissolved in water, the solution dialysed against water for 36 h, centrifuged, and the insoluble material discarded. Solid ammonium sulphate was added to the solution to 25 % (w/v) saturation, the solution centrifuged, and the precipitate discarded. Additional ammonium sulphate was then added to the supernatant liquid to 45 % (w/v) saturation, the solution centrifuged, and the precipitate retained. The precipitate was dissolved in water, and the solution dialysed against water overnight. The glucanase activity of the solution was assayed, and the solution freeze dried. The activity of the freeze-dried material was about 130 units/mg protein, and varied slightly with the batch. The enzyme was dissolved in water for preparing sphaeroplasts.

Osmotic lysis measurements. The susceptibility of sphaeroplasts to osmotic lysis was measured at 20 °C by adding 0.1 portions of sphaeroplast suspension (containing 10 mg dry wt equiv./ml) to 2.9 ml portions of citrate-phosphate buffer (50 mM; pH 6.0) containing MgCl_2 (10 mM) and sorbitol at concentrations ranging from 1.0 to 0.1 M. After adding the sphaeroplast suspension, the contents of each tube were gently shaken, maintained at room temperature for 10 min, and the absorbance ($E_{1\text{cm}}^{600}$) of the suspension measured.

Extraction and analysis of lipids. Lipids were extracted from organisms, and the total lipid content of the extract determined, as described by Hunter & Rose (1972). Total phospholipids were determined by assaying the phosphorus content of the lipid extract using the method of Chen, Toribara & Warner (1956). Phosphorus contents were multiplied by a factor of 25 to give a value for the total phospholipid content. The fatty-acid composition of the lipid extracts was determined by gas-liquid chromatography (g.l.c.) of the methyl esters of the fatty acids. Fatty-acid methyl esters were prepared by evaporating a portion of lipid

Fig. 2. Time-course of anaerobic growth of *Saccharomyces cerevisiae* NCYC 366 in medium containing oleic acid (○), linoleic acid (▲) or γ -linolenic acid (●).

extract to dryness and dissolving the residue in benzene (0.5 ml) and 5.0% (v/v) methanolic HCl (4.0 ml) (Stoffel, Chu & Ahrens, 1959). The solution was refluxed for 2 h on a steam bath, the condenser being fitted with a guard tube containing anhydrous CaCl_2 . After cooling, water (10 ml) was added to the solution, and the methyl esters extracted with diethyl ether (3×10 ml). The ether extracts were dried over anhydrous Na_2SO_4 for 1 h and concentrated. Methyl esters were separated by g.l.c. with a Pye 104 model and a flame-ionization detector. Stainless-steel columns (5 ft $\times$ 0.25 in) were packed with 15% EG SS-X supported on Gas Chrom P (100 to 200 mesh) at 175 °C and a nitrogen gas flow of 50 ml/min. The detector was maintained at 200 °C. Samples (1 μ l) were injected into the column as solutions in chloroform. Peak areas were measured, and the relative amounts of fatty-acid methyl esters calculated by the method of Carrol (1961).

Chemicals. Oleic acid, linoleic acid and γ -linolenic acid were supplied by Sigma Chemical Co. (London) Ltd. All three acids were shown to be 99% pure by gas chromatography. Standard mixtures of fatty-acid methyl esters, and 15% EG SS-X on Gas Chrom P, were obtained from Applied Science, State College, Pennsylvania, U.S.A. Silica gel for thin-layer chromatography was supplied by Merck. Chloroform, methanol and ethanol were re-distilled before use. All other chemicals used were AnalaR or of the highest purity available commercially.

RESULTS

Growth and composition of organisms grown anaerobically in media containing different unsaturated fatty acids

The rates of growth of *Saccharomyces cerevisiae* NCYC 366 grown anaerobically in media containing oleic, linoleic or γ -linolenic acid were about the same (Fig. 2). However, the duration of the lag phase of growth was extended by about 4 h when linoleic acid replaced oleic acid, and by about the same period of time when linolenic acid replaced linoleic acid

Table 1. *Fatty-acid composition and total lipid and phospholipid contents of Saccharomyces cerevisiae* NCYC 366 grown anaerobically in media containing oleic, linoleic or γ -linolenic acid.

