Effects of ethanol on the plasma membrane of Saccharomyces cerevisiae

Cartwright, Charles Paul

Award date:
1986

Awarding institution:
University of Bath

EFFECTS OF ETHANOL ON THE PLASMA MEMBRANE OF SACCHAROMYCES CEREVISIAE

Submitted by CHARLES PAUL CARTWRIGHT

for the degree of Ph.D.

of the University of Bath

1986

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

CONTENTS

	Page No.
SUMMARY	i
ACKNOWLEDGEMENTS	iii
INTRODUCTION	1
THE YEAST PLASMA MEMBRANE	3
Composition of the Plasma Membrane of *Saccharomyces cerevisiae*	4
Structure of the Plasma Membrane	8
The Proton-Motive Force across the Yeast Plasma Membrane	11
The Plasma-Membrane ATPase of *Saccharomyces cerevisiae*	22
Solute Transport in *Saccharomyces cerevisiae*	37
ETHANOL AND MEMBRANES	47
Effects of Ethanol on Micro-Organisms other than *Saccharomyces cerevisiae*	47
Effects of Ethanol on *Saccharomyces cerevisiae*	55
Effects of Ethanol on Animal and Model Membranes	66
METHODS	76
ORGANISM	76
EXPERIMENTAL CULTURES	76
PREPARATION OF SPHEROPLASTS	78
DETERMINATION OF THE MAGNITUDE OF ΔP	
Measurements of Rates of Proton Flux	78
Accumulation of Propionic Acid, Tetraphenylphