JOURNAL OF BACTERIOLOGY, May 1970, p. 311-319

Vol. 102, No. 2
Printed in U.S.A.

Osmotic Properties of Spheroplasts from *Saccharomyces cerevisiae* Grown at Different Temperatures

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Received for publication 31 January 1970

Spheroplasts were prepared from cells of *Saccharomyces cerevisiae* NCYC 366, grown at 30 or 15 C, by incubating cells with snail-gut juice after pretreatment with 2-mercaptoethanol. Walls of cells grown batchwise or in continuous culture at 15 C were more resistant to digestion with snail juice than walls on cells grown under the same conditions as 30 C. Spheroplasts lysed when suspended in hypotonic solutions of mannitol. The resistance of spheroplasts to osmotic lysis tended to increase when the test temperature was lowered below 30 C. The increased resistance was greater with spheroplasts from cells grown at 15 C. Cations, especially Ca^{2+}, protected spheroplasts against osmotic lysis. In general, the protective effects, measured at 30 C, were smaller with spheroplasts from cells grown at 15 C compared with 30 C. Citrate and ethylenediaminetetraacetate (EDTA) decreased the resistance of spheroplasts to osmotic lysis. On the whole, the decrease was greater with spheroplasts from cells grown at 30 C rather than 15 C. In the presence of EDTA, spheroplasts from cells grown at 30 C were less resistant to osmotic lysis at 5 C than at 30 C; when spheroplasts from cells grown at 15 C were similarly examined, they were more resistant to lysis at 5 C than at 30 C. Spheroplast membranes from cells grown at 15 C had slightly but significantly greater contents of Mg^{2+}, Ca^{2+}, K^{+}, and Na^{+} compared with spheroplast membranes from cells grown at 15 C. Mg^{2+} and Ca^{2+} were more easily extracted with EDTA from membranes of 30 C-grown cells than from 15 C-grown cells.

Osmotically fragile bodies can be obtained from many strains of yeast by incubating cells with snail-gut juice (10, 17, 21) or with enzymes produced by various microorganisms (16, 26, 38). Analyses of membranes obtained by subjecting these bodies to osmotic lysis show that they contain about 10% carbohydrate which consists of glucan and mannan (22). Although this carbohydrate may represent residual cell wall material, the osmotically fragile bodies are nevertheless usually referred to as protoplasts. Some workers, however, dispute this designation and prefer to recognize them as spheroplasts (38).

Microbial protoplasts furnish an ideal system with which to examine the effect of chemical composition on the behavior and function of membranes, for, by growing microorganisms under different environmental conditions, it is possible to vary the composition of the protoplast membrane and also probably of other membranes in eucaryotic microorganisms. In this respect, microbial protoplasts offer a decided advantage over erythrocytes on which the majority of studies on membrane structure and function have been done. In this Laboratory, we have examined the effect of growth temperature on several aspects of the physiology of microorganisms (11, 12), particularly on the composition and function of the spheroplast membrane (13, 14). The present paper describes the effect of growth temperature-induced changes in the composition of the spheroplast membrane of a strain of *Saccharomyces cerevisiae* on the osmotic properties of spheroplasts obtained from the cells.

MATERIALS AND METHODS

Organism. The organism used in this work was *S. cerevisiae* NCYC 366, a strain which can readily be converted into spheroplasts (9). The yeast was maintained on slopes of malt wort-agar as described by Dixon and Rose (7), or of glucose-salts-vitamins medium supplemented with 2% (w/v) agar.

Batch cultures. One-liter portions of a glucose-salts-vitamins medium (*p*H 4.5; reference 30) supplemented with D-biotin (0.195 pg per liter) were dispensed into 2-liter round, flat-bottomed flasks which were then covered with glass caps and sterilized at

115 C for 10 min. Portions of medium were inoculated with the equivalent of 10 mg (dry weight) of cells, either as a portion from a logarithmic-phase culture grown in glucose-salts-vitamins medium at 30 C in a shaker incubator or as a suspension in 67 mM KH_2PO_4 of cells harvested from a slope culture. Stirred cultures were incubated at the stated temperature as described by Stanley and Rose (35). Cells were harvested by centrifuging mid-exponential-phase cultures (0.20 to 0.25 mg, dry weight, per ml) at 2,000 × *g*. They were washed three times with 67 mM KH_2PO_4 and suspended in citrate-phosphate buffer (5 mM; *p*H 5.8; reference 8) containing 0.8 M mannitol and 10 mM $MgCl_2$ to a concentration of about 10 mg (dry weight) per ml before being converted into spheroplasts.

Continuous cultures. Chemostat cultures of the yeast were grown with stirring in a glass vessel (2-liter working volume; Taylor-Rustless Fittings Co. Ltd., Leeds, England) fitted with a device for controlling the dissolved oxygen tension in the culture as described by Maclennan and Pirt (24) and by Brown and Rose (2). The basal medium had the composition given by Rose and Nickerson (30), except that the concentrations were: 2.0 g of glucose per liter, 2 g of $(NH_4)_2SO_4$ per liter, and 1.0 g of KH_2PO_4 per liter. This medium supported growth under conditions of glucose limitation. Cultures were grown at 30 or 15 C, and at rates of 0.2, 0.1, or 0.05/hr. The *p*H value of cultures was 4.5, and the dissolved oxygen tension was 84 mm of Hg.

