CHAPTER 2

ETHANOL TOXICITY AND ETHANOL TOLERANCE IN YEASTS

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I. INTRODUCTION

Negative effects of fermentation end-products on the performance of the fermenting microorganism, as well as the degree of tolerance for its end-products the microorganism may display, constitute problems of fundamental biological interest. Central questions are the biochemical and biophysical mechanisms that underly the toxic effects, the evolutionary changes on the molecular level that may have led to increases in end-product tolerance and the physical and chemical factors of the extracellular environment that modulate the toxicity of the end-products.

In the case of yeasts capable of conducting alcoholic fermentations of economic interest, the problem of end-product toxicity has also a practical dimension of considerable importance. Obviously the selection or construction of ethanol-resistant strains or the manipulation of environmental factors that increase tolerance may contribute to process optimization. While great improvements have been achieved over the centuries by empirical means in the fermentation technology of alcoholic beverages, which are being applied also in other and more modern uses of alcoholic fermentation, sooner or later limits are reached beyond which further improvements become dependent on intimate knowledge of the biology of the process on the molecular level. This is particularly true when the use of genetic engineering is envisioned as a means for strain improvement.

The current world-wide interest in the industrial production of ethanol from renewable carbon sources for use as a fuel and for other purposes, has increasingly stimulated research on the toxicity of ethanol for yeasts. Research has centered on *Saccharomyces cerevisiae* and narrowly related yeasts which due to their great fermentative capacity and their high tolerance for ethanol are used in most traditional and modern alcoholic fermentations. In recent years some attention has also been given to the effects of ethanol on yeasts other than *S. cerevisiae* which are capable of fermenting polysaccharides and sugars not accessible to the latter such as inulin, starch, lactose, cellobiose, and D-xylose.

In the present review an attempt is made to systematize the results that have so far been obtained in the research on ethanol effects on yeast performance while special attention is given to the mechanisms that underlie these effects. For a recent review of alcohol effects on microorganisms, including prokaryotes, see Ingram and Buttke (1a).

II. KINETICS OF THE INHIBITION OF YEAST GROWTH AND FERMENTATION BY ETHANOL

In 1946 Hinshelwood (1) wrote the following linear equation to account for product inhibition of the specific growth rate of a microbial population

$$\mu_x = \mu_o - qX \tag{1}$$

where μ_x and μ_o are the specific growth rates in the presence and absence of product, q an empirical constant and X the product concentration[1].

Holzberg et al. (2) studying the kinetics of growth and fermentation of a strain of *S. cerevisiae* var. *elipsoideus* in grape juice, established a linear relationship between the specific growth rate and the ethanol concentration above a critical value and modified the Hinshelwood equation accordingly

$$\mu_x = \mu_o[1 - 0.235(X - 2.6)] \tag{2}$$

which may be generalized as

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[1] *The symbols used in the equations were uniformized and are often different from those used by the original authors.*

$$\mu_x = \mu_o - a(X - X_{min}) \tag{3}$$

where a is an empirical constant and X_{min} the alcohol concentration above which inhibition became measurable.

On the other hand Egamberdiev and Ierusalimsky (3) using a strain of *Saccharomyces vini* established a hyperbolic relationship such as is found in pure non-competitive inhibition of enzymes

$$\mu_x = \mu_o \frac{K}{K + X} \tag{4}$$

where K is the inhibition constant. However, at high ethanol concentrations μ was more strongly inhibited than was predicted by Eq. (4). Whenever an equation with the form of Eq. (4) applies, the reciprocal of the specific growth rate should be linear function of the ethanol concentration

$$\frac{1}{\mu_x} = \frac{1}{\mu_o} + \frac{1}{\mu_o \cdot K} X \tag{5}$$

Exponential relations were encountered by Nagatani et al. (4) who studied the effects of ethanol on the specific rates of growth (in batch culture) and of fermentation (resting cells in Warburg apparatus) of a respiration-deficient strain of bakers' yeast

$$\mu_x = \mu_o e^{-k_1 X} \tag{6}$$

and

$$V_x = V_o e^{-k_2 X} \tag{7}$$

where k_1 and k_2 are the exponential inhibition constants of growth and fermentation respectively. Whenever Eqs. (6) and (7) apply semilog plots of the experimental data should yield straight lines, such as

$$\ln \mu_x = \ln \mu_o - k_1 X \tag{8}$$

Eqs. (1)-(8) refer to inhibition kinetics in batch processes or, more generally, to conditions in which the fermentable sugar and all other nutrients have saturating concentrations. An important refinement in the study of ethanol toxicity for yeast was achieved by Aiba et al. (5). Using the strain earlier used by Nagatani et al., (4) they determined the effects of ethanol on the kinetics of growth and fermentation in a chemostat culture. In both cases ethanol affected the capacity of the cell system (i.e., the maximum specific rates of growth and fermentation) but not its affinity for the fermentable sugar. Thus Lineweaver-Burk plots of specific growth rates and specific fermentation rates against the steady state glucose concentration in the presence of ethanol at several concentrations were of the type as encountered in the non-competitive inhibition of enzymes. However, the inhibition of the capacity by ethanol followed exponential rather than hyperbolic kinetics. Consequently they wrote

$$\mu_x = \mu_o e^{-k_1 X} \frac{S}{K_s + S} \tag{9}$$

and

$$V_x = V_o e^{-k_2 X} \frac{S}{K_m + S} \tag{10}$$

where S is the steady state glucose concentration, K_s the half-saturation constant of glucose for growth and K_m the analogous constant for fermentation.

Aiba and Shoda (6) replotted the data of Aiba et al. (5) and concluded that the relations were hyperbolic rather than exponential

$$\mu_x = \mu_o \frac{K_1}{K_1 + X} \frac{S}{K_s + S} \tag{11}$$

and

$$V_x = V_o \frac{K_2}{K_2 + X} \frac{S}{K_m + S} \tag{12}$$

This somewhat surprising reversal may indicate that in the system studied (5,6) the capacity inhibition of growth and fermentation by ethanol was neither clearly exponential nor clearly hyperbolic. Such a situation was encountered later by Roman et al. (7) in the ethanol inhibition of D-xylulose fermentation by a strain of *Schizosaccharomyces pombe*. The results fitted linear, hyperbolic and exponential equations equally well and the authors chose the linear relationship to model end-product inhibition in their system.

Bazua and Wilke using *S. cerevisiae* ATCC 4126 in chemostat cultures with a complex glucose medium also observed non-competitive inhibition by ethanol of growth and fermentation. However, in their system the capacity inhibition was neither linear, nor exponential, nor hyperbolic and they proposed a fourth type of equation

$$\mu_x = \mu_o - \frac{aX}{b - X_{max}} \tag{13}$$

where a and b are empirical constants and X_{max} is the ethanol concentration above which no measurable growth occurred. With respect to the inhibition of fermentation they proposed an analogous equation.

Also Ghose and Tyagi (9) using *S. cerevisiae* NRL-Y-132 found non-competitive inhibition of growth and fermentation. In their case the capacity inhibition was linear and could be described by the following equations:

$$\mu_x = \mu_o(1 - \frac{X}{X_{max}}) \tag{14}$$

and

$$V_x = V_o(1 - \frac{X}{X'_{max}}) \tag{15}$$

where X_{max} and X'_{max} represent the ethanol concentrations above which growth and fermentation respectively were no longer measurable. Significantly, X'_{max} (114 g L^{-1}) was much higher than X_{max} (87 g L^{-1}) i.e., fermentation was more resistant to ethanol than growth, a finding that has been confirmed by others (10-13) and is common knowledge among enologists.

A generalized form of Eq. (14) was introduced by Levenspiel (14)

$$\mu_x = \mu_o(1 - \frac{X}{X_{max}})^n \tag{16}$$

Levenspiel (14) described procedures for the evaluation of the "toxic power", n. Applying this technique to the experimental data of Bazua and Wilke (8), he obtained a good fit for a value of n = 0.41. Indeed, Eq. (16) may be fitted to linear

Table 1. The Principal Equations That Have Been Proposed to Express the Kinetics of Inhibition of Growth and Fermentation of Yeasts by Ethanol

Strain	*Equations*	*Original Authors*	*Other Authors Who Found Or Assumed Similar Relations*
S. cerevisiae var. *elipsoideus*	$\mu_x = \mu_O - a(X - X_{min})$	Holzberg et al. (2)	
S. vini	$\mu_x = \mu_O \frac{K}{K + X}$	Egamberdiev and Ierusalimski (3)	(6, 10, 12, 18)
Baker's yeast (respiration deficient)	$\mu_x = \mu_O e^{-k_1 X}$ $v_x = v_O e^{-k_2 X}$	Nagatani et al.(4)	(15, 16, 17)
Baker's yeast (respiration deficient)	$\mu_x = \mu_O e^{-k_1 X} \frac{S}{K_s + S}$ $v_x = v_O e^{-k_2 X} \frac{S}{K_m + S}$	Aiba et al. (5)	(19)

Table 1. *(continued)*

Strain	*Equations*	*Original Authors*	*Other Authors Who Found Or Assumed Similar Relations*
Baker's yeast (respiration deficient)	$\mu_x = \mu_O \frac{K_1}{K_1 + X} \frac{S}{K_s + S}$ $v_x = v_O \frac{K_2}{K_2 + X} \frac{S}{K_m + S}$	Aiba and Shoda (6)	(18, 20)
S. cerevisiae ATCC 4126	$\mu_x = \mu_O - \frac{aX}{b - X_{max}}$	Bazua and Wilke (8)	
S. cerevisiae NRL-Y-132	$\mu_x = \mu_O(1 - \frac{X}{X_{max}})$ $v_x = v_O(1 - \frac{X}{X'_{max}})$	Ghose and Tyagi (9)	(7, 13, 21, 22, 23)
Data of Bazua and Wilke (8)	$\mu_x = \mu_O(1 - \frac{X}{X_{max}})^n$	Levenspiel (14)	(24)

and non-linear instances of inhibition kinetics and as such may be useful for modeling the behavior of a given strain under defined conditions. It does not express, however, nor pretend to express, molecular mechanisms that underlie ethanol effects on yeast performance.