Fatty acid (%) total	Organisms grown in medium containing		
	Oleic acid	Linoleic acid	Linolenic acid
12:0	2.5 $\pm$ 0.5	1.7 $\pm$ 0.4	2.3 $\pm$ 0.5
13:0	1.8 $\pm$ 0.4	1.1 $\pm$ 0.3	1.2 $\pm$ 0.4
14:0	3.6 $\pm$ 0.2	4.8 $\pm$ 0.3	8.5 $\pm$ 1.2
14:1	tr	1.5 $\pm$ 0.8	—
16:0	18.5 $\pm$ 0.7	24.2 $\pm$ 0.6	22.8 $\pm$ 1.5
16:1	4.0 $\pm$ 0.8	2.1 $\pm$ 0.4	2.4 $\pm$ 0.2
18:0	2.3 $\pm$ 0.2	2.7 $\pm$ 0.4	5.7 $\pm$ 0.9
18:1	65.1 $\pm$ 1.1	5.2 $\pm$ 1.0	1.3 $\pm$ 0.4
18:2	1.6 $\pm$ 0.4	56.7 $\pm$ 0.9	1.9 $\pm$ 0.6
18:3	—	—	53.9 $\pm$ 2.9
Total lipid (mg/100 mg dry wt equiv.)	10.8 $\pm$ 0.3	9.8 $\pm$ 0.4	10.9 $\pm$ 0.4
Total phospholipid (mg/100 mg dry wt equiv.)	2.7 $\pm$ 0.1	2.8 $\pm$ 0.0	2.8 $\pm$ 0.1

Values quoted are $\pm$ standard errors of the mean of at least four separate determinations. tr, trace.

(Fig. 2). Organisms grown in media containing any one of these unsaturated fatty acids did not differ in size or shape.

Varying the fatty-acid composition of media did not cause changes in the contents of total lipids or total phospholipids in organisms (Table 1). The fatty-acid composition of lipids extracted from organisms varies with the nature of the fatty acid in the medium (Table 1), the major fatty-acid residue being that included in the medium. The extent of enrichment with the exogenously provided acid ranged from 65 % of the total fatty acid in organisms grown in oleic acid-containing media to 54 % in organisms grown in the presence of linolenic acid.

Actidione and chloramphenicol were added to anaerobic cultures before organisms were harvested to minimize synthesis of aerobically induced enzymes during harvesting. Since these antibiotics were removed from suspensions when the organisms were washed, it was conceivable that, when the organisms were incubated aerobically in the glucanase-containing digest to form sphaeroplasts, the fatty-acid composition of the yeast lipids changed. Parallel studies on *Saccharomyces cerevisiae* NCYC 366 grown anaerobically in media containing Tween 80 to supply unsaturated fatty acid (Wheeler & Rose, 1973) and one of several different sterols (Hossack, Wheeler & Rose, 1973) have shown that the extent of enrichment of the yeast lipids with exogenously provided sterol decreases by about 10 % when organisms are incubated for 20 min in buffered sorbitol-MgCl₂ conditions that simulate those used in sphaeroplast formation (J. A. Hossack & A. H. Rose, unpublished observations). When organisms that had been grown anaerobically in medium containing ergosterol and oleic acid were incubated under similar conditions, the fatty-acid composition of the lipids extracted from the organisms was not changed. The ability of the organisms to modify their sterol composition, but not their fatty-acid composition, when incubated under aerobic conditions may be explained by the ability of the organisms to accumulate significant quantities of squalene, the long-chain sterol precursor which is cyclized to form lanosterol in a reaction that requires molecular oxygen. Precursors of fatty-acid synthesis are not accumulated during growth under anaerobic conditions.