Measurement of growth. Routine measurements of cell density were made by determining the optical density (OD) of culture in a Hilger Spekker absorptiometer (model H 760) with neutral green-grey filters and a water blank; OD readings were related to dry weight of cells by a calibration curve. Concentrations of cells in culture were also determined by filtering portions through weighed membrane filters (Metricel, type GA-3, pore size 1.2 μm; Gelman Instrument Co. Ltd., Ann Arbor, Mich.), washing the cells on the filter with two 5-ml portions of water at room temperature, and drying the filters and cells to constant weight at 90 C.

Preparation of spheroplasts. Spheroplasts were prepared by incubating cells with snail-gut juice. The commercial juice, supplied in 1-ml ampoules, was diluted with an equal volume of water, and centrifuged at 25,000 × *g* at 0 C for 20 min. The supernatant liquid, with a protein content of 35 to 45 mg/ml (corresponding to 100 units of β-glucuronidase and 800 units of sulfatase per ml), depending on the batch, was supplemented with one-tenth of its volume of 1% (w/v) cysteine hydrochloride to inactivate the preservative added to the commercial preparation (37). Before mixing with dilute snail juice, suspensions of cells in citrate-phosphate buffer containing 0.8 M mannitol and 10 mM 2-mercaptoethanol were shaken at 150 rev/min for 15 min at 37 C. Pretreatment with 2-mercaptoethanol has been shown by Davies and Elvin (Biochem. J. **93**: 8P–9P) to increase the rate of spheroplast formation from yeast cells. The cells were removed by centrifugation at 1,000 × *g*, washed twice in 0.8 M mannitol containing 10 mM $MgCl_2$, and suspended in citrate-phosphate buffer containing 1.0 M mannitol and 10 mM $MgCl_2$ to a concentration of 10 mg (dry weight) per ml. Suspensions were then supplemented with one-fifth volume of snail juice to give the required protein concentrations, and incubated at 37 C in a shaker incubator (150 rev/min). A control suspension was supplemented with buffered mannitol instead of snail juice. To follow the progress of spheroplast formation, a sample (0.1 ml) of suspension was added to 2.9 ml of water, shaken, and left at room temperature (18 to 20 C) for 10 min before the absorbance was measured at 660 nm in a Unicam SP 500 spectrophotometer by using 1-cm cuvettes. A decrease in absorbance indicated the formation of osmotically fragile bodies in the suspension. Spheroplast formation was also followed by examining samples under the phase-contrast microscope and was shown to be complete when the absorbance of the diluted sample remained constant. Spheroplasts were washed twice with 0.8 M mannitol containing 10 mM $MgCl_2$, care being taken to suspend the spheroplasts gently. Spheroplasts suspended in 0.8 M mannitol-10 mM $MgCl_2$ (about 10 mg, dry weight, per ml) remained stable, as judged by their response to osmotic stress, for at least 3 days when stored at 4 C.

Osmotic lysis measurements. Buffer containing mannitol and, where indicated, other compounds at the concentrations stated, was dispensed in 5-ml portions in test tubes and preincubated at the stated temperature. A portion (0.1 ml) of stock suspension of spheroplasts was added to each tube, the contents were immediately shaken, and the suspensions were incubated at the stated temperature. Under each of the conditions used, lysis was complete after 5 min of incubation. The absorbance of each suspension was measured at 660 nm by using a 1-cm cuvette in a Unicam SP 500 spectrophotometer fitted with a constant-temperature cuvette housing through which was circulated water from a refrigerated water bath (Grant Instruments Ltd., Cambridge, England; model LB 4). The temperature of the cuvette contents was monitored by inserting a hypodermic thermistor along the inside of the cuvette and coupling the thermistor to an automatic chart recorder (Rustrak Instrument Co. Ltd., Manchester, N.H.; reference 28).

Preparation of spheroplast membranes. Spheroplasts (10 mg, dry weight, per ml) suspended in 0.8 M mannitol containing 10 mM $MgCl_2$ were squirted into 20 volumes of ice-cold phosphate buffer (pH 4.5) containing 10 mM $MgCl_2$, and the suspension was centrifuged at 1,500 × *g* for 30 min at 3 C. The supernatant liquid was removed, and the membranes were suspended with gentle stirring in 10 mM $MgCl_2$ (3 C). The membranes were washed twice with 10 mM $MgCl_2$ at 3 C and suspended in 10 mM $MgCl_2$ to the desired concentration. The concentration of membranes in the suspension was determined by filtering portions through Metricel filters, washing, and drying to constant weight at 90 C. Routine determinations were also made on freshly prepared suspensions by measuring the absorbance of the membrane suspension and relating this to dry weight of membrane by a calibration curve. Suspensions of membranes in 10 mM $MgCl_2$ were stored for up to 24 hr at 3 C.

Analytical methods. Contents of Mg^{2+} and Ca^{2+} in

membranes and washings were determined by atomic absorption spectrophotometry. Portions (20 mg, dry weight) of membrane were washed carefully three times with glass-distilled water and suspended in 0.3 ml of a 1:1 mixture of 3 M trichloroacetic acid and glacial acetic acid (34). The suspension was placed in a boiling-water bath for 3 to 5 min, supplemented with 3 ml of glass-distilled water, and again placed in a bath of boiling water. After cooling to room temperature, 0.1 ml of a solution containing 6 mg each of $CaCl_2$ and $SrCl_2$ was added to eliminate interference from phosphates and divalent ions. The suspension was then made up to 5 ml, centrifuged at 3,500 $\times$ *g* for 15 min at room temperature to remove protein, and the divalent cation content of the supernatant liquid was determined by using a model 140 EEL atomic absorption spectrophotometer with an air-acetylene flame. Determinations of Mg^{2+} were made by using the 285.2-nm line, and of a Ca^{2+} by using the 422.7-nm line.