Table 1 lists the kinetic equations that have been proposed to express ethanol effects on yeast performance and indicates the reception these equations have received by subsequent authors. The heterogeneity of the kinetic relations so far proposed led to the hypothesis (55) that the overall kinetics of ethanol inhibition of growth and fermentation in *S. cerevisiae* is composite and that a number of underlying inhibitory mechanisms contribute to the overall kinetics. The relative weights of the different kinetic contributions may vary with the strain, the concentration of the ethanol, other chemical or physical factors (such as the temperature and the oxygen tension) and the physiological state of the cells. For these reasons the overall kinetics varies from case-to-case and no explicit equation exists that can be universally applied.

III. EFFECTS OF ETHANOL AND OTHER ALKANOLS ON THE TEMPERATURE RELATIONS OF S. CEREVISIAE AND OTHER YEASTS

Since the early observation by Gray (25) that "rise in temperature is accompanied by decrease in ability to tolerate alcohol", many authors have reported that ethanol toxicity for *S. cerevisiae* and similar yeasts may be enhanced by rises in the process temperature and that the enhanced toxicity may lead to the depression of growth and to loss of viability of the yeast cell population (21,26-38). Similar observations on the temperature dependence of ethanol toxicity have been reported for *Candida krusei* (39), *Kluyveromyces fragilis* (34-35) and *K. marxianus* (40).

The temperature-enhanced toxicity of ethanol may lead to so-called "heat sticking" of fermentations when the process temperature becomes too high (41). As we shall see, the enhancement of ethanol toxicity by high temperatures not only affects cell viability but also the optimum and the maximum temperatures for growth of *S. cerevisiae* and other yeasts.

Only recently it has become known that low temperatures may also enhance the apparent toxicity of ethanol for such yeasts. The brewing industry, when involved in the production of high-alcohol beers, is now feeling the need for yeasts that are sufficiently alcohol tolerant at low temperatures and are viable at the end of fermentation and suitable for reuse in subsequent fermentation (42). Also at low temperatures ethanol affects both cell viability and the relevant cardinal growth temperature, in this case the minimum temperature for growth (35).

A. *Effects on the Temperature Profile of Growth*

When strains of *S. cerevisiae* and of *K. fragilis* were grown at different temperatures and varying concentrations of added ethanol, it was found that T_{max} (the maximum temperature for growth) decreased while T_{min} (the minimum temperature for growth) increased with increasing concentrations of ethanol (35). Thus these yeasts are characterized by a temperature profile of maximum ethanol tolerance (Fig. 1).

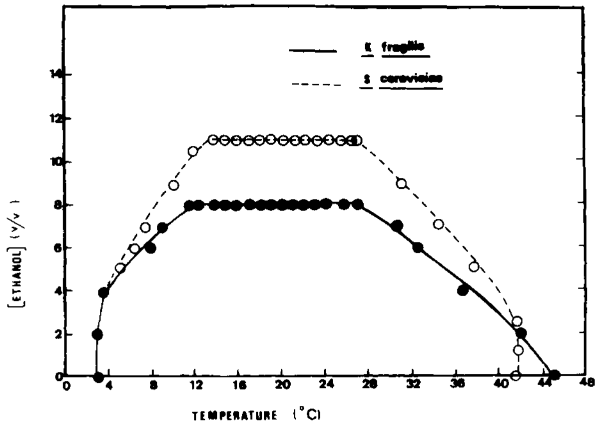

Figure 1. Temperature profiles of maximum ethanol tolerance of *Saccharomyces cerevisiae* and *Klyveromyces fragilis*. Experimental points indicate the concentrations of ethanol above which growth could not be detected. (35)

In *S. cerevisiae* a temperature plateau of maximum ethanol tolerance ranged from 13-27°C at 11% (v/v) ethanol. In the less ethanol-tolerant strain of *K. fragilis* a similar plateau occurred at 8% (v/v) ethanol. In both yeasts the maximum ethanol tolerance with respect to growth decreased at temperatures that are higher or lower than the limits of the plateau. Thus high temperature fermentations (red wine and fuel ethanol in warm countries) and low temperature fermentations (high alcohol beer, champagne) may be more ethanol-sensitive with respect to yeast growth than fermentations at intermediate temperatures.

In *S. cerevisiae* growth and thermal death are associated in the supraoptimal temperature range for growth (for a review see (43)). The Arrhenius plots of thermal death and growth intersect at biologically significant values giving rise to a second branch in the growth plot that represents the net specific growth rate as a function of the temperature when death and growth concur. This leads to the establishment of two maximum temperatures for growth, T_{max_i} (the initial maximum temperature for growth) and T_{max_f} (the final maximum temperature for growth).

As is shown in Fig. 2, ethanol shifted the temperature profile of growth and death to a lower temperature, as a whole and without disrupting it, affecting simultaneously in a coordinated way the maximum and the optimum temperatures for growth as well as the parameters of thermal death (31). The extent of the shift depended on the ethanol concentration.

The results suggested that the following temperature relations govern yeast growth and thus fermentation performance in an alcohol batch fermentation at relatively high temperatures (higher twenties to lower thirties). Initially, while the ethanol concentrations is sufficiently low, the yeast population has its normal cardinal temperatures (T_{op}, the optimum temperature for growth; T_{max_i} and T_{max_f}). During fermentation, while the ethanol concentration increases, the three cardinal temperatures decrease. At a certain critical ethanol concentration, which depends on the strain and the process temperature, T_{op} will become identical with the latter. Further increase of the ethanol concentration due to continuing fermentation will lead the population into the second exponential period of the supraoptimal temperature range during which exponential death concurs with exponential growth (43-44). As long as T_{max_f} does not decrease to the level of the process temperature, the specific growth rate will be greater than the specific death rate and the viable population continues to increase while dead cells accumulate.

At ethanol concentrations that depress T_{max_f} below the process temperature, death will proceed at higher specific rates than growth, leading to the extinction of the viable population. This sequence of events may take place wholly, partly or not at all depending on the alcohol tolerance of the strain, the final ethanol concentration and the process temperature. At high process temperatures such as may occur in red wine fermentations and in the industrial production of fermentation ethanol in warm countries, so-called "heat-sticking" due to the events described above may stop the fermentation prematurely.

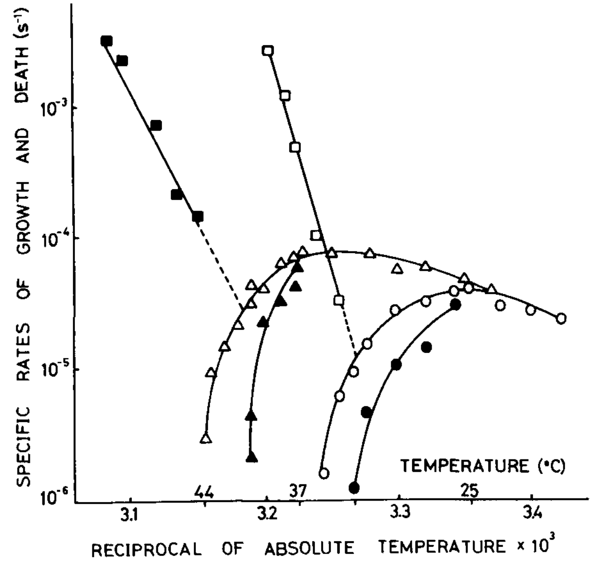

Figure 2. Temperature profile of a strain of *Saccharomyces cerevisiae*. Growth and thermal death experiments in liquid mineral medium with vitamins and glucose. To the left, profile in the absence of added ethanol: ■ Specific death rates; Δ specific growth rates of the first exponential period; ▲ net specific growth rates of the second exponential period. To the right, profiles in the presence of 6% (w/v) added ethanol: □ specific death rates; ○ specific growth rates of the first exponential period; ● net specific growth rates of the second exponential period. (31)

As is shown in Fig. 1, ethanol increased the minimum temperature for growth of *S. cerevisiae* and of *K. fragilis* (35). Recent results (45) indicate that ethanol leads to exponential death of populations of *S. cerevisiae* at temperatures below the ethanol-increased minimum temperature for growth while at temperatures above this critical value ethanol prolongs the lag phase in batch culture of non-adapted cells during which exponential death takes place until growth takes over.