Fig. 3. Time-course of formation of sphaeroplasts from *Saccharomyces cerevisiae* NCYC 366, grown anaerobically in medium containing oleic acid (○), linoleic acid (▲) or γ -linolenic acid (●), by means of a digestion mixture containing 0.7 mg glucanase protein/ml. Details of the cultural methods and of the method for preparing sphaeroplasts are described in Methods. The dotted line indicates the change in absorbance of a suspension of linolenic acid-grown organisms in buffered sorbitol-MgCl₂ lacking glucanase. Suspensions of other organisms in glucanase-free buffered sorbitol-MgCl₂ behaved in an almost identical manner.

Formation of sphaeroplasts

Organisms grown in media containing different fatty acids vary in their susceptibility to sphaeroplast formation. In a digestion mixture containing 0.7 mg glucanase protein/ml, a concentration which is optimal for complete conversion of a population of aerobically grown *Saccharomyces cerevisiae* NCYC 366 into sphaeroplasts (Wheeler & Rose, 1973), organisms grown anaerobically in oleic acid-containing medium were readily converted into sphaeroplasts in about 30 min (Fig. 3). In the presence of this concentration of glucanase, formation of sphaeroplasts from organisms grown in linoleic acid-containing medium was incomplete even after 60 min of incubation (Fig. 3). Also under these conditions, very few organisms grown in linolenic acid-containing medium were converted into osmotically sensitive forms (Fig. 3). By increasing the concentration of glucanase protein to 1.75 mg/ml, it was possible to obtain complete conversion to sphaeroplasts of a population of organisms grown in linoleic acid-containing medium in about 40 min. However, even in digestion mixtures containing 3.5 mg glucanase protein/ml, it was not possible to prepare sphaeroplasts from organisms grown in linolenic acid-containing medium during 60 min of incubation, which we consider to be the maximum permissible incubation period.

It seemed possible that our failure to produce sphaeroplasts from organisms grown in medium containing linolenic acid may have been a result of rupture of the extremely fluid plasma membrane when the sphaeroplast emerged following wall digestion. Microscopic examination of suspensions of organisms grown in linolenic acid-containing medium and

Fig. 4. Effect of sorbitol concentration on lysis of sphaeroplasts from *Saccharomyces cerevisiae* NCYC 366 grown anaerobically in medium containing oleic acid (a), linoleic acid (b) or linolenic acid (c). —, behaviour of sphaeroplasts formed in glucanase digests lacking spermine; ----, behaviour of sphaeroplasts formed in glucanase digests supplemented with 10 mM-spermine. Glucanase digests contained the following concentrations of glucanase (mg/ml): a (0.7); b (1.75); c (2.8).

incubated in a digestion mixture containing 3.5 mg glucanase protein/ml showed that intact sphaeroplasts were formed but that they rapidly lysed. Cations are known to stabilize bacterial protoplasts (Mager, 1959; Tabor, 1962; Harold, 1964) and an examination was therefore made of the effect of including cations in the glucanase-containing digestion mixture. Including Ca^{2+} or increasing the concentration of Mg^{2+} did not permit formation of sphaeroplasts from organisms grown in medium containing linolenic acid. However, when spermine (10 mM) was included in a digestion mixture containing 2.8 mg glucanase protein/ml, sphaeroplasts were formed from organisms grown in linolenic acid-containing medium in 40 to 60 min. The presence of spermine (10 mM) in the digestion mixture did not alter the time-course pattern of sphaeroplast formation from organisms grown in medium containing oleic or linoleic acid.

Osmotic lysis of sphaeroplasts

Sphaeroplasts from organisms grown in media containing different fatty acids varied in their sensitivity to osmotic lysis (Fig. 4a, b, c). Those formed from organisms grown in medium containing linoleic acid were more easily lysed in hypotonic solutions of sorbitol than sphaeroplasts from organisms grown in the presence of oleic acid (Fig. 4a, b). Lysis in hypotonic solutions of sorbitol of sphaeroplasts formed (in the presence of spermine) from organisms grown in linolenic acid-containing medium followed a similar course to that of sphaeroplasts formed in the absence of spermine from linoleic acid-grown organisms (Fig. 4b, c). Including spermine (10 mM) in the glucanase digest conferred some measure of protection on sphaeroplasts against osmotic lysis, the effect being greater with sphaeroplasts from organisms grown in the presence of oleic acid than those from organisms grown in linoleic acid-containing medium (Fig. 4a, b). The possibility that this effect was caused by retention of spermine on the surface of the sphaeroplast was dismissed after the discovery that including spermine (10 mM) in buffered sorbitol solutions had no effect on the kinetics of lysis of sphaeroplasts formed from organisms grown in medium containing any of the three fatty acids.

