Effects of Sodium Chloride on Steady-state Growth and Metabolism of *Saccharomyces cerevisiae*

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SUMMARY

Sodium chloride decreased the maximum specific growth rate of *Saccharomyces cerevisiae*. Chemostat experiments showed this to be largely due to an increased requirement for energy-yielding substrate, apparently linked to maintenance and leading to a decrease in the yield. The increased maintenance requirement is probably concerned with maintaining an intracellular Na⁺ concentration ten times lower than the extracellular concentration. NaCl caused much higher concentrations of glucose to be required to maintain any particular glucose-uptake rate; it also increased the production of glycerol.

INTRODUCTION

Sodium chloride affects many parameters of yeast growth, including growth rate (Norkrans, 1966; Combs, Guarneri & Pisano, 1968), yield of biomass (Ross & Morris, 1962; Norkrans, 1966; Combs *et al.* 1968), lag phase of growth (Phaff, Mrak & Williams, 1952; Ross & Morris, 1962; Norkrans, 1966) and cell composition (Combs *et al.* 1968). The tolerance of various yeast species to NaCl varies greatly (Phaff *et al.* 1952; Ross & Morris, 1962; Norkrans, 1968; van Uden & Buckley, 1970; van Uden & Vidal-Leiria, 1970). The two most important factors appear to be the presence of a mechanism for retaining a low concentration of NaCl within the organism (Norkrans & Kylin, 1969) and the ability of enzymes to operate in the presence of high concentrations of NaCl (Ingram, 1957). The present paper is an attempt to interpret various effects of NaCl on growth of *Saccharomyces cerevisiae* in the chemostat through an analysis of growth kinetics and energetics.

METHODS

Organisms used were *Saccharomyces cerevisiae* IGC 3507 from the culture collection of this laboratory and a respiratory-deficient mutant of it. Experiments were carried out using the mutant unless otherwise indicated in the text. The mutant was chosen to eliminate effects due to variation in respiratory quotient with either change in dilution rate or presence of NaCl and thus to facilitate the computation of yield and maintenance parameters.

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Growth conditions. Cultures were grown in a chemostat at 20° (Watson, 1969). The culture medium was similar to that used by van Uden (1967*a*) except that 1 % (w/v) glucose and in certain experiments 1.0 M-NaCl were used. Under these conditions the cultures were carbon-limited. Samples of culture containing approximately 20 mg. dry wt organisms were filtered through preweighed filters (1.2 μ m. pore size; Millipore Filter Corporation, Bedford, Massachusetts). The filters were washed with three volumes of distilled water and dried to constant weight at 80°.

Determination of glucose and glycerol. Samples of culture were rapidly filtered through a Millipore membrane. Glucose and glycerol in the filtrates were estimated by the glucose oxidase and glycerol kinase methods (Biochemica Test Combinations, Boehringer Mannheim GmbH).

Production of carbon dioxide by cultures was measured using an infrared carbon dioxide analyser (M.S.A. Lira Infrared Analyser Model 300; Mine Safety Appliances Company, Glasgow; Watson, 1969).

Sodium and potassium contents of organisms. A portion of culture containing approximately 40 mg. dry wt of organisms was rapidly filtered and washed with 5 $\times$ 10 ml. of 0.1 M-MgCl₂ (pH 6.0 to 6.5; Tempest, Dicks & Hunter, 1966). The filter was placed in approximately 7 ml. of de-ionized water and boiled for 10 min. The suspension was centrifuged, washed with a small volume of de-ionized water, recentrifuged and the combined extracts made up to 10 ml. with de-ionized water. Analyses of Na⁺ and K⁺ were carried out using an atomic absorption Spectrometer (Perkin-Elmer Model 290, Perkin-Elmer Corp., Norwalk, Connecticut).

RESULTS

Effect of NaCl on yeast growth

In batch cultures of the yeast, the maximum specific growth rate (μ)_{max} and the yield with respect to glucose (Y^a) but not the rate of glucose uptake decreased with 0.25 to 1.50 M-NaCl. An extension in the duration of the lag phase of growth was also observed. Subsequent experiments were carried out in the chemostat under conditions, of glucose limitation.