B. Effects on Cell Viability

Leão and van Uden (32) prepared Arrhenius plots of thermal death of *S. cerevisiae* without and with various concentrations of ethanol, isopropanol, propanol and butanol. The Arrhenius plots of the specific death rates constituted families of straight lines which were statistically parallel. These results implied that the alkanols affected only the vertical intercepts of the plots or, in other words, they affected $\Delta S^{\neq}$, the entropy of activation of thermal death, but did not significantly change $\Delta H^{\neq}$, the enthalpy of activation of thermal death. To calculate the $\Delta S^{\neq}$ values, use was made of the theory of absolute reaction rates:

$$\ln \frac{k_d}{T} = \ln \frac{k_B}{h} + \frac{\Delta S^{\neq}}{R} - \frac{\Delta H^{\neq}}{R} \frac{1}{T} \qquad (17)$$

where T is absolute temperature, k_B is Boltzmann's constant, h is Planck's constant, and R is the gas constant. The calculation of $\Delta S^{\neq}$ was based on the mean value of $\Delta H^{\neq}$ (32).

$\Delta S^{\neq}$ of thermal death depended on the concentrations of the alkanols. The relationship appeared to be linear in the cases of isopropanol, propanol, and butanol, and linear over the range of higher concentrations (above ±0.3 M) in the case of ethanol. The following equation was written to express these linear relations:

$$\Delta S^{\neq}_{x} = \Delta S^{\neq}_{o} + C^{A}_{E}\, X \qquad (18)$$

where $\Delta S^{\neq}_{o}$ and $\Delta S^{\neq}_{x}$ represent the entropies of activation of thermal death at concentration zero and X, respectively, of the alkanol. For constant C^{A}_{E} the name "entropy coefficient for the aqueous phase" was introduced. It is defined as the increase in entropy of activation of thermal death per unit concentration of alkanol in the aqueous phase (the culture medium).

The values of C_A^E for the four alkanols are listed in Table 2. Their values increased with the lipid solubility of the respective alkanol. Division by the respective lipid-butter partition coefficients led to very similar values, i.e., C_E^M, the entropy coefficient of the alkanols for the lipid phase expressed as the increase in $\Delta S^{\neq}$ per unit concentration of alkanol in the membrane had nearly the same value.

The parallelism of the Arrhenius plots implied that the specific death rates under isothermic conditions should be exponential functions of the alkanol concentration. From the application of Eq. (17) to the parallel Arrhenius plots follows directly that under isothermic conditions

$$\ln k_d^x = \ln k_d^o + \frac{\Delta\Delta S^{\neq}}{R} \tag{19}$$

where k_d^x and k_d^o are the specific death rates at concentrations X and zero of the alkanol.

From Eq. (18) follows that

$$\Delta\Delta S_x^{\neq} = C_E^A X \tag{20}$$

and substitution of (20) in (19) leads to

$$\ln k_d^x = \ln k_d^o + \frac{C_E^A}{R} X \tag{21}$$

and

$$k_d^x = k_d^o \, e^{\frac{C_E^A}{R} X} \tag{22}$$

Eq. (21) was tested experimentally and it was found that under isothermic conditions k_d was indeed an exponential function of the alkanol concentration (32). Figure 3 shows the results obtained with ethanol and isopropanol. The slopes of the semilog plots increased with the lipid solubility of the alkanols and as prediced by Eq. (sl) were independent of the temperature. The estimates of C_E^A calculated from the slopes by the use of Eq. (21) were similar in values with the estimates of C_E^A obtained from the $\Delta S^{\neq}$ plots (Table 2).

The results indicate that the alkanols enhanced thermal death by acting in a non-specific way (i.e., only dependent on the lipid solubility of the compound and, if at all, its molecular size but not its chemical structure) on hydrophobic cell regions.

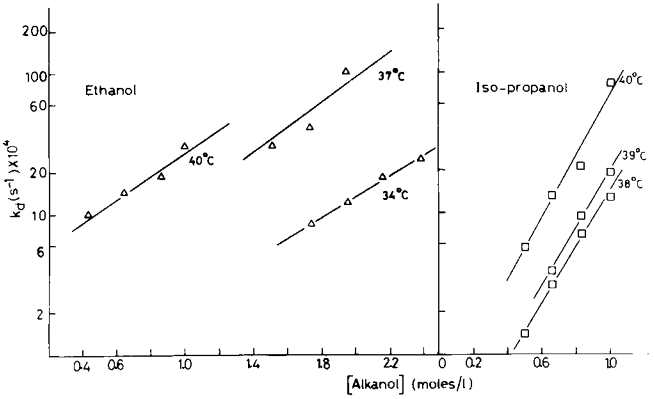

Figure 3. Dependence at constant temperature of the specific rates of thermal death in a strain of *Saccharomyces cerevisiae* on the concentration of ethanol and isopropanol.(32)

The results also suggest that the thermosensitive sites of *S. cerevisiae,* the inactivation of which leads to death, are located in a cell membrane. The expression "enhancement of thermal death" implies that the alkanols turned the targets of thermal death more heat sensitive rather than acting on a death target of their own. This is not necessarily true. Indeed, though cell death through the action of a chemical is normally a function of the temperature, the underlying molecular mechanism may be quite distinct from that of thermal death. In the case of temperature-dependent death of yeast in the presence of alkanols the following evidence suggested that its molecular mechanism is similar or identical with that of thermal death: 1) ethanol enhanced death and depressed the maximum and the optimum temperatures for growth without disrupting the temperature profile of the yeasts (31), and 2) the alkanols did not significantly affect $\Delta H^{\neq}$ of death or, using the language of students of desinfection, the temperature coefficient of death was the same without and with alkanols (32).

Table 2. Increase of the Entropy of Activation of Thermal Death of Saccharomyces cerevisiae *per mol of Alkanol in One Liter of the Aqueous Phase (C_E^A) or in One kg of Membrane (C_E^M)*

Alkanol	*Lipid-buffer partition coefficient*[a]	*Entropy coefficients*[b] *From Eq. (18)* C_E^A	C_E^M	*From Eq. (21)* C_E^A	C_E^M
Ethanol	*0.14*	*5.1*	*36.4*	*3.6*	*25.7*
Isopropanol	*0.276*	*10.2*	*37.0*	*9.6*	*34.8*
Propanol	*0.45*	*17.4*	*38.7*	*15.3*	*34.0*
Butanol	*1.5*	*58.3*	*38.9*	*60.0*	*40.0*

[a] *From (46)*

[b] C_E^A *- entropy units* $mol^{-1}\ l^{-1}$ *(aqueous phase)*

C_E^M *- entropy units* $mol^{-1}\ kg^{-1}$ *(membrane)*

Furthermore, it was found (37,47,48) that ethanol enhances "petite" mutation in *S. cerevisiae* and that at any given ethanol concentration the temperature profile of petite mutation is located between the T_{op} and the T_{max} that are established at the same ethanol concentration (37). This is an indication that the thermosensitive death sites of *S. cerevisiae* may be located in the inner mitochondrial membrane. Since thermal death and T_{max} are associated in *S. cerevisiae* and are affected in a correlated way, it is likely that the thermosensitive T_{max} sites are identical with or have a thermosensitive site in common with the death sites and are therefore also likely to be located in the inner mitochondrial membrane (for a discussion see (43)).

While available kinetic evidence suggests that cell death at high temperatures in the presence of alkanols is caused by the alkanol enhancement of thermal death and not by the action of alkanols on specific death targets of their own, the existence of such targets cannot be ruled out. Brown et al. (10) working with a commercial yeast and a laboratory strain of *S. uvarum* at 23°C, i.e., well within the temperature range of maximum ethanol tolerance found in similar yeasts (35), found that cell death concurred with exponential growth in batch culture and that the specific death rate was a function of the alcohol concentration. Correction of the specific growth rates for cell death uncovered hyperbolic non-competitive inhibition kinetics.

Watson and associates (49,50) working with four strains of *S. cerevisiae* also observed ethanol-induced death at 23°C, at high ethanol concentration (12-16.5%, w/v). Resistance to this toxic effect transitorily increased after subjection of the cells to "primary and secondary heat shock" (49).

Thomas, Hossack and Rose (51) working with a strain of *S. cerevisiae* at 30°C, observed loss of viability of cell suspensions in phosphate buffers containing 1 M ethanol. When the plasma membranes of the yeast were enriched with linoleyl residues, the rate of death was slower than for cells with membranes enriched in oleyl residues. Moreover this protective effect of multiple unsaturated fatty-acyl residues was enhanced when the membranes contained a sterol with an unsaturated side chain (ergosterol, stigmasterol) rather than one with a fully saturated chain (campesterol, cholesterol). These findings suggest that the target sites for ethanol-enhanced death of *S. cerevisiae* at intermediate temperatures are located in the plasma membrane.

C. *A Model*

The effects of alkanols on the temperature profile of *S. cerevisiae* and on the activation parameters of thermal death are displayed diagramatically in Fig. 4. Based on these relations Loureiro and van Uden (33) described and tested the following model.