DISCUSSION

Our data on the composition of organisms grown in each of the three fatty acid-containing media show that exploiting the anaerobically induced requirement for an unsaturated acid constitutes a valuable technique for effecting specific alterations in the fatty-acid composition of yeast lipids providing care is taken to prevent, or at least minimize, changes in lipid composition following exposure of the anaerobically grown organisms to oxygen. The degree of enrichment of the lipids of *Saccharomyces cerevisiae* with the fatty acid supplied in the medium was similar to the values reported by Eletr & Keith (1972) with an aerobically grown fatty acid-requiring mutant of *Sacch. cerevisiae*. Nevertheless, the extent of enrichment with the exogenously supplied fatty acid is lower with *Sacch. cerevisiae*, grown anaerobically or aerobically, than with fatty acid-requiring strains of *Acholeplasma laidlawii* (68 to 75 %; McElhaney & Tourtellotte, 1969) or with fatty-acid auxotrophs of *Escherichia coli* (60 to 70 %; Silbert, Ruch & Vagelos, 1968). This lower level of enrichment may be associated with a need by *Sacch. cerevisiae* to accommodate a greater proportion of saturated fatty acids in membranes for optimal membrane function.

Alterations to the fatty-acid composition of membrane lipids in *Acholeplasma laidlawii* cause changes in the morphology of the organisms. An increase in the content of oleic-acid residues induces the mycoplasmas to grow as long branching filaments whereas, when the membranes contain a predominance of palmitic acid and stearic acid residues, filament formation is not observed (Razin, Cosenza & Tourtellotte, 1966; Razin, Tourtellotte, McElhaney & Pollack, 1966). Variations in the susceptibility of *Saccharomyces cerevisiae* NCYC 366 for conversion into sphaeroplasts after growth in the presence of different fatty acids might be attributed to alterations in the activities of wall-synthesizing enzymes in the yeast plasma membrane. Since our data show differences in the susceptibility of the walls to digestion with a β -glucanase, one possibility is that walls contain different amounts of, or a differently structured, β -glucan (Phaff, 1971). Alternatively, the walls may differ in their architecture, so that accessibility of the β -glucanase to the wall glucan is affected. Synthesis of one of the major wall polysaccharides in *Sacch. cerevisiae*, namely the α -linked mannan, involves the action of a lipid intermediate (isoprenoid alcohol) and of an isoprenoid alcohol kinase (Sentandreu & Lampen, 1971). The activity of an isoprenoid alcohol kinase in *Staphylococcus aureus* is influenced by the degree of unsaturation in the bacterial plasma membrane, the activity being greater the lower the proportion of unsaturated fatty-acid residues in the membrane (Higashi & Strominger, 1970; Higashi, Strominger & Sweeley, 1970). If activity of the yeast plasma-membrane isoprenoid alcohol kinase is similarly affected by lipid unsaturation, an increase in the proportion of unsaturation in the plasma-membrane phospholipid might lead to a diminished synthesis of mannan which in turn could be accompanied by an increase in the proportion of β -glucan in the wall. This speculative explanation for the differences in the susceptibility of *Sacch. cerevisiae* NCYC 366 to the action of β -glucanase could be examined by analysis of walls from the organisms. Another possibility is that the protein composition of the yeast plasma membrane may be modified as a result of alterations in lipid composition. This effect was found in *Mycoplasma* sp. by Pisetsky & Terry (1972) who reported that the content of one major protein was considerably greater in membranes enriched in saturated fatty-acid residues.