Contents of Na^+ in membranes and washings were also determined by atomic absorption spectrophotometry by using an air-acetylene flame. Portions (20 mg, dry weight) of membrane were carefully washed with solutions described in the Results section and suspended in 10 ml of 5% (w/v) perchloric acid (39); the suspension was boiled until it was water-clear. Some samples gave a straw-colored solution due to the presence of carbon, and these were cleared by adding one to two drops of 10% (v/v) hydrogen peroxide solution and heating until the color disappeared. The solution was allowed to cool, supplemented with 0.1 ml of a solution containing 3 mg each of $LaCl_3$ and $SrCl_2$ per ml, and the Na^+ content of the solution was determined at 589 nm by using the model 140 EEL atomic absorption spectrophotometer. Potassium contents of membranes and washings were determined by using an EEL flame photometer with a coal gas-air flame. Membranes were treated exactly as for determination of Na^+ content by atomic absorption spectrophotometry. DNA contents of cells were determined by the method of Burton (3), with herring-sperm DNA as a standard, and RNA contents were determined by the method of Schneider (31), with acid-hydrolyzed yeast RNA as a standard. Protein contents of cells were determined by the method of Lowry et al. (23) with bovine plasma albumin as a standard. Ninhydrin-positive compounds in suspending liquids were determined by the Yemm and Cocking method (40) with DL-leucine as a standard.

Chemicals. All chemicals used were reagent grade or of the highest purity available commercially. Herring-sperm DNA, yeast RNA, and bovine plasma albumin were supplied by Sigma Chemical Co., London. Snail-gut juice, the digestive juice of *Helix pomatia*, was supplied by L'Industrie Biologique Francaise, S.A., Gennevilliers, Seine, France.

RESULTS

Effect of growth temperature on susceptibility of cell walls to digestion with snail juice. The temperature at which cells were grown in batch culture affected the susceptibility of their walls to digestion with snail juice. Cells grown at 30 C were rapidly converted into spheroplasts in reaction mixtures containing about 20 mg of snail-juice protein per ml (Fig. 1). When the concentration of snail-juice protein in the reaction mixture was decreased to about 2 mg/ml, the rate of spheroplast formation was slightly lowered. Pretreatment of cells with 2-mercaptoethanol increased the rate of spheroplast formation in all reaction mixtures. When the growth temperature was decreased below 30 C, cells became increasingly resistant to digestion with snail juice (Fig. 1). In reaction mixtures containing 20 mg of snail-juice protein per ml, about 3 hr of incubation at 37 C was required to convert cells grown at 10 C completely into spheroplasts,

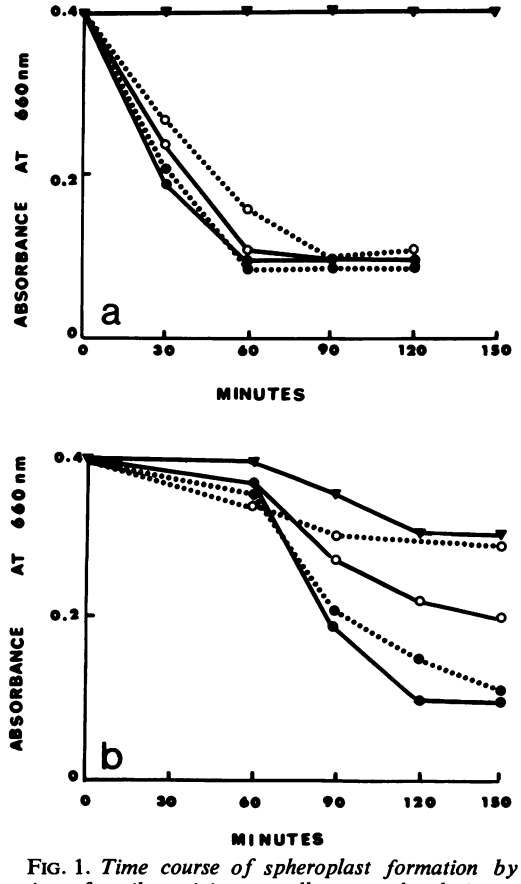

FIG. 1. *Time course of spheroplast formation by action of snail-gut juice on cells grown batchwise at 30 C (a) or 15 C (b).* ●, *Formation of spheroplasts in suspensions containing 20 mg of snail-juice protein per ml;* ○, *formation in suspensions containing 2 mg of snail-juice protein per ml; solid line, behavior of cells pretreated with 2-mercaptoethanol; dotted line, behavior of cells not pretreated;* ▼, *behavior of cells in suspensions not supplemented with snail-gut juice.*

even after pretreatment with 2-mercaptoethanol. There was no significant decrease in the absorbance of suspensions obtained by diluting samples from reaction mixtures containing cells grown at 30 C but lacking snail juice. However, when comparable experiments were done with cells grown at temperatures below 30 C, there was a decrease in absorbance of the diluted sample which was greater the lower the temperature at which the cells had been grown in the range 30 to 10 C (Fig. 1). The decrease in absorbance was not due to autolysis of cells in the hypertonic mannitol solution since only small amounts of ninhydrin-positive compounds and of compounds that absorb at 260 and 280 nm were released into the suspending liquid; also, there was only a very small decrease in the contents of cell protein and RNA, and no effect on the DNA contents of cells. That the decrease in absorbance was probably due to the formation of osmotically fragile bodies, presumably as a result of limited autolysis (27), was indicated by experiments in which dilution of samples of the suspension into 0.8 M mannitol was shown not to cause a decrease in absorbance. The increased autolysis in cells grown at low temperatures may be due to an increased synthesis of autolytic enzymes, to production of a wall that is more susceptible to digestion by these enzymes, or to both.