Applying Eq. (17) to the relations depicted in Fig. 4, it can easily be shown that

$$T^{x}_{\max_f} = \frac{\Delta H^{\neq}}{C^{A}_{E}\, x + \dfrac{\Delta H^{\neq}}{T^{o}_{\max_f}}} \qquad (23)$$

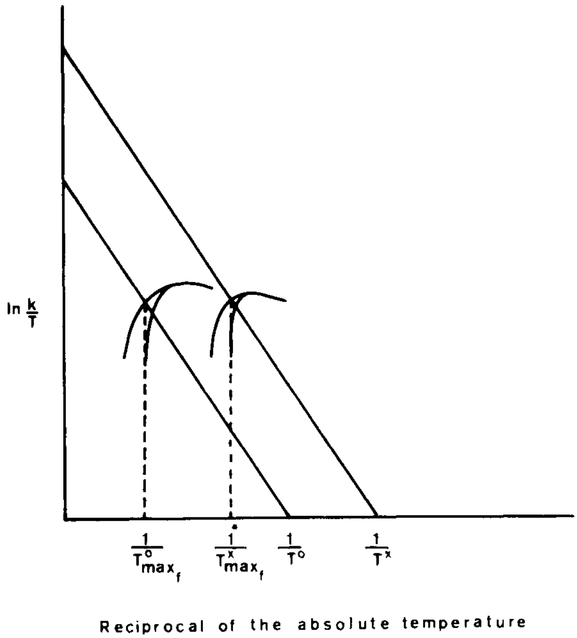

Figure 4. Diagram of the relations between alkanol-enhanced thermal death and the maximum temperature for growth in *S. cerevisiae*. Straight lines represent modified Arrhenius plots according to Eq. (1) of thermal death at concentrations 0 and X of alkanol; $1/T_0$ and $1/T_X$: intersection of these lines with the horizontal zero axis; curved lines modified Arrhenius plots according to Eq. (1) of growth at concentrations 0 and X of alkanol; $T^0_{max_f}$ and $T^X_{max_f}$: final maximum temperatures for growth (in degrees Kelvin) at concentrations 0 and X of alkanol; k, specific rate of growth or death; T, absolute temperature.(33)

where $T^{x}_{max_f}$ and $T^{o}_{max_f}$ are the final maximum temperatures for growth at concentration X and zero of the alkanol, $\Delta H^{\neq}$ the enthalpy of activation of thermal death and C^{A}_{E} the entropy coefficient for the respective alkanol.

The model was tested on an industrial wine yeast in a liquid growth medium with added ethanol and a reasonable good fit was obtained. The use of Eq. (23) for the prediction of the effect of ethanol on the maximum temperature for growth in an industrial fermentation may require the introduction of additional coefficients. The main difficulty is that the response of the cell population to the extracellular concentration of the ethanol produced in a batch fermentation changes in a more complex way that the responses to added ethanol in laboratory experiments (52-53). This question will be discussed in section VI.

IV. EFFECTS OF ETHANOL AND OTHER ALKANOLS ON THE MEMBRANE TRANSPORT OF NUTRIENTS IN S. CEREVISIAE AND OTHER YEASTS

While interference with the inner mitochondrial membrane and other target sites may underlie the effects of alkanols on the cardinal temperatures for growth and on cell viability of *S. cerevisiae* and other yeasts, the mechanisms that underlie the inhibition by alkanols of growth and fermentation at any permissive temperature are probably distinct from the former. So far, interference with nutrient transport across the plasma membrane or with glycolytic enzymes (see section V) has been proposed to explain the toxic effects of alkanols on growth and fermentation not specifically linked to extreme temperatures.

In 1979 Thomas and Rose (54) reported that ethanol depressed the rates of uptake of glucose, glucosamine, lysine, arginine and dihydrogen phosphate by cells of *S. cerevisiae* suspended in buffer. Significantly, the uptake (with the exception of phosphate) could be modulated by changing the lipid composition of the yeast. The inhibitory power of ethanol was more pronounced when cells had been grown (anaerobically) in the presence of oleic rather than linoleic acid. The inhibitory effect of ethanol on the specific growth rate in anaerobic batch cultures responded in an analogous way to the incorporation of oleic and linoleic acid. This

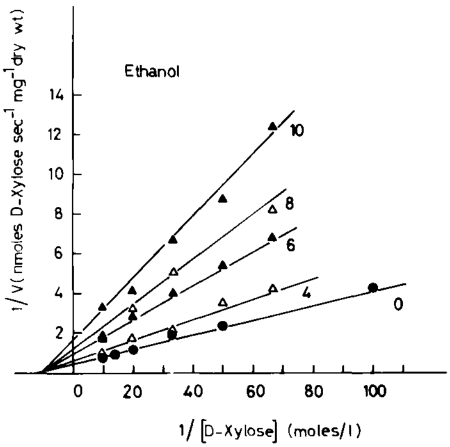

Figure 5. Double reciprocal plots of the initial uptake rates of D-xylose by *S. cerevisiae* in the absnece and the presence of ethanol. Numbers indicate ethanol concentrations (% (w/v)).(55)

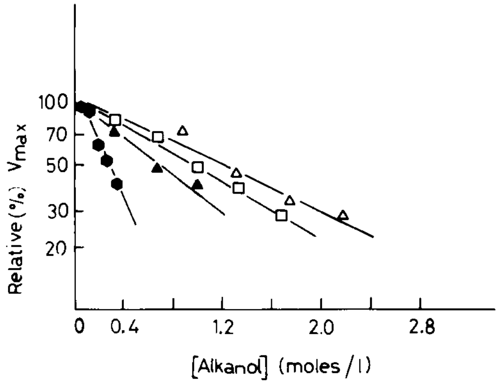

Figure 6. Semilog plots of the relative (%) maximum initial uptake rates of D-xylose by *S. cerevisiae* as a function of alkanol concentration. (△) Ethanol, (□) isopropanol, (▲) propanol and (⬢) butanol.(55)

Table 4. Significance of true and apparent sensitivity coefficients

Value	True sensitivity coefficient	Apparent sensitivity coefficient
Smaller than unity	Indicates the relative weight of the step under consideration	True relative weight may be smaller, never greater.
Equal to unity	Weight 100%."Master Reaction"	Possibly a "Master Reaction." True weight may be less than 100%.
Greater than unity	Not possible	Indeterminate

finding strongly suggested that in *S. cerevisiae* the inhibition of glucose uptake and fermentation by ethanol may largely be due to interference with transport mechanisms located in the plasma membrane. In subsequent years the kinetics of ethanol-inhibited transport in yeasts was worked out in some detail.

A. *Effects on Sugar Transport*

Ethanol, isopropanol, propanol and butanol inhibited the transport of D-xylose in *S. cerevisiae* (55). This non-metabolizable analogue of glucose uses the glucose transport system (56-57) of *S. cerevisiae*. Ethanol was also found to inhibit transport of D-xylose in *K. fragilis* (58). The inhibition of D-xylose transport in the two yeast species followed non-competitive kinetics, i.e., V_{max}, the maximum uptake rate was affected, but not the affinity for the sugar as expressed by K_m, the Michaelis constant (Fig. 5). Similar kinetics were encountered with respect to the inhibition of maltose transport by ethanol and other alkanols in *S. cerevisiae* (59).

The effects of the alkanols on the maximum velocity of D-xylose transport and maltose transport in *S. cerevisiae* and on D-xylose transport in *K. fragilis* followed exponential kinetics rather than the hyperbolic kinetics that is found in non-competitive enzyme inhibition. Fig. 6 shows the effects of several alkanols on the V_{max} of D-xylose transport in *S. cerevisiae*. Thus the inhibition kinetics could be described by the following equation:

$$V_x = V^o_{max}\, e^{-k(X-X_{min})}\, \frac{S}{K_m + S} \tag{24}$$

where S is the concentration of the sugar, K_m is the respective Michaelis constant, V_x is the initial uptake rate under defined conditions in the presence of alkanol, V^o_{max} is the maximum uptake rate under these conditions in the absence of alkanol, X is the alkanol concentration, X_{min} is the minimum inhibitory concentration of the alkanol, and k is the exponential inhibition constant characteristic for the alkanol. Table 3 lists estimates of X_{min} and k for alkanols with respect to nutrient transport in *S. cerevisiae*. The effects of the alkanols on the glucose (55) and maltose (59) transport system of *S. cerevisiae* increased with their lipid solubility indicating hydrophobic regions of the plasma membrane as the target sites.

B. *Effects on Ammonium Transport*

The transport system of *S. cerevisiae* for ammonium can be studied without interference from metabolism by the use of methylammonium which is a non-metabolizable analogue of ammonium (60,61). Ethanol, isopropanol, propanol and butanol had a more powerful effect on ammonium transport in *S. cerevisiae* than on the transport of sugars (62). Again, the inhibition was non-competitive, the effect on the maximum transport rate followed exponential kinetics and the values of the exponential inhibition constant increased with the lipid solubility of the alkanols.

Though the inhibition by alkanols of the transport systems of *S. cerevisiae* for glucose, maltose, ammonium and amino acids displayed similar non-competitive, exponential kinetics, the minimum inhibitory concentrations and the exponential inhibition constants were different with respect to the three transport systems (Table 3). Thus, ethanol at concentrations above 0.33 M inhibited the glucose transport system with an exponential inhibition constant of 0.62 ℓ/mol, while the

Table 3. Parameters of ethanol-inhibition of the maximum velocities of transport of glucose (D-xylose) (13), maltose (59), ammonium (62) and amino-acids (63) in S. cerevisiae IGC 3507.