Introduction of unsaturation into the fatty-acyl chains of phospholipids perturbs the close packing of the chains. Studies on monolayers of pure phospholipids show that the area occupied by each molecule increases as additional unsaturation is introduced into the acyl chains (Demel, Geurts van Kessel & van Deenen, 1972). Moreover, the permeability of

lecithin-containing liposomes to a variety of solutes increases as the degree of unsaturation in the fatty-acyl chains of the lecithin is increased (Demel, Geurts van Kessel & van Deenen, 1972), presumably as a result of the increased mobility of the chains that results from a less tight packing. The most plausible explanation of the increased susceptibility of sphaeroplasts from *Saccharomyces cerevisiae* NCYC 366 to osmotic lysis as the degree of unsaturation in the plasma-membrane lipids is raised is that the greater mobility of the fatty-acyl chains in the membrane phospholipids restricts the capacity of the membrane to stretch in hypotonic solutions of sorbitol. Monolayer studies have also shown that mixing a sterol with a phospholipid reduces the area occupied by each molecule of the phospholipid (Demel, van Deenen & Pethica, 1967; Demel, Bruckdorfer & van Deenen, 1972), and it is suggested that sterols interact with unsaturated fatty-acyl chains on phospholipids and cause them to pack more closely. It would be interesting to incorporate sterols other than ergosterol into the lipids of *Sacch. cerevisiae* NCYC 366 and to ascertain whether changes in the susceptibility of the sphaeroplasts to osmotic lysis, with a given fatty acid supplied, can be correlated with a change in the magnitude of the condensing effect with monolayers. Demel, Geurts van Kessel & van Deenen (1972) reported that the condensing effect of sterols varies with the structure of the side chain on the sterol molecule.

Working with protoplasts of *Bacillus megaterium*, Corner & Marquis (1969) concluded that osmotic lysis of protoplasts results not from brittle fracture of the plasma membrane but as a result of pores being formed in the membrane as it stretched. Solute molecules enter these pores and cause lysis of the protoplast. It is conceivable that a similar explanation holds for osmotic lysis of yeast sphaeroplasts. As additional unsaturation is introduced into the fatty-acyl chains of the plasma-membrane phospholipids, the less tight packing of the chains could most easily give rise to the formation of pores, which might explain the different susceptibility of osmotic lysis observed in our experiments.

The polyamine, spermine, binds a variety of acidic compounds (Bachrach, 1970). The protective effect of spermine on sphaeroplasts emerging from *Saccharomyces cerevisiae* NCYC 366 grown in the presence of linolenic acid is probably explained by the capacity of the polyamine to bind to negatively charged groups on the surface of the yeast plasma membrane. Much more difficult to explain is the diminished susceptibility to osmotic lysis observed in sphaeroplasts formed in glucanase digests containing spermine. One possibility is that, during sphaeroplast formation in the absence of the polyamine, there is a loss from the membrane of phospholipids or other membrane components, a loss which is prevented when spermine is present. Evidence is not available in support or otherwise of this suggestion. But if lipids are lost from sphaeroplast membranes in this manner, it would cast serious doubts on the justification for considering sphaeroplasts to be physiologically identical with the structure which lies beneath the cell wall in micro-organisms.

The research described in this paper was supported by a grant from the Science Research Council (B/RG/182). One of us (F. A.) is grateful to the Fundação de Amparo a Pesquisa do Estado de São Paulo for provision of a fellowship. We also acknowledge the competent technical assistance of Mrs Zena Newman, and the kind gift of basidiomycete QM 806 from Dr E. T. Reese of the Department of the Army, Natick, Massachusetts, U.S.A. R. I. Illingworth gave timely assistance with analysis of the yeast lipids.