The increased resistance of cell walls to digestion with snail juice as the growth temperature was lowered below 30 C is probably due to growth temperature-induced changes in cell-wall composition and structure. These changes may be a direct result of the lowering of the growth temperature or a result of the decrease in growth rate that accompanies a lowering of the growth temperature in batch cultures (11). To decide between these possibilities, cells were grown in a chemostat in which it was possible to maintain the *p*H value, nutrient concentration, and dissolved oxygen tension constant, and to vary the growth temperature and growth rate independently of each other. Cells grown continuously at 30 C at different dilution rates (equal to growth rates) differed little in their susceptibility to spheroplast formation; moreover, these cells behaved very similarly to cells grown batchwise at 30 C. However, when the growth temperature was decreased to 15 C, the susceptibility of cells grown continuously at a fixed rate was less than those grown at the same rate at 30 C.

Susceptibility of spheroplasts to osmotic lysis. When suspensions of spheroplasts in 0.8 M mannitol-10 mM $MgCl_2$ were added to hypotonic solutions of mannitol (*p*H 4.5 to 4.8), the absorbance of suspensions decreased over the first 5 min and then remained constant. The decrease in absorbance was greater the lower the concentration of mannitol in the suspending liquid; it was greatest when the spheroplasts were suspended in water (the final concentration of mannitol being 0.016 M; Fig. 2). Lysis curves were sigmoid, and it was necessary to submit the data to probit analysis (15) by which the sigmoid curves were converted into straight lines. Data analyzed in this way (Fig. 2) showed that spheroplasts from cells grown at 30 C were more sensitive to osmotic lysis than those from cells grown at 15 C. Changes in absorbance of suspensions of spheroplasts may result from swelling as well as lysis. That the changes in absorbance observed when spheroplasts were suspended in hypotonic solutions of mannitol were due mainly to lysis was demonstrated by restoring the mannitol concentration to 0.8 M in suspensions containing initially 0.2 or 0.6 M mannitol, which caused only a small increase in absorbance. To study the effect of test temperature on osmotic lysis of spheroplasts, dilutions were made into solutions containing different concentrations of mannitol, and the course of lysis followed at temperatures in the range 30 to 1 C. In general, a decrease in test temperature below 30 C led to an increase in the resistance of spheroplasts to osmotic lysis. Change in resistance to lysis is expressed in the form of

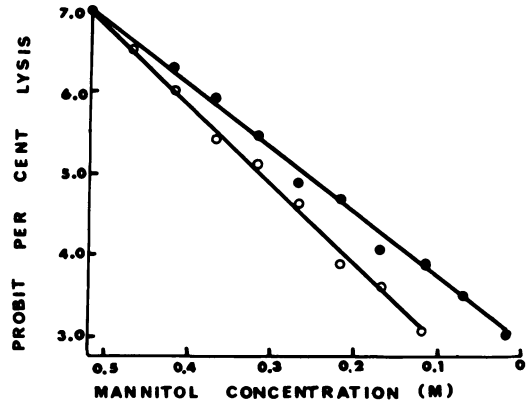

FIG. 2. *Osmotic lysis of spheroplasts from cells grown batchwise at 30 C (○) or 15 C (●). Cells were grown as described in Materials and Methods. Spheroplasts were prepared from cells grown at 30 C by incubating with 2 mg of snail-juice protein per ml for 1 hr, and from cells grown at 15 C by incubating with 20 mg of snail-juice protein per ml for 2 hr. All cells were pretreated with 2-mercaptoethanol. Lysis data were submitted to probit analysis by using the methods of Finney (15). The slope of the line for spheroplasts from 30 C-grown cells was 0.097 ± 0.0023 standard deviation, and for spheroplasts from 15 C-grown cells it was 0.119 ± 0.0025 standard deviation. The slopes of the lines are significantly different at a 5% level. Probit 5.0 indicates 50% lysis.*

osmotic shift or C_m values (19), which are equal to the decrease or increase in concentration of mannitol required to obtain 50% lysis of spheroplasts. An increase in C_m value indicates a greater resistance to lysis in spheroplasts as compared with the control, and a decrease in lowered resistance.

Since the lysis curves were sigmoid, the data were submitted to probit analysis (15). In the range of test temperatures, 30 to about 6 C (Table 1), the increased resistance was greater with spheroplasts from cells grown at 15 C rather than 30 C, but at lower test temperatures (6 to 1 C), there was little difference in the behavior of the two types of spheroplasts. The slopes of the lysis curves obtained after probit analysis differed to some extent, but no consistent pattern was discernible in these variations.