Nutrients	*Exponential inhibition constant of ethanol (ℓ/mol)*	*Minimum inhibitory concentration of ethanol (mol/ℓ)*
Glucose	*0.62*	*0.33*
Maltose	*0.65*	*0.54*
Ammonium	*1.48*	*0.87*
Glycine	*1.69*	*0.71*
Alanine	*2.11*	*0.58*
Phenylalanine	*2.16*	*0.53*
Tyrosine	*2.04*	*0.66*
Tryptophan	*1.76*	*0.56*

ammonium transport system was inhibited only at concentrations above a much higher minimum inhibitory concentration of ethanol (0.87 M). However, at ethanol concentrations at which the transport system for both glucose and ammonium were inhibited, the inhibition by the latter was much stronger ($k = 1.48$ ℓ/mol) than by the former ($k = 0.62$ ℓ/mol). These findings imply that the overall composite inhibitory effect of ethanol on growth of the strain in glucose-ammonium medium is unlikely to follow exponential kinetics. The results also suggested that fermentation may still proceed at ethanol concentrations at which growth in glucose-ammonium medium is already completely inhibited, which is consistent with the frequently observed fact that fermentation is more ethanol resistant than growth (10-13).

C. Effects on the General Amino Acid Permease (GAP)

Similar results were obtained in a study of the effects of ethanol, isopropanol, propanol and butanol on the GAP of a strain of *S. cerevisiae* (63). The alkanols above X_{min}, their minimum inhibitory concentrations, inhibited the transport of glycine and other amino acids. The values of X_{min} decreased with the lipid solubility of the alkanols (Table 3). Again, the inhibition was of the non-competitive type while the effects on the maximum uptake rate followed exponential kinetics. As in the cases of the transport systems for glucose, maltose and ammonium, Eq. (24) applied though the constants had distinct values (Table 3).

As was found with respect to the alkanol-inhibition of the transport system of *S. cerevisiae* for glucose (55), maltose (59) and ammonium (62), also in the case of the GAP the inhibitory potency of the alkanols increased with their lipid solubility, pointing to interaction between the alkanols and the transport system in hydrophobic regions of the plasma membrane.

D. Uncoupling of Secondary Active Transport

In the case of the glucose transport system which in *S. cerevisiae* is an electroneutral uniport (for a discussion see (64)), the inhibitory effects of alkanols may be explained exclusively by interference with the porter protein, either directly or by changing its lipid environment in the plasma membrane. In the case of the ammonium transport system which in all likelihood is an electrogenic uniport (65) possible effects of the alkanols on the membrane potential should also be taken into account, while in the case of maltose transport and the general amino acid permease which in *S. cerevisiae* are electrogenic proton symports (66-67), possible effects of alkanols on the membrane potential as well as on the transmembrane proton gradient should be considered in addition to alkanol interference with the porter proteins. The membrane potential and the proton gradient may in principle be affected by alkanols either through inhibition of active proton extrusion or through enhancement of passive proton influx (uncoupling). The latter mechanism was found to occur in *S. cerevisiae* and was analyzed in some detail (68).

Ethanol, isopropanol, propanol and butanol exponentially enhanced the passive influx of protons into cells of *S. cerevisiae* de-energized by pretreatment with 2-deoxyglucose. The influx followed first-order kinetics over an initial period of time with a rate constant that increased exponentially with the alkanol concentration:

$$-\frac{d[H^+]}{dt} = C_0\, e^{kX}[H^+] \qquad (25)$$

where X is the alkanol concentration, C_0 the rate constant without added alkanol, k the enhancement constant and $[H^+]$ the extracellular proton concentration. Eq. (25) implies the following linear relation when X is constant:

$$pH_t = pH_0 + C_0\, e^{kX}(\log_{10} e)t \qquad (26)$$

where pH_t and pH_0 are the extracellular pH values at time zero and after time t.

The exponential enhancement constants increased with the lipid-solibility of the alkanols (Table 4) which indicated hydrophobic membrane regions as the target sites. While the enhancement constants were independent of pH, the rate constants decreased linearly with the extracellular proton concentration indicating the presence of an additional surface barrier against proton penetration the effectiveness of which increased with protonation.

The alkanols affected the acidification curves of energized yeast suspensions in such a way that the final pH values were linear functions of the alkanol concentration. To explain these results Leão and van Uden (68) formulated the following model which is based on the assumption of balance between opposing proton movements at the final pH:

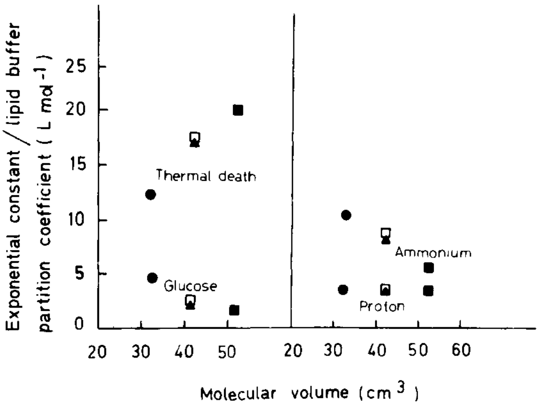

Figure 7. Effects of molecular volume on the exponential inhibition/enhancement of membrane-bound processes in *S. cerevisiae* by alkanols. (●) Ethanol, (□) isopropanol, (▲) propanol and (■) butanol.

$$k_p = C_0 \, e^{kX} [H^+]_f \tag{27}$$

where k_p is the rate of active proton extrusion and $[H^+]_f$ the proton concentration at the final pH. Taking logs and rearranging, the following relation between the alkanol concentration and the final pH is obtained

$$pH_f = \log_{10} C_0 - \log_{10} k_p + kX \log_{10} e \tag{28}$$

Under the conditions that the effects of the alkanols on active proton extrusion over the range of final pH values are not too great so that $\log_{10} k_p$ is nearly a constant and the variation of C_0 over this range is small so that $\log_{10} C_0$ is also nearly a constant, Eq. (28) should be linear or nearly so. The relations were indeed linear and the estimates of enhancement constant k calculated from the slopes had nearly identical values and relations with the lipid-buffer partition coefficients of the alkanols as the estimates obtained from the experiments with de-energized cells. This was held to indicate that passive proton influx takes place in energized cells with similar rates as in de-energized cells and that uncoupling contributes to the overall kinetics of alkanol-inhibited secondary active transport across the yeast plasma membrane. This implies that alkanols should decrease the potential ratios of substrate accumulation in cases of secondary active transport. Indeed the presence of alkanols decreases the ratio of methylammonium accumulation in energized *S. cerevisiae* (69).

E. Relative Weight of Transport Inhibition by Alkanols in the Overall Inhibition Kinetics in S. cerevisiae

The identification in a given yeast strain of a cell constituent (an enzyme or a permease for instance) and of a corresponding mechanism (a glycolytic step or transport of a nutrient for instance) does not constitute immediate proof that this constituent is a principal target of ethanol toxicity and that the inhibition of the corresponding mechanism contributes in a significant way to the overall kinetics of inhibition by ethanol of the performance of that strain.

Sometimes strong qualitative evidence may allow one to exclude an inhibition mechanism as kinetically significant. Thus in *Schizosaccharomyces pombe* ethanol up to 6% (w/v) had little or no effect on the specific rate of D-xylulose uptake while the specific rate of ethanol production was inhibited indicating that in this case effects on transport could hardly contribute significantly to the overall inhibition kinetics (7). It is desirable to have a method for evaluating in a more quantitative way the relative weights of underlying mechanisms. In the reviewer's laboratory limited use has been made, for this purpose, of the so-called "sensitivity coefficient."

Kacser and Burns (70) expressed the rate-controlling weight of an enzyme or a permease in the overall kinetics of growth (or fermentation) by the sensitivity coefficient. Applying these relations to the weight of transport in growth or fermentation we have

$$\frac{d\mu}{dE}\,\frac{E}{\mu} = R_1 \qquad (29)$$

and

$$\frac{dv}{dE}\,\frac{E}{v} = R_2 \qquad (30)$$

where R_1 and R_2 are sensitivity coefficients, μ the specific growth rate, v the specific fermentation rate and E the capacity of the transport system under consideration.

When over a given range of alkanol concentrations the inhibitory effects on the specific growth rate and on the capacity (V_{max}) of the transport step are exponential we may write for that range

$$\mu_X = \mu_0 \, e^{-k_1 X} \tag{31}$$

and

$$E_x = E_0 \, e^{-k_2 X} \tag{32}$$

which on differentiation gives

$$-\frac{d\mu}{dX} = k_1 \mu \tag{33}$$

and

$$-\frac{dE}{dX} = k_2 E \tag{34}$$

Dividing (33) by (34) and rearranging gives

$$\frac{d\mu}{dE} \, \frac{E}{\mu} = \frac{k_1}{k_2} = R_{ap} \tag{35}$$

where R_{ap} is the apparent sensitivity coefficient of the transport step inhibited by alkanol over a range of alkanol concentrations over which the inhibition of growth and transport is exponential and constant. Similar results are obtained by applying this treatment to the inhibition of fermentation.