REFERENCES

- ANDREASEN, A. A. & STIER, T. J. B. (1954). Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. *Journal of Cellular and Comparative Physiology* **43**, 271–281.
- BACHRACH, U. (1970). Metabolism and function of spermine and related polyamines. *Annual Review of Microbiology* **24**, 109–134.
- BASTIDE, J.-M., BASTIDE, M., BONTE, M., TRAVÉ, P. & TORRALBA, PR. A. (1972). Étude de la culture du basidiomycete QM 806 en fermenteur: Production d'une exo- β -(1-3)-D-glucanase. *Travaux de la Société de Pharmacie de Montpellier* **32**, 93–106.
- CARROL, K. K. (1961). Quantitative estimation of peak areas in gas-liquid chromatography. *Nature, London* **191**, 377–381.
- CHEN, P. S., TORIBARA, T. Y. & WARNER, H. (1956). Microdetermination of phosphorus. *Analytical Chemistry* **28**, 1756–1758.
- CORNER, T. R. & MARQUIS, R. E. (1969). Why do bacterial protoplasts burst in hypotonic solution? *Biochimica et biophysica acta* **183**, 544–558.
- DEMEL, R. A., VAN DEENEN, L. L. M. & PETHICA, B. A. (1967). Monolayer interaction of phospholipids and cholesterol. *Biochimica et biophysica acta* **135**, 11–19.
- DEMEL, R. A., BRUCKDORFER, K. R. & VAN DEENEN, L. L. M. (1972). Structural requirements of sterols for the interaction with lecithin at the air-water interface. *Biochimica et biophysica acta* **255**, 311–320.
- DEMEL, R. A., GEURTS VAN KESSEL, W. S. M. & VAN DEENEN, L. L. M. (1972). The properties of polyunsaturated lecithin in monolayers and liposomes and the interactions of these lecithins with cholesterol. *Biochimica et biophysica acta* **255**, 26–40.
- DIAMOND, R. J. & ROSE, A. H. (1970). Osmotic properties of sphaeroplasts from *Saccharomyces cerevisiae* grown at different temperatures. *Journal of Bacteriology* **102**, 311–319.
- EDDY, A. A. & RUDIN, A. D. (1958). The structure of the yeast cell wall. II. Degradative studies with enzymes. *Proceedings of the Royal Society, B* **149**, 419–432.
- ELETR, S. & KEITH, A. D. (1972). Spin-label studies of dynamics of lipid alkyl chains in biological membranes; role of unsaturated sites. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 1353–1357.
- HAROLD, F. M. (1964). Stabilisation of *Streptococcus faecalis* protoplasts by spermine. *Journal of Bacteriology* **88**, 1416–1420.
- HENRY, S. A. & KEITH, A. D. (1971). Membrane properties of saturated fatty acid mutants of yeast revealed by spin labels. *Chemistry and Physics of Lipids* **7**, 245–265.
- HIGASHI, Y. & STROMINGER, J. L. (1970). Biosynthesis of the peptidoglycan of bacterial cell walls. XX. Identification of phosphatidylglycerol and cardiolipin as cofactors for isoprenoid alcohol phosphokinase. *Journal of Biological Chemistry* **245**, 3691–3696.
- HIGASHI, Y., STROMINGER, J. L. & SWELEY, C. C. (1970). Biosynthesis of the peptidoglycan of bacterial cell walls. XXI. Isolation of free C₅₅-isoprenoid alcohol and of lipid intermediates in peptidoglycan synthesis from *Staphylococcus aureus*. *Journal of Biological Chemistry* **245**, 3697–3702.
- HOSSACK, J. A., WHEELER, G. E. & ROSE, A. H. (1973). Environmentally induced changes in the lipid composition of cells and membranes of *Saccharomyces cerevisiae*. *Proceedings of the 3rd International Symposium on Yeast Protoplasts* (in the press).
- HUNTER, K. & ROSE, A. H. (1972). Lipid composition of *Saccharomyces cerevisiae* as influenced by growth temperature. *Biochimica et biophysica acta* **260**, 639–663.
- HUOTARI, F. I., NELSON, T. E., SMITH, F. & KIRKWOOD, S. (1958). Purification of an exo-B-(1-3) glucanase from basidiomycete species QM 806. *Journal of Biological Chemistry* **243**, 952–956.
- LIGHT, R. J., LENNARZ, W. J. & BLOCH, K. (1962). The metabolism of hydroxystearic acids in yeast. *Journal of Biological Chemistry* **237**, 1793–1800.
- McELHANEY, R. N. & TOURTELLOTE, M. E. (1969). Mycoplasma membrane lipids: Variations in fatty acid composition. *Science, New York* **164**, 433–434.
- MAGER, J. (1959). The stabilising effect of spermine and related polyamines on bacterial protoplasts. *Biochimica et biophysica acta* **36**, 529–531.
- PATCHING, J. W. & ROSE, A. H. (1969). The effects and control of temperature. In *Methods in Microbiology*, vol. 2, pp. 23–28. Edited by J. R. Norris and D. W. Ribbons, London: Academic Press.
- PHAFF, H. J. (1971). Structure and biosynthesis of the yeast cell envelope. In *The Yeasts*, vol. 2, pp. 135–210. Edited by A. H. Rose and J. S. Harrison. London: Academic Press.