Effect of ions on osmotic lysis of spheroplasts. Incorporation of chlorides of NH_4^+, Mn^{2+}, Cu^{2+}, Al^{3+}, and Fe^{3+} into mannitol solutions had no effect on the resistance of spheroplasts to osmotic lysis, but incorporation of chlorides of alkali and alkaline earth metals increased the resistance of spheroplasts to lysis. The magnitude of the protective effect increased as the concentration was increased up to 2 to 5 mM, depending on the cation, but decreased at higher concentrations. A detailed examination was made of the protective effect of Na^+, K^+, Mg^{2+}, and Ca^{2+}, at 30 C in the concentration range 10^{-2} to 10^{-4} M, on spheroplasts from cells grown at 30 or 15 C. Again, lysis curves were sigmoid, and calculation of C_m values was possible only after the data were submitted to probit analysis (15). These data show that, on the whole, the protective effect was greater with Ca^{2+} than with any of the other cations tested (Table 2). However, the protective effects, measured at 30 C, of each of the cations were often less with spheroplasts from cells grown at 15 C compared with 30 C. The protective effects of cations were also measured at 5 C instead of 30 C (Table 2), but no consistent pattern of effects was discernible from the data. Incorporation of alkali and alkaline-earth chlorides into mannitol solutions had little if any effect on the *p*H value of the solution.

A decrease in the resistance of spheroplasts to osmotic lysis was obtained when either citrate or ethylenediaminetetraacetate (EDTA) was incorporated into mannitol solutions at concentrations in the range 50 to 0.1 mM (Table 3). The decrease in resistance (expressed as C_m values)

TABLE 1. *Effect of test temperature on osmotic lysis of spheroplasts from cells grown at 30 or 15 C*[a]

Growth temp	Osmotic shift value[b] (mM mannitol) at test temp (C)								
	30	20	15	10	8	6	4	2	1
C									
30	0	3	−11	10	6	35	85	74	83
15	0	10	25	43	40	53	58	61	75

[a] Spheroplasts were prepared from cells grown at 30 C by incubating with 2 mg of snail-juice protein per ml for 1 hr, and from cells grown at 15 C by incubating with 20 mg of snail-juice protein per ml for 2 hr. All cells were pretreated with 2-mercaptoethanol.

[b] Osmotic shift values are equal to the change in mannitol concentration required to obtain 50% lysis of spheroplasts at the stated temperature as compared with 30 C. A positive value indicates that the spheroplasts were more resistant at the test temperature than at 30 C; a negative value indicates that they were less resistant. The values quoted are means from triplicate determinations on at least two different batches of spheroplasts. Standard errors of the mean of these values are in the range ±9.2 to 9.7 mM.

TABLE 2. *Effect of cation concentration on osmotic lysis of spheroplasts from cells grown at 30 or 15 C*[a]

Concn of cation[c]	Test temp	Osmotic shift value[b] (mM mannitol)							
		Growth temp with Na^+		Growth temp with K^+		Growth temp with Mg^{2+}		Growth temp with Ca^{2+}	
		30 C	15 C	30 C	15 C	30 C	15 C	30 C	15 C
mM	C								
1	30	37	24	49	27	22	34	51	14
	5	63	35	29	32	0.5	21	12	17
5	30	57	37	87	33	22	57	60	21
	5	24	40	41	42	58	39	71	15
10	30	6	27	71	19	17	36	50	21
	5	27	30	45	30	70	37	42	10

[a] Spheroplasts were prepared from cells grown at 30 C by incubating with 2 mg of snail-juice protein per ml for 1 hr, and from cells grown at 15 C by incubating with 20 mg of snail-juice protein per ml for 2 hr. All cells were pretreated with 2-mercaptoethanol.

[b] Osmotic shift values are equal to the decrease in mannitol concentration required to obtain 50% lysis of spheroplasts in the presence of the stated concentration of cation compared with the value in suspensions lacking the cation. The greater the value, the greater the resistance of the spheroplasts to lysis. The values shown are the means of triplicate determinations on at least two different batches of spheroplasts. The standard errors of the mean of these values are in the range ±9.1 to 9.8 mM.

[c] Cations were incorporated in the form of chlorides.

TABLE 3. *Effect of concentration of EDTA and citrate on osmotic lysis of spheroplasts from cells grown at 30 or 15 C*[a]

Concn of anion	Test temp	Osmotic shift value[b] (mM mannitol)			
		Growth temp with EDTA[c]		Growth temp with citrate[d]	
		30 C	15 C	30 C	15 C
mM	*C*				
1	30	49	31	62	37
	5	57	17	3	11
5	30	111	54	93	67
	5	181	27	143	20
10	30	132	60	112	72
	5	175	32	142	40
50	30	157	72	120	81
	5	171	54	147	56

[a] Spheroplasts were prepared from cells grown at 30 C by incubating with 2 mg of snail-juice protein per ml for 1 hr, and from cells grown at 15 C by incubating with 20 mg of snail-juice protein per ml for 2 hr. All cells were pretreated with 2-mercaptoethanol.

[b] Osmotic shift values are equal to the increase in mannitol concentration required to obtain 50% lysis of spheroplasts in the presence of the stated concentration of anion compared with the value in the absence of the anion. The greater the value the smaller the resistance of the spheroplast to lysis. The values quoted are the means of triplicate determinations on at least two different batches of spheroplasts. The standard errors of the mean of these values are in the range 9.4 to 9.7 mM.

[c] Incorporated in the form of the disodium salt.

[d] Incorporated in the form of the trisodium salt.

was greater with spheroplasts from cells grown at 30 C than with cells grown at 15 C. When spheroplasts from cells grown at 30 C were lysed at 5 C instead of 30 C in the presence of EDTA or citrate, they were generally less resistant to osmotic stress. However, when spheroplasts from cells grown at 15 C were similarly examined, they were more resistant to lysis at 5 C than at 30 C.