The sensitivity coefficients obtained in this way are apparent and not identical with the true sensitivity coefficients. The latter refer to a theoretical situation in which the capacity of E, under consideration, is varied, while the capacities of all other enzymes and permeases are maintained constant. In a real situation, such as the inhibition of growth by an alkanol, more than one enzyme or permease simultaneously suffer reductions in their capacity. Consequently the information that may be obtained by determining R_{ap} is less than one would obtain if the true R were available

(Table 4). Nevertheless when, over a given alkanol range, transport of a given nutrient and growth (or fermentation) display exponential inhibition kinetics and when the apparent sensitivity coefficient for that range approaches unity we have circumstantial evidence that the transport step weighs heavily in the overall inhibition kinetics. Applying this technique to a respiration deficient strain of *S. cerevisiae* it was found that for concentrations of less than 6% (w/v) ethanol the inhibition of glucose transport while at higher concentrations the inhibition of ammonium transport governed the overall inhibition kinetics of growth (71).

V. EFFECTS OF ETHANOL ON GLYCOLYTIC ENZYMES

The toxic effects of ethanol and other alkanols on growth, fermentation and viability of yeasts are correlated with their lipid solubility (72-73, 55, 59, 62, 68). Thus ethanol, though less soluble in lipids than in water, (lipid-buffer partition coefficient around 0.14), appears to exert at least in part its toxic effects on the yeast cell by interaction with hydrophobic regions. Such regions are constituted not only by the lipid core of biomembranes but also by hydrophobic regions of proteins, be they membrane-bound or soluble. One expects therefore that ethanol at high enough concentrations may inhibit and/or inactivate some if not all enzymes.

Key glycolytic enzymes of the alcohol-sensitive yeast *Kloeckera apiculata* suffered a marked irreversible denaturation in vivo in the presence of 10% ethanol while the same enzymes in an ethanol-tolerant strain of *S. oviformis* were hardly affected (74).

Nagodawithana et al (75), detected non-competitive inhibition by ethanol of hexokinase and α-glycerophosphate dehydrogenase in a strain of *S. uvarum* and suggested that this mechanism may contribute to the regulation of glycerol and ethanol production. At 15% (v/v) ethanol, hexokinase and α-glycerophosphate dehydrogenase activities were 45% respectively 64% of the control values while at these high ethanol concentrations phosphofructokinase and aldolase were not affected at all. The proposition that inhibition of glycolytic enzymes by ethanol may contribute to the overall kinetics of ethanol inhibition of *S. cerevisiae* and similar ethanol tolerant yeasts appeared attractive under the assumption that the ethanol concentration within the yeast cell may be much higher than in the surrounding medium. This assumption, as we shall see in the next chapter, is probably not valid. Furthermore, even a reduction of the hexokinase capacity by 50% might not reduce glycolytic flux noticeably due to the excess capacity of the enzyme as compared with the glucose transport system (76).

The question of ethanol effects on glycolytic enzymes was reexamined by Miller et al. (76), who studied twelve glycolytic enzymes in cell-free extracts of baker's yeast. The ethanol concentrations necessary to irreversibly reduce the activities of these enzymes by 50% within 30 min at 30°C were high and varied between 17 and more than 35% (w/v). With respect to reversible inhibition none of the enzymes was measurably affected by ethanol concentrations below 5% (w/v). Above this concentration some enzymes were inhibited in a competitive way other non-competitively. Miller et al. (76), concluded that cessation of alcohol production in their *Saccharomyces* strain could be due to the combined inhibition/denaturation of certain glycolytic enzymes, "especially if the concentration of ethanol inside the cell is significantly higher than the 10-12% (w/v) in the medium when fermentation normally ceases."

Under the condition that a significant accumulation of ethanol inside the yeast cell does *not* occur (see next section) it appears unlikely that the inhibition of glycolytic enzymes or of other soluble enzymes rather than of transport mechanisms has a significant weight in the overall kinetics of ethanol inhibition of *S. cerevisiae* and similarly ethanol tolerant yeasts. This may not be true at high temperatures however. It was shown by Foster and Hofmeyr (76a) that at 34°C the hexokinase activity of a strain of *S. cerevisiae* was abolished by 10% v/v ethanol in about ten minutes while at 15 and 25°C extremely high ethanol concentrations were necessary to achieve similar effects.

While membrane effects of alkanols affecting transport mechanisms and viability in *S. cerevisiae* appear to be the primary mechanism of alkanol inhibition of this and similar ethanol-resistant yeasts, it does not follow that this is the case in all yeasts, much less in all microorganisms. It may well be that membranes are in general more ethanol resistant than some other components and become only determinant for the toxic alkanol effects when evolution towards ethanol tolerance has turned these other components even more resistant than the membranes, the latter constituting a barrier more difficult to overcome by genetic evolution towards ethanol tolerance than the former. This may have occurred in *S. cerevisiae* and other highly ethanol-tolerant microorganisms.

In other less ethanol-tolerant microorganisms, cell components other than membranes may be determinant for the toxicity, such as glycolytic enzymes in the yeast *K. apiculata* (73) and in the thermophilic anaerobic *Clostridium thermocellum* (77). Conceivably, ethanol-sensitive cell components, other than biomembranes, may be responsible for the low degree of ethanol tolerance that is observed in many of the yeasts that have been considered in recent years for the industrial fermentation of D-xylose, cellobiose and starch (78-86). Should this be the case, it should be comparatively easy to improve the ethanol tolerance of such yeasts by simple mutations until a point is reached where their biomembranes become determinant for ethanol tolerance.

VI. THE QUESTION OF THE INTRACELLULAR ACCUMULATION OF ETHANOL

Nagodawithana and Steinkraus (29) reported that added ethanol was less toxic for *S. cerevisiae* than ethanol produced by yeast. The death rates were lower in the presence of added ethanol than those measured at similar external ethanol concentrations endogenously produced. As is shown in Table 5,

Table 5. Inhibitory effect of ethanol on the specific growth rate of S. cerevisiae expressed as K_i the ethanol concentration in the medium that reduces the specific growth rate by 50%.(20)

K_i $g\ell^{-1}$	^0C	Origin of the ethanol	Ref.
5.2	30	endogenous	20
15.2	30	endogenous	105
4.9	35	endogenous	106
20.6	28	added	3
55	30	added	6
40	35	added	8
105.2	30	added	18
3.8	30	endogenous	18

also the inhibitory effects on the specific growth rate as reported by many authors (3,6,8,18,20,105,106) showed a similar pattern. Nagodawithana and Steinkraus (29) proposed that, due to an unbalance between the rates of production and the net outflux of ethanol, there would be an intracellular accumulation of ethanol which in turn would explain the apparently greater inhibitory potency of endogenously produced ethanol present in the medium. This hypothesis was supported by the findings of several authors (18,24,30,53,54,84,90) who reported that the intracellular concentrations of ethanol, in the course of a batch fermentation may be much higher than its concentration in the extracellular medium.

Loureiro and Ferreira (92) performed dialysis experiments which labeled ethanol in a strain of *S. cerevisiae* and observed that equilibrium between extra-and intracellular ethanol was established in less than 25 s. On theoretical grounds they predicted that for cells producing 0.6 µmol ethanol mg^{-1} dry weight min^{-1} and with the permeability to ethanol exhibited by the strain used in their work, the maximum ethanol gradient to be expected is less than 0.1M.

Beavan et al. (87), found that ethanol is very rapidly released when the fermenting yeast is washed with water or buffer. Their findings confirm that ethanol diffuses rapidly through the plasma membrane and that the latter is unlikely to constitute a barrier against which ethanol accumulates inside the cell. Even so they also found that during a batch fermentation the intracellular ethanol concentration was higher than the extracellular one and that in the earlier stages the ethanol gradient was of the order of 1.0 M.

Dasari et al. (93), found that estimates of intracellular ethanol concentrations in batch cultures of *S. cerevisiae* are significantly affected by continued fermentation during processing of the sample. Precooling of the samples to 4°C and reduction of centrifugation time significantly reduced the apparent intracellular ethanol concentrations. Under their conditions during the early stages of fermentation the intracellular concentration was higher than the extracellular one with a maximum ethanol gradient of the order of 0.2 M while in the later stages the situation was reversed with the maximum inside-outside gradient exceeding 0.3 M. A similar inversion had been reported earlier by Goma et al. (94).

For the purpose of evaluating the possible mechanisms of ethanol toxicity in *S. cerevisiae* the significant finding of (93) and (94) was that at high extracellular ethanol concentrations (in the later stages of batch fermentation) the intracellular one was not higher. This implies that effects of ethanol on ethanol-resistant intracellular components such as glycolytic enzymes are not likely to play a significant role in the overall inhibition kinetics (with the possible exception of high temperature conditions (76a)).

From a physical chemical point of view these findings are puzzling. Based on what is known on ethanol diffusion through biomembranes (92) one would expect that at any extracellular ethanol concentration, the intracellular one might be slightly higher, never much higher and never lower. Their findings would make sense if at low extracellular ethanol concentrations during the early stages of fermentation a mechanism would exist that opposes the free diffusion of ethanol while at high extracellular concentrations during the later stages of fermentation another mechanism - active transport of ethanol (93-94) - would enhance ethanol export. While the first mechanism would require a high degree of membrane impermeabilization against free ethanol diffusion - difficult to envision from a biochemical and a physiological point of view - the second mechanism would be energetically very wasteful unless *both* mechanisms were active during the later stages of fermentation. Of course there is the possibility that further analytical refinements may reveal that at all active stages of a batch fermentation the intracellular ethanol concentrations are always only slightly higher than the extracellular ones.