Pirt (1965) derived the following equation relating yield (Y), maximum yield ($Y_{\max}$), specific uptake rate of energy-yielding substrate specifically for maintenance (k_m), and specific growth rate (μ)

$$\frac{1}{Y} = \frac{1}{Y_{\max}} + \frac{k_m}{\mu}. \quad (1)$$

If $Y_{\max}$ and k_m are invariant with μ , a plot of $1/Y$ against $1/\mu$ will give a straight line with slope k_m and intercept on the 'y' axis of $1/Y_{\max}$. Fig. 1 shows such a plot for chemostat cultures grown in the presence and absence of NaCl. A glucose-limited culture without NaCl showed a small increase in yield with increase in dilution rate as predicted by equation (1). Deviation from linearity occurred at high dilution rates; a k_m value of approximately 0.2 μ mole glucose/mg. dry wt/h. was calculated from the linear part. A glucose-limited culture grown in the presence of 1.0 M-NaCl showed still less linearity in this plot, but a very pronounced decrease was obtained in the yield following a decrease in dilution rate, indicating a high maintenance requirement of approximately 2 μ moles glucose/mg./h.

Effect of NaCl on energy metabolism

As pointed out by van Uden (1968), concepts of maintenance and yield may also be applied to the products of energy metabolism. Fig. 2 shows results for the evolution of CO_2 . The approximate k_m values obtained were, without NaCl, $0.4 \mu\text{mole CO}_2/\text{mg.}/\text{h.}$, and with NaCl, $2.0 \mu\text{moles CO}_2/\text{mg.}/\text{h.}$ Since the ratio of the k_m values gives the direct stoichiometry for the net conversion of energy substrate during maintenance without growth, the ratio $k_m \text{ glucose} : k_m \text{ CO}_2$ of 1:2 obtained for the culture lacking NaCl suggests the conversion of 1 mole of glucose to 2 moles of CO_2 . The stoichiometry for the culture containing NaCl was 1 mole of glucose producing 1 mole of CO_2 and suggested incomplete fermentation of substrate for maintenance, due to the production of end products in addition to CO_2 and ethanol.

Fig. 1

Fig. 2

Fig. 1. Reciprocal plots relating yield (based on glucose consumed) to dilution rate in carbon-limited continuous cultures of a respiratory-deficient mutant of *Saccharomyces cerevisiae* IGC 3507. ●, Data for culture with 1.0 M-NaCl; ○, data for culture without NaCl.

Fig. 2. Reciprocal plots relating yield (based on CO_2 produced) to dilution rate in carbon-limited continuous cultures of a respiratory-deficient mutant of *Saccharomyces cerevisiae* IGC 3507. ●, Data for culture with 1.0 M-NaCl; ○, data for culture without NaCl.

The culture grown without NaCl produced glycerol. *Saccharomyces cerevisiae* grown anaerobically produces glycerol (Nordström, 1966, 1968) probably due to the generation of excess NADH_2 which is removed through the conversion of dihydroxyacetone phosphate to glycerol phosphate. The yield with respect to glycerol production in this culture was constant for most dilution rates studied and suggested a surplus of NADH_2 directly proportional to biomass production (Fig. 3). The culture containing NaCl also produced glycerol. However, the presence of NaCl resulted in a lowering of the yield of organisms with respect to glycerol (Fig. 3). Similar results were found in

batch culture (Table 1). Respiratory-sufficient *S. cerevisiae* grown aerobically does not normally produce glycerol. Glycerol production was, however, induced when the wild-type was grown in the presence of NaCl (Table 1). Similar results have been recorded for other yeasts (Ōnishi, 1963). It would appear, then, that enhanced glycerol production under the influence of NaCl cannot be explained directly in terms of the maintenance of a certain intracellular NAD/NADH₂ ratio.

Fig. 3

Fig. 4

Fig. 3. Reciprocal plots relating yield (based on glycerol produced) to dilution rate in carbon-limited continuous cultures of a respiratory-deficient mutant of *Saccharomyces cerevisiae* IGC 3507. ●, Data for culture with 1.0 M-NaCl; ○, data for culture without NaCl.

Fig. 4. Reciprocal plots relating calculated yield (based on ATP formed) to dilution rate in carbon-limited continuous cultures of a respiratory-deficient mutant of *Saccharomyces cerevisiae* IGC 3507. ●, Data for culture with 1.0 M-NaCl; ○, data for culture without NaCl.