- PISETSKY, D. & TERRY, T. M. (1972). Are mycoplasma membrane proteins affected by variations in membrane fatty acid composition? *Biochimica et biophysica acta* **274**, 95-104.
- PROUDLOCK, J. W., WHEELDON, L. W., JOLLOW, D. J. & LINNANE, A. W. (1968). Role of sterols in *Saccharomyces cerevisiae*. *Biochimica et biophysica acta* **152**, 434-437.
- RAZIN, S. (1973). Physiology of mycoplasmas. *Advances in Microbial Physiology* **10**, 1-80.
- RAZIN, S., COSENZA, B. J. & TOURTELLOTTE, M. E. (1966). Variations in mycoplasma morphology induced by long-chain fatty acids. *Journal of General Microbiology* **42**, 139-145.
- RAZIN, S., TOURTELLOTTE, M. E., MCELHANEY, R. N. & POLLACK, J. D. (1966). Influence of lipid components of *Mycoplasma* membranes on osmotic fragility of cells. *Journal of Bacteriology* **91**, 609-616.
- REESE, E. T. & MANDELS, M. (1959). β (1-3) Glucanases in fungi. *Canadian Journal of Microbiology* **5**, 173-185.
- RESNICK, M. A. & MORTIMER, R. K. (1966). Unsaturated fatty acid mutants of *Saccharomyces cerevisiae*. *Journal of Bacteriology* **92**, 597-600.
- SCHWEIZER, E. & BOLLING, H. (1970). A *Saccharomyces cerevisiae* mutant defective in saturated fatty acid biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **67**, 660-666.
- SCHWEIZER, E., KÜHN, L. & CASTORPH, H. (1971). A new gene cluster in yeast: The fatty acid synthetase system. *Hoppe-Seyler's Zeitschrift für physiologische Chemie* **352**, 377-384.
- SENTANDREU, R. & LAMPEN, J. O. (1971). Participation of a lipid intermediate in the biosynthesis of *Saccharomyces cerevisiae* LK2G12 mannan. *Federation of European Biochemical Societies Letters* **14**, 109-113.
- SILBERT, D. F., RUCH, F. & VAGELOS, P. R. (1968). Fatty acid replacements in a fatty acid auxotroph of *Escherichia coli*. *Journal of Bacteriology* **95**, 1658-1665.
- STOFFEL, W., CHU, F. & AHRENS, E. H. (1959). Analysis of long chain fatty acids by gas-liquid chromatography. Micromethod for preparation of methyl esters. *Analytical Chemistry* **31**, 1101-1111.
- TABOR, C. W. (1962). Stabilization of protoplasts and sphaeroplasts by spermine. *Journal of Bacteriology* **83**, 1101-1111.
- WHEELER, G. E. & ROSE, A. H. (1973). Location and properties of an esterase activity in *Saccharomyces cerevisiae*. *Journal of General Microbiology* **74**, 189-192.
- WISNIESKI, B. J. & KIYOMOTO, R. Y. (1972). Fatty acid desaturase mutants of yeast: growth requirements and electron spin resonance spin-label distribution. *Journal of Bacteriology* **109**, 186-195.