The question why the apparent toxicity of added ethanol is less than that of ethanol produced by the yeast remains an open one. Among the many variables, other than the intracellular ethanol concentration, that may contribute to changes in the apparent ethanol toxicity during a batch fermentation the following have received some attention. Growth inhibitors other than ethanol may be introduced with the inoculum (95) or accumulate during the fermentation (particularly in recycle fermentors with low bleed rates). Such inhibitors may include salts and proteins (96), organic acids, aldehydes and higher alcohols (97) and fatty acids (C_6, C_8, C_{10}) produced by yeasts in grape musts (98). Osmotic pressure (88) and the sugar concentration (9,15,17,23,99-101) may affect the specific growth rate and thus simulate enhanced ethanol toxicity. During a batch fermentation the specific growth rate is gradually depressed by the increasing ethanol concentration and possibly by other factors. This changes the physiological state of the cells as expressed for example by the lipid composition of the plasma membrane (87), the resistance to thermal death (43), the resistance to ethanol-enhanced thermal death (102) and by changes in the sensitivity coefficients of metabolic and transport steps. Also decreases in the concentration of dissolved oxygen during a batch fermentation may affect the physiological state of the cell (28) and shift the relative kinetic weights of ethanol-sensitive steps or enhance ethanol-induced death possibly due to a decrease in sterol content (103,104,104a).

VII. INTERACTION OF ETHANOL AND OTHER ALKANOLS WITH MEMBRANES

A. Exponential Enhancement and Inhibition Constants of Alkanols

A number of alkanol-sensitive rate processes have been identified that underly the alkanol-sensitivity of growth, fermentation and viability in *S. cerevisiae* and other yeasts. These processes include: thermal death (32), passive proton influx across the plasma membrane (68), glucose transport (55, 58), maltose transport (59), ammonium transport (62) and amino-acid transport (63). The effects of ethanol and other alkanols on these processes have a number of characteristics in common and it has now become possible to make some generalizations regarding ethanol tolerance in *S. cerevisiae*.

In all cases the alkanols affected the processes in an exponential way as may be expressed by the following generalized equation:

$$C_X = C_0 \, e^{\pm k(X - X_{min})} \qquad (36)$$

where C_0 is the rate constant of the process in the absence of alkanol, C_X the rate constant in the presence of alkanol, X the alkanol concentration, X_{min} the minimum concentration above which the toxic effect is measurable, and k the exponential constant characteristic for the alkanol. In some cases alkanols exponentially enhance the process (thermal death, passive proton influx) and the sign in the exponent of Eq. (36) is then positive. In other cases the alkanols exponentially inhibit the process (the maximum velocity of the transport of glucose, maltose, ammonium and amino-acids) and the exponential sign is then negative. As is shown in Table 6

Table 6. Parameters of the Exponential inhibition/enhancement by alkanols of thermal death (32), transport of glucose (55), maltose (59), ammonium (62) and glycine (63), and the passive influx of protons in S. cerevisiae (68).

Alkanol	*Thermal death*		*Glucose*		*Maltose*		*Ammonium*		*Glycine*		*Proton diffusion*	
	k	C_m	k	C_m	k	C_m	k	C_m	k	C_m	k	C_m
Ethanol	1.81	n.d.	0.62	0.33	0.65	0.54	1.48	0.87	1.69	0.71	0.48	≅0
Isopropanol	4.83	n.d.	0.77	≅0	1.19	0.29	2.45	0.33	2.25	0.37	1.03	≅0
Propanol	7.70	n.d.	0.93	≅0	1.65	0.28	3.88	0.17	3.20	0.13	1.46	≅0
Butanol	30.19	n.d.	2.79	≅0	9.55	0.10	8.50	0.05	13.49	0.05	5.25	≅0

k = *exponential inhibition/enhancement constant (ℓ/mol)*

C_m = *minimum inhibitory concentration*

n.d. = not determined

the exponential constants of enhancement or inhibition of all alkanol-sensitive rate processes so far identified in *S. cerevisiae* are of the same order of magnitude. This suggests that the molecular mechanisms of alkanol interference with these processes are similar in nature.

In all alkanol-sensitive rate processes so far identified in *S. cerevisiae* the effects of the alkanols are correlated with their lipid-buffer partition coefficients suggesting that the alkanols interfere with hydrophobic membrane regions. This, in principle, is quite plausible in the cases of proton diffusion and nutrient transport across the plasma membrane, while there is a body of circumstantial evidence pointing to the inner mitochondrial membrane as the target region of thermal death in *S. cerevisiae,* and its enhancement by alkanols (37,43,47,48).

Though the similarity in the values of the exponential constants and the correlation with the lipid-buffer partition coefficients of the alkanols point to alkanol-induced changes in the physical state of biomembranes as the ultimate cause of their toxic effects on *S. cerevisiae,* there may be differences in the fine mechanisms of the alkanol-membrane interactions that underly the enhancement or inhibition of individual membrane-bound rate processes. That such differences may exist is suggested by the differences in value of two parameters: the minimum inhibitory concentrations of the alkanols and the effects of molecular volume. As is shown in Table 6 the minimum inhibitory concentrations vary considerably from process to process. With respect to the importance of molecular volume three cases may be distinguished (Fig. 7): the toxic effect depends only on the lipid solubility of the alkanol and not on its molecular volume (enhancement of passive protein diffusion); for equal membrane concentrations the toxic effects of the alkanols increase with their molecular volume (enhancement of thermal death) or decrease with their molecular volume (inhibition of transport of sugars, ammonium and amino acids).

B. Alkanol Effects on Membranes and Adaptive Responses

Interest in the problems of anesthesia has produced a body of research much of which is relevant to the problems of alkanol effects on yeasts (for a review see Seeman (46)). The concentrations of alkanols that provided 50% protection to red blood cells from hypotonic hemolysis were found to be identical to the concentrations that caused nerve blocking in the frog sciatic nerve. The potency of the alkanols strongly correlated with their lipid solubility. This led to the concept that the interaction of alkanols and other anesthetics with hydrophobic membrane regions is non-specific, i.e., rather than binding to specific receptor sites the alkanols would act through their physical presence in the membrane. There are some indications of chemical specificity however in the case of the alkanols: the C_1-C_4 alcohols would act predominantly through interaction of their hydroxyl functions with membrane sites presumably near the surface, while in the longer chain alcohols interaction of the acyl chain with the lipid core would dominate (107,108). While it has been shown that alkanols shift the phase transition temperature of artificial bilayers, with different transition profiles in the presence of ethanol, isopropanol, propanol and butanol as compared with the higher alkanols (107), the precise sites of ethanol action in natural membranes are still a matter for more or less informed speculation. Suggestions have included the lipid core (109-111), hydrophobic regions of membrane proteins (112,113) or both (114). It has also been proposed (115) that alkanols induce anesthesia by displacing the lipids from membrane proteins, the so-called "annular lipid hypothesis."

Curtain et al. (116), using electron-spin resonance spectroscopy with appropriate spin labels, studied the effect of ethanol on the plasma membranes of protoplasts from two strains of *Saccharomyces* as compared with ethanol effects on vesicles with the same phospholipid composition as the *Saccharomyces* membranes. Increasing ethanol concentration had a much stronger fluidizing effect on the plasma membranes of the yeast protoplasts than on the protein-less membrane vesicles. They interpreted the fluidization as being due to displacements of annular phospholipids from membrane proteins.

Responses of yeasts, protozoans and bacteria, growing in batch culture, to the presence of alkanols were found to consist in changes in the lipid and phospholipid composition of their membranes (108,117-130). L.O. Ingram and associates, working in the University of Florida, used *Escherichia coli* as a model organism for studying the responses of the microbial cell to the presence of ethanol and other alkanols (108,117-126). Though one should not readily extrapolate from the prokaryotic *E. coli* to eukaryotic yeast, their findings may well be relevant for understanding ethanol effects on the latter. They may be summarized as follows. The presence of alkanol in batch cultures of *E. coli* led to marked changes in the composition of the plasma membrane. In the case of ethanol and alkanols up to C_4, their presence led to increases in fatty acid unsaturation (vaccenic acid), in acyl chain length and in the relative abundance of phosphatidyl glycerol while the relative phosphatidyl ethanolamine concentration suffered a marked decrease. Long-chain alcohols (C_5-C_8) on the other hand led to an increase in fatty acid saturation (palmitic acid) while their effects on phospholipid composition were similar with those of ethanol. The changes in membrane lipid composition induced by ethanol and effects on lipid metabolism were analogous with those caused by chaotropic agents and by a decrease in growth temperature. (Since these chemical and physical agents may decrease the specific growth rate of *E. coli* in batch culture, it might be worthwhile to use the chemostat to verify whether changes in the specific growth rate *per se* have an independent effect on membrane lipid composition). Some, if not all, of the ethanol induced changes in membrane composition appeared to be truly adaptive. Indeed, the increase in unsaturated fatty acids which occurs in *E. coli* membrane during growth in the presence of ethanol was beneficial for cell growth and survival. A mutant unable to make vaccenic acid and unable to increase its proportion of unsaturated fatty acids during growth with ethanol was hypersensitive to growth inhibition and killing by ethanol. This hypersensitivity was relieved by supplementing the cultures with vaccenic or oleic acid. In other experiments it was found that ethanol-induced membrane leakage and subsequent cell death, as well as ethanol-inhibition of leucine transport were reduced in cells enriched in vaccenic acid and enhanced in cells enriched in palmitic acid.