Table 1. *Production of glycerol by wild-type and mutant strains of Saccharomyces cerevisiae* IGC 3507 grown batchwise in media containing sodium chloride

Strain	NaCl concentration in medium (M)						
	0	0.25	0.50	0.75	1.00	1.25	1.50
	Yield (g. dry wt organisms/mole glycerol produced)						
Wild-type strain	> 100,000	64,000	850	330	140	89	55
Mutant strain	66	56	43	34	27	24	18

Organisms grown continuously in the presence of NaCl showed greatly increased production of glycerol per unit of biomass formed as the dilution rate was decreased. The plot of $1/Y$ (glycerol) against $1/D$ was not linear; glycerol production is apparently not a simple function of energy metabolism and variations in $Y_{\max}$ (glycerol) may therefore be expected to occur with dilution rate. The value of k_m with respect to

glycerol was $1 \mu\text{mole/mg./h.}$ (Fig. 3). In the NaCl-free culture there was no production of glycerol linked to maintenance.

Since any deviations from linearity in the plot of $1/Y$ (glycerol) against $1/D$ will cause deviations in the plot for yield based on glucose, and since there was production of glycerol during maintenance in the NaCl-containing culture, a more satisfactory analysis of the results would be obtained in terms of ATP utilized for biomass production (Y^{ATP}) eliminating completely the 'glycerol effect'. Fig. 4 shows a reciprocal plot for Y^{ATP} assuming that each mole of glucose utilized produces 2 moles of ATP, unless it is converted to glycerol when it consumes 2 moles of ATP (or 1 mole of ATP/mole of glycerol formed). The calculated values are probably low with respect to $Y_{\text{max}}^{\text{ATP}}$ as some glucose will certainly not enter the Embden-Meyerhof pathway but may enter biosynthetic pathways. This, however, should not affect the slope of the line as it is reasonable to assume that under carbon-limitation the partial contributions of processes leading to biomass production are not affected too greatly by growth rate. Hence an evaluation of the ATP turnover for maintenance may be obtained and was $0.52 \mu\text{mole of ATP/mg. dry wt/h.}$ for organisms grown without NaCl, and $2.2 \mu\text{moles of ATP/mg./h.,}$ with NaCl. Virtually the same $Y_{\text{max}}^{\text{ATP}}$ of $11 \text{ mg. dry wt organisms/mmole of ATP}$ was obtained for both cultures, suggesting that the effect of NaCl on the ATP yield is solely due to an increased requirement of maintenance energy.

Cation contents of organisms

Organisms grown with and without NaCl contained the same K^+ content. Some penetration of Na^+ occurred in the yeast grown with NaCl (approximately 0.1 M intracellular Na^+ concentration) but an extracellular:intracellular Na^+ ratio of $10:1$ was maintained (Table 2).

Table 2. *Sodium and potassium contents of a respiratory-deficient mutant of Saccharomyces cerevisiae 1GC 3507 grown in carbon-limited continuous culture*

Dilution rate	Ion content (m-equiv./g. dry wt)			
	Without NaCl		With NaCl (1.0 M)	
	Na^+	K^+	Na^+	K^+
< 0.02	$0.012 \pm 0.001(2)$	$0.58 \pm 0.07(2)$	$0.25 \pm 0.06(3)$	$0.67 \pm 0.06(3)$
$0.06-0.07$	—	—	$0.29 \pm 0.05(3)$	$0.65 \pm 0.02(3)$
> 0.09	$0.012 \pm 0.001(2)$	$0.76 \pm 0.10(2)$	—	—

Means $\pm$ standard errors are quoted with the number of observations in parenthesis. Since yeast contains approximately twice its dry wt as intracellular water, the above results may be expressed as molar concentrations upon multiplication by 0.5 .

Kinetics of glucose uptake

The kinetics of glucose uptake appeared to be changed by NaCl. The yeast is transport-limited in glucose-limited continuous culture (van Uden, 1967*b*) as shown by the unidirectional Michaelis-Menten kinetics of the rate of sugar uptake without NaCl (Fig. 5*a*). The culture containing NaCl did not show unidirectional uptake kinetics (Fig. 5*b*); in addition, much higher concentrations of glucose were required to maintain a particular rate of glucose uptake.

Fig. 5. Reciprocal plots relating specific glucose uptake rate to glucose concentration in carbon-limited continuous cultures of a respiratory-deficient mutant of *Saccharomyces cerevisiae* IGC 3507. (a) Shows data for a culture containing 1.0 M-NaCl; (b) for a NaCl-free culture.