Cation contents of spheroplast membranes. Table 4 shows that there is a small difference in the contents of Mg^{2+} and Ca^{2+} in membranes from spheroplasts of cells grown at 30 C compared with 15 C. Moreover, these divalant cations were less easily extracted by EDTA from membranes of spheroplasts from cells grown at 15 C compared with 30 C. Membranes from spheroplasts of cells grown at 15 C contained slightly but significantly larger amounts of Na^+ and K^+ compared with membranes from cells grown at 30 C (Table 5). Repeated washing of the membranes with water removed all of the Na^+ and K^+ but there was no significant difference in the ease of extraction of these ions from membranes of cells grown at 30 C compared with 15 C. Washing with 10 mM $MgCl_2$ also extracted Na^+ and K^+ from membranes, although Na^+ was more easily extracted than K^+ from both types of membrane. Washing with 1 mM NaCl led to a small increase in the Na^+ content of membranes, and washing with 2 mM KCl had a similar effect on the K^+ content. However, membranes from spheroplasts of cells grown at 30 C did not behave differently from those from cells grown at 15 C (Table 5).

DISCUSSION

The main alterations in lipid composition caused by lowering the growth temperature of microorganisms are an increased synthesis of unsaturated fatty acids (12, 20, 25) and, in yeasts, an increased synthesis of phospholipids at the expense of triglycerides (20). It has been confirmed in this Laboratory (K. Hunter and A. H. Rose, *unpublished observations*) that these changes take place when the growth temperature of *S. cerevisiae* NCYC 366 is decreased from 30 to

TABLE 4. *Effect of EDTA concentration on extraction of Mg^{2+} and Ca^{2+} from membranes of spheroplasts from cells grown at 30 or 15 C*[a]

Growth temp	EDTA concn	Amt of Mg^{2+} in membrane[b]	Mg^{2+} extracted	Amt of Ca^{2+} in membrane[b]	Ca^{2+} extracted
C	*M*		%		%
30	0	247 ± 12.6	0	26 ± 1.4	0
	10^{-4}	174 ± 14.7	29.6	10 ± 2.0	61.5
	10^{-3}	168 ± 16.2	32.0	0	100
	2×10^{-3}	171 ± 15.8	30.8	0	100
	5×10^{-3}	152 ± 13.2	38.5	0	100
	10^{-2}	149 ± 16.7	39.7	0	100
15	0	285 ± 16.2	2.0	28 ± 1.8	0
	10^{-4}	278 ± 12.1	2.5	23 ± 1.6	17.9
	10^{-3}	250 ± 12.3	12.3	0	100
	2×10^{-3}	231 ± 12.1	18.9	0	100
	5×10^{-3}	200 ± 12.5	29.8	0	100
	10^{-2}	207 ± 13.1	26.4	0	100

[a] Spheroplasts were prepared from cells grown at 30 C by incubating with 2 mg of snail-juice protein per ml for 1 hr, and from cells grown at 15 C by incubating with 20 mg of snail-juice protein for 2 hr. Membranes were obtained from spheroplasts. Extractions were carried out by suspending membranes (8 mg, dry weight, per ml) for 15 min in solutions of disodium EDTA at the stated concentrations. The suspensions were then centrifuged, the membranes were washed three times with water, and the cation content of the membranes was determined. Neither of the cations could be detected in the third water-washing. The total amounts extracted were equal to the amount lost from the membranes.

[b] The values quoted are the number of micrograms per gram (dry weight) of membrane; they are averages, with standard errors of the mean of triplicate determinations on at least two different batches of membrane.

TABLE 5. *Elution of Na^+ and K^+ ions from spheroplast membranes from cells grown at 30 or 15 C*[a]

Eluent	Treatment	Amt of Na^+ in cells grown at[b]		Amt of K^+ in cells grown at[b]	
		30 C	15 C	30 C	15 C
Water	Before elution	47 ± 5.4	57 ± 6.0	85 ± 3.2	97 ± 5.1
	After one washing	Tr	10 ± 2.3	42 ± 2.8	40 ± 4.2
	After two washings		Tr	21 ± 2.5	17 ± 3.1
	After three washings			Tr	Tr
$MgCl_2$ (10 mM)	Before elution	52 ± 6.4	62 ± 5.3	82 ± 3.7	100 ± 4.2
	After one washing	Tr	Tr	45 ± 4.2	52 ± 5.0
NaCl (1 mM)	Before elution	50 ± 5.7	58 ± 5.7		
	After one washing	56 ± 6.0	65 ± 6.0		
	After two washings	59 ± 6.0	67 ± 6.2		
	After three washings	52 ± 5.8	70 ± 6.3		
KCl (2 mM)	Before elution			80 ± 3.5	103 ± 4.7
	After one washing			49 ± 2.8	49 ± 3.6
	After two washings			42 ± 4.3	52 ± 4.3
	After three washings			39 ± 3.6	46 ± 3.9

[a] Spheroplasts were prepared from cells grown at 30 C by incubating with 2 mg of snail-juice protein per ml for 1 hr, and from cells grown at 15 C by incubating with 20 mg of snail-juice protein per ml for 2 hr. Membranes were obtained from spheroplasts. Membranes (50 mg, dry weight) were washed by suspending in 10 to 15 ml of eluent and then centrifuging at 1,500 × *g* for 10 min at room temperature.

[b] The values quoted are the number of micrograms per gram (dry weight) of membrane; they are averages, with standard errors of the mean of triplicate determinations on at least two different batches of membrane. Tr, trace.