C. Manipulation of Membrane Composition and Ethanol Tolerance

Anthony H. Rose and his colleagues working in the University of Bath, England, pioneered the study of the relationship between composition and function in the plasma membrane of *S. cerevisiae* (131). They devised an elegant technique that exploits the anaerobically induced requirement in *S. cerevisiae* for a sterol and an unsaturated fatty acid (132, 133). Since both requirements are fairly broad (134,135), they were able, using appropriately supplemented media under anaerobic conditions, to enrich the plasma membrane of *S. cerevisiae* NCYC 366 with exogenously supplied sterol to the extent of about 70% of the total sterol, and with residues of exogenously supplied fatty acids to about 55% of the total residues (51,136). Using this technique they showed that the effect of ethanol on cell viability was influenced by the lipid composition of the plasma membranes (51). They also found that when anaerobically growing cultures of *S.*

cerevisiae were supplemented with 1.5 M ethanol, the growth inhibiting effect was less pronounced in cells enriched with linoleic acid than in cells enriched with oleic acid (54). Similar results were obtained with respect to the effects of ethanol on nutrient uptake in yeast cells with different membrane lipid composition. Using the same enrichment technique, Watson (137) found that cell populations of *S. cerevisiae* enriched in unsaturated fatty acid produced higher concentrations of ethanol, under anaerobic conditions, than control cells.

From the point of view of fermentation technology the improvement of yeast performance and the enhancement of ethanol resistance by supplementing the broth with unsaturated fatty acids has yielded promising results. Success has been reported with the use of a number of different additives containing unsaturated fatty acids, such as Koji mold proteolipid (138,139), albumin or methylcellulose complexed with unsaturated phospholipids (140), *Aspergillus oryzae* proteolipid (141-143), ergosteryl oleate (144), albumin complexed with phosphatidyl choline (145), mycelium from *Aspergillus awamori* var. *kawachi* (146,147), ergosterol-tween-linoleic acid mixture (147), albumin complexed with ergosterol and tween (148) and a combination of yeast extract, ergosterol and tween (149). The beneficial effect of heat pretreatment or grapes on the subsequent wine fermentation (more rapid, more complete and more temperature resistant) has been attributed to the release of activators, including sterols, from the grape pellicles (150).

The further improvement by recombinant DNA techniques (151) of ethanol tolerance in highly ethanol-resistant yeasts such as *S. cerevisiae* in which ethanol effects on membranes are determinant for the overall toxicity, may require the previous elucidation of the genetic control of membrane lipid composition, of lipid and phospholipid biosynthesis and of lipid-protein interaction. In ethanol-sensitive yeasts of industrial interest such as fermenters of xylose, cellobiose and starch (78-86) (several inulin fermenters approach the ethanol tolerance of *S. cerevisiae* (13,35)) the determinant ethanol-sensitive targets should also be identified and their genetic control elucidated before rational genetic improvement strategies can be outlined. However, in cases in which a single or a few soluble enzymes constitute the main targets, the empirical selection of spontaneous or induced mutants may produce significant results. Even in highly ethanol-tolerant yeasts some further improvement may be achieved by such relatively simple methods as was shown by Brown and Oliver (152) who devised an elegant continuous selection technique for ethanol-resistant mutants and applied it with some success to a strain of *S. uvarum.*

VIII. CONCLUSIONS

1. The inhibition by ethanol of growth and fermentation in *S. cerevisiae* and related yeasts is non-competitive. The capacity inhibition may be linear, exponential, hyperbolic or more complex, depending on the strain and the growth conditions. Fermentation is less sensitive to ethanol than growth. The overall kinetics of ethanol inhibition of growth and fermentation is composite and depends on the relative weights of the kinetic contributions of the underlying inhibitory mechanisms, which may vary with the strain, the physiological state of the cells, the ethanol concentration, the concentration of other growth inhibitors, the composition of the growth medium, the oxygen tension, the temperature and other chemical and physical variables. No single, explicit equation is universally applicable to the overall inhibition kinetics.

2. Ethanol and other alkanols profoundly affect the temperature relations of growth and death of *S. cerevisiae, Klyveromyces fragilis* and other yeasts. These effects underlie the "heat sticking" of high temperature fermentations (ethanol and red wine in warm countries) and difficulties with yeast viability in low temperature fermentations (high alcohol beer, champagne). Ethanol depresses the maximum and the optimum temperatures for growth and increases the minimum temperature. The extent of the effects depend on the ethanol concentration. As a consequence these yeasts display a temperature profile of maximum ethanol tolerance which is characterized by a plateau of intermediate temperatures at which ethanol tolerance is highest that falls off at the higher and lower temperature extremes.

Ethanol and other alkanols exponentially enhance thermal death. The degree of enhancement depends on the concentration of the alkanol and its solubility in lipids. Effects of ethanol on the temperature profile of "petite" mutation in *S. cerevisiae* point to the inner mitochondrial membrane as the target site of ethanol effects on thermal death and on the maximum and optimum temperatures for growth.

Ethanol also induces death during the lag phase or concurrently with growth at intermediate and low growth temperatures and at temperatures below the ethanol increased minimum temperature for growth. The relation of this type of death to ethanol-enhanced thermal death is an open question.

3. At all permissive growth and fermentation temperatures ethanol and other alkanols inhibit the transport of glucose, maltose, ammonium and amino acids across the plasma membrane of *S. cerevisiae*. The inhibition kinetids is of the non-competitive type. However, the capacity inhibition is exponential and not of the hyperbolic type that is found in non-competitive enzyme inhibition. The exponential inhibition constants of the alkanols are positively correlated with their lipid-buffer partition coefficients which indicates hydrophobic membrane regions as the target sites. Over the lower range of inhibitory alkanol concentrations glucose transport is more strongly inhibited than that of ammonium and amino acids, while the opposite occurs in the upper concentration range. These relations are consistent with the fact that in *S. cerevisiae* fermentation is more ethanol-resistant than growth.

In addition to alkanol effects on the porter proteins, either direct or through interference with their lipid environment within the membrane, alkanols uncouple secondary active transport by exponentially enhancing the passive diffusion of protons into the yeast cell. The exponential enhancement constants increase with the lipid solubility of the alkanols as does their uncoupling capacity expressed as their effect on the steady state accumulation ratios of charged substrates.

While in yeasts that are less ethanol-tolerant than *S. cerevisiae,* ethanol may affect membrane transport in a similar way, cell processes other than transport may be more ethanol-sensitive than membrane transport and contribute significantly to the overall inhibition kinetics. Many of the yeasts that have been proposed for the industrial fermentation of D-xylose, cellobiose and starch may belong to this category.

4. Glycolytic enzymes are less resistant to ethanol in ethanol-sensitive yeasts such as *K. apiculata* than in *S. cerevisiae* and related ethanol-tolerant yeasts. In the latter, the combined inhibition/denaturation of glycolytic enzymes by ethanol could only contribute significantly to the overall inhibition kinetics if the concentrations of ethanol inside the cells were much higher than the concentrations that can

be achieved in the broth (with the possible exception of high temperature conditions). Many authors have reported that intracellular accumulation of ethanol occurs in *S. cerevisiae* during batch fermentation. Work on the kinetics of ethanol diffusion across the yeast plasma membrane and critical modifications of the methods used for the estimation of intracellular ethanol have made it unlikely that intracellular accumulation of ethanol takes place. The often reported fact that endogenously produced ethanol in the broth has a greater apparent toxicity than ethanol added to the medium is probably due to factors other than intracellular ethanol accumulation such as the physiological state of the cells and the production of toxic compounds other than ethanol.

5. Ethanol and other alkanols affect in a similar way a number of membrane-bound processes in *S. cerevisiae* (thermal death, passive proton influx, transport of sugars, ammonium and amino acids). The kinetics is exponential, the values of the constants of exponential enhancement (thermal death, proton diffusion) and exponential inhibition (transport) are of the same order of magnitude and are correlated with the lipid-buffer partition coefficients of the alkanols. Minimizing the values of these constants by environmental or genetic manipulations of membrane composition is equivalent to maximizing ethanol tolerance. Environmental manipulation to increase ethanol tolerance in *S. cerevisiae* has achieved some success by the use of unsaturated fatty acids as additives to the broth. Studies on the effects of alkanols on membrane composition and lipid metabolism in yeast and other microorganisms, particularly in *E. coli* are beginning to throw some light on the more likely avenues for future genetic improvements by DNA recombination techniques.

In the ethanol-sensitive yeasts that have been proposed for the industrial fermentation of D-xylose, cellobiose and starch, the main alcohol-sensitive targets are conceivably more amenable to genetic improvement by classical methods than is membrane composition.

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