DISCUSSION

The decrease in the maximum specific growth rate in the presence of NaCl can be largely explained in terms of an increased requirement for energy-yielding substrate, apparently linked to maintenance, and leading to a decrease in the yield. It is probable that the increased maintenance requirement associated with growth in the presence of higher NaCl concentrations was concerned with maintaining an electrochemical gradient of sodium ions, the extracellular Na^+ concentration being ten times greater than the intracellular concentration. There is probably a passive or facilitated diffusion into the organisms (i.e. independent of metabolic energy), balanced by an active outward transport of Na^+ (see Stein, 1967). If the chloride ions move in both directions by either simple or facilitated diffusion then, in order to maintain equilibrium with respect to Cl^- , a membrane potential, E , must be generated (negative to the inside) sufficient to balance the opposing chemical potential difference (Nernst equation)

$$E = \frac{RT}{F} \ln \frac{[\text{Cl}]_0}{[\text{Cl}]_i}, \quad (2)$$

where F is the Faraday, R is the gas constant, $[\text{Cl}]_0$ and $[\text{Cl}]_i$ are the extracellular and intracellular concentrations (activities) of chloride. The sodium ions, however, will be subjected to both an adverse chemical and electrical potential gradient. The free energy change for the movement of 1 mole of sodium across the membrane will therefore be

$$\Delta G_{\text{Na}^+} = RT \ln \frac{[\text{Na}]_0}{[\text{Na}]_i} + EF, \quad (3)$$

where $[\text{Na}]_0$ and $[\text{Na}]_i$ are extracellular and intracellular concentrations (activities) of

sodium. Assuming $[Cl]_i$ and $[Cl]_o$ to be equal to $[Na]_i$ and $[Na]_o$ respectively, equation (3) reduces to

$$\Delta G_{Na+} = 2RT \ln \frac{[Na]_o}{[Na]_i}. \quad (4)$$

To maintain active transport of sodium ions, a metabolic free energy decrease (ΔG_{met}) must be linked to the overall process in such a way that

$$\Delta G_{Total} = (\Delta G_{Na+} + \Delta G_{met}) < 0. \quad (5)$$

In the present example, ΔG_{Total} must be sufficiently negative to assure an active Na^+ efflux that will compensate for the passive influx of Na^+ , thus maintaining a steady-state. If the increase of ATP turnover for maintenance, in the culture with NaCl, is concerned with Na^+ transport, the decrease of metabolic free energy for transport may be estimated. The difference between the k_m^{ATP} of organisms grown with and without NaCl is $1.7 \mu\text{moles ATP/mg./h.}$ Taking the standard free energy change of ATP hydrolysis as a measure, the metabolic free energy decrease linked to Na^+ transport would be $-5 \times 10^{-2} \text{ J/mg. organisms/h.}$

Substitution of $[Na]_o = 1.0$ and $[Na]_i = 0.1$ into equation (4) shows that the rate of active transport of Na^+ out of the cell (or the diffusion rate into the cell) must be less than $5 \mu\text{moles/mg. dry wt of organisms/h.}$ (equation 5). If, further, a direct stoichiometric relationship exists between the sodium transport rate and the turnover rate of ATP, e.g. through an ATPase (see Glynn, 1968), the above result only allows two possible values, 1 or 2 Na^+ /ATP molecule hydrolysed or a Na^+ transport rate of 1.7 or $3.4 \mu\text{moles } Na^+/\text{mg./h.}$ Measurement of the initial uptake rate of labelled Na^+ into *Saccharomyces cerevisiae* from 1.35 M-NaCl by Norkrans & Kylin (1969) gave a value of approximately $1 \mu\text{mole } Na^+/\text{mg./h.}$ showing a reasonable agreement.

The present study reveals no information about the mechanism of the active transport process or whether it is linked directly to the metabolic energy of ATP. The significance of increased glycerol production is also obscure. Its production could be a result of the activation of certain enzymes on the pathway leading to glycerol by NaCl (Schoeffeniels, 1969); alternatively, it might be a byproduct of increased organic acid production necessary for NAD/NADH₂ regulation (a fall in pH value would be advantageous as it decreases the velocity of Na^+ uptake; Armstrong & Rothstein, 1964). It is, however, difficult to explain why the increased production of glycerol expressed itself primarily as a maintenance effect.

The present finding may be significant in a marine environment containing low concentrations of energy source (Duursma, 1960; Vaccaro, Hicks, Jannasch & Carey, 1968). Not only will the yield of organisms, with similar Na^+ requirements, be severely decreased at the low growth rates imposed but, in some cases, the concentration of assimilable carbohydrate may not be high enough to sustain growth, or to allow competition with better adapted organisms using lower concentrations for an equal growth rate (Jannasch, 1968; van Uden & Fell, 1968).

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