15 C. This decrease in incubation temperature causes an approximately 11% increase in the proportion of unsaturated fatty acids in the cell lipids. It appears, however, that this increased synthesis of unsaturated fatty acids occurs mainly in intracellular lipids, including mitochondrial lipids, and that the increase in the proportion of unsaturation in the lipids of the outer spheroplast membrane is small. The increased synthesis of phospholipids after a decrease in growth temperature from 30 to 15 C amounts to about 20%. The increase is mainly in phosphatidylcholine.

Most of the findings reported in this paper can be explained by the increase in the proportion of phospholipids in spheroplast membranes from cells grown at 15 C instead of 30 C. The possibility that differences in the ion-binding capacities of spheroplast membranes were due to an increased retention of cell-wall material was discounted, since membranes from spheroplasts of cells grown at 30 C contain, at 15 C, approximately the same amounts of anthrone-positive material (22). Several workers, using pure phospholipids or phospholipids in mixed monolayers, have shown that one of the main properties of these lipids is their ability to bind cations, especially divalent cations (18, 29, 32). This would explain the small but significant increase in the contents of Ca^{2+}, Mg^{2+}, K^+, and Na^+ in membranes of spheroplasts from cells grown at 15 C instead of 30 C. The finding that spheroplasts from cells grown at 15 C were more resistant to osmotic lysis compared with those from cells grown at 30 C suggests that the presence of an increased proportion of phospholipids and cations in the membranes may confer this increased resistance. Support for this contention comes from the discovery that spheroplasts from cells grown at 15 C were more resistant to the action of the chelating agents EDTA and citrate compared with those from cells grown at 30 C. Additional support comes from the finding that cations, especially Ca^{2+}, have a greater protective effect on lysis of spheroplasts from cells grown at 30 C than on those from cells grown at 15 C. The fact that cations have this effect suggests that not all of the ion-binding sites on the membrane are occupied in intact spheroplasts. Some ions may have been removed after digestion of the wall during spheroplast formation, a suggestion which implies that the cell wall may have a role in regulating the ionic environment around the outside of the spheroplast membrane in the intact cell.

The manner in which phospholipids, together with cations, confer resistance to osmotic lysis in spheroplast membranes is not known. Intramolecular and intermolecular cross-linking with

divalent ions is known to stabilize macromolecular systems and may therefore be important in biological membranes (33). In membranes containing a high proportion of phospholipids, this type of cross-linking would be more extensive, assuming that a suitable concentration of divalent ions is also present. Since the diameters of the hydrated Ca^{2+} and Mg^{2+} are almost identical, it would be expected that these two ions would have identical effects in membranes. The finding that Ca^{2+} is slightly more effective than Mg^{2+} in protecting spheroplasts against osmotic lysis suggests that the former ion may act, to a limited extent, in a more specific role at certain membrane loci. Cross-linking with divalent ions suggests that a major function for phospholipid-ion complexes in biological membranes may be to confer a measure of extensibility on the membrane. This may explain why some microorganisms (e.g., gram-positive bacteria) contain a greater proportion of phospholipids in their membranes than others, since these organisms have higher internal osmotic pressures and hence may be more susceptible to osmotic lysis during cell-wall extension and growth.

The increased resistance to osmotic lysis of spheroplasts from cells grown at 15 C rather than 30 C, as the test temperature was lowered below 30 C, could be explained by the small increase in the proportion of unsaturated fatty acids in the spheroplast membranes from cells grown at the lower temperature. The presence of an increased proportion of unsaturation in lipids lowers the temperature at which the endothermic phase transition takes place (4, 5, 6). This phase transition is primarily associated with a "melting" of the hydrocarbon chains in the phospholipid, and the greater mobility in these chains in membranes from cells grown at the lower temperature could explain the greater resistance to osmotic lysis at low test temperatures.

ACKNOWLEDGMENTS

This investigation was supported by a grant (B/SR/5724) from the Science Research Council (United Kingdom) for which we express our thanks.

We are extremely grateful to K. Hunter, of this Laboratory, for permission to refer to some of his unpublished data, and to B. J. R. Taylor, of the School of Biological Sciences at Bath University, for assistance with the probit analysis of our data.

LITERATURE CITED

1. Anderson, F. B., and J. W. Millbank. 1966. Protoplast formation and yeast cell-wall structure. Biochem. J. 99:682–686.
2. Brown, C. M., and A. H. Rose. 1969. Fatty-acid composition of *Candida utilis* as affected by growth temperature and dissolved-oxygen tension. J. Bacteriol. 99:371–378.
3. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315–323.
4. Byrne, P., and D. Chapman. 1964. Liquid crystalline nature of phospholipids. Nature (London) 202:987–988.
5. Chapman, D., and D. T. Collin. 1965. Differential thermal analysis of phospholipids. Nature (London) 206:189.
6. Chapman, D., P. Byrne, and G. G. Shipley. 1966. The physical properties of phospholipids. 1. Solid state and mesomorphic properties of some 2,3-diacyl-DL-phosphatidyl ethanolamines. Proc. Roy. Soc. A 290:115–142.
7. Dixon, B., and A. H. Rose. 1964. On the synthesis of ornithine carbamoyltransferase in biotin-deficient *Saccharomyces cerevisiae*. J. Gen. Microbiol. 34:229–240.
8. Eddy, A. A. 1958. The structure of the yeast cell wall. II. Degradative studies with enzymes. Proc. Roy. Soc. B 149:425–440.
9. Eddy, A. A., and A. D. Rudin. 1958. The structure of the yeast cell wall. I. Identification of charged groups at the surface. Proc. Roy. Soc. B 149:419–432.
10. Eddy, A. A., and D. H. Williamson. 1957. A method of isolating protoplasts from yeast. Nature (London) 179: 1252–1253.
11. Farrell, J., and A. H. Rose. 1967. Temperature relationships among microorganisms. p. 147–218. *In* A. H. Rose (ed.), Thermobiology. Academic Press Inc., London.
12. Farrell, J., and A. H. Rose. 1967. Temperature effects on microorganisms. Annu. Rev. Microbiol. 21:101–120.
13. Farrell, J., and A. H. Rose. 1968. Cold shock in a mesophilic and a psychrophilic pseudomonad. J. Gen. Microbiol. 50:429–439.
14. Farrell, J., and A. H. Rose. 1970. Temperature effects on solute accumulation by *Candida utilis*. Arch. Mikrobiol. *in press*.
15. Finney, D. J. 1962. Probit analysis: a statistical treatment of the sigmoid response curve, 2nd ed. Cambridge University Press, London.
16. Garcia Mendoza, C., and J. R. Villaneuva. 1962. Production of yeast protoplasts by an enzyme preparation of *Streptomyces* sp. Nature (London) 195:1326–1327.
17. Giaja, J. 1922. Sur la levure dépouillée de membrane. C. R. Soc. Biol. Paris 86:708–709.
18. Gordon, R. C., and R. A. Macleod, 1966. Mg^{++} phospholipids in cell envelopes of a marine and a terrestrial pseudomonad. Biochim. Biophys. Res. Commun. 24:684–688.
19. Indge, K. J. 1968. The effects of various anions and cations on the lysis of yeast protoplasts by osmotic shock. J. Gen. Microbiol. 51:425–432.
20. Kates, M., and R. M. Baxter. 1962. Lipid composition of mesophilic and psychrophilic yeasts (*Candida* species) as influenced by environmental temperature. Can. J. Biochem. Physiol. 40:1218–1227.
21. Kraut, H., F. Eichhorn, and H. Rubenbauer. 1927. Über eine Darstellung des Hefegummis durch enzymatischen Abbau und über den Nachweis eines hefegummi-spaltenden Enzyms der Hefe. Chem. Ber. 60B:1644–1649.
22. Longley, R. P., A. H. Rose, and B. A. Knights. 1968. Composition of the protoplast membrane from *Saccharomyces cerevisiae*. Biochem. J. 108:401–412.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
24. Maclennan, D. G., and S. J. Pirt. 1966. Automatic control of dissolved oxygen concentration in stirred microbial cultures. J. Gen. Microbiol. 45:289–302.
25. Marr, A. G., and J. L. Ingraham. 1962. Effect of temperature on the composition of fatty acids in *Escherichia coli*. J. Bacteriol. 84:1260–1267.
26. Nagasaki, S., N. P. Neumann, P. Arnow, L. D. Schnable, and J. O. Lampen. 1966. An enzyme which degrades the walls of living yeast. Biochem. Biophys. Res. Commun. 25:158–164.
27. Nečas, O. 1956. Regeneration of yeast cells from naked protoplasts. Nature (London) 177:898–899.
28. Patching, J. W., and A. H. Rose. 1970. *In* J. R. Norris and

D. W. Ribbons (ed.), Methods in microbiology, vol. 2. Academic Press Inc., London, *in press*.

29. Rojas, E., and J. M. Tobias. 1965. Membrane model: association of inorganic cations with phospholipid monolayers. Biochim. Biophys. Acta **94**:394–404.
30. Rose, A. H., and W. J. Nickerson. 1956. Secretion of nicotinic acid by biotin-dependent yeasts. J. Bacteriol. **72**:324–328.
31. Schneider, W. C. 1960. Determination of nucleic acids in tissue by pentose analysis. p. 680–684. *In* S.P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3, Academic Press Inc., New York.
32. Shah, D. O., and J. H. Schulman. 1965. Binding of metal ions to monolayers of lecithins, plasmalogen, cardiolipin and diacetyl phosphate. J. Lipid Res. **6**:341–349.
33. Singer, I., and I. Tasaki. 1968. Nerve excitability and membrane macromolecules. p. 347–410. *In* D. Chapman (ed.), Biological membranes. Academic Press Inc., London.
34. Sparrow, M. P., and B. M. Johnstone. 1964. A rapid method for extraction of Ca and Mg from tissue. Biochim. Biophys. Acta **90**:425–426.
35. Stanley, S. O., and A. H. Rose. 1967. On the clumping of *Corynebacterium xerosis* as affected by temperature. J. Gen. Microbiol. **48**:9–23.
36. Streiblová, E. 1968. Surface structure of yeast protoplasts. J. Bacteriol. **95**:700–707.
37. Sutton, D. D., and J. O. Lampen. 1962. Localization of sucrose and maltose fermenting systems in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **55**:303–312.
38. Tanaka, H., and H. J. Phaff. 1965. Enzymatic hydrolysis of yeast cell walls. 1. Isolation of wall-decomposing organisms and separation and purification of lytic enzymes. J. Bacteriol. **89**:1570–1580.
39. Willis, J. B. 1961. Determination of calcium and magnesium in urine by atomic absorption spectroscopy. Anal. Chem. **33**:556–559.
40. Yemm, E. W., and E. C. Cocking. 1955. The determination of amino-acids with ninhydrin. Analyst **80**:209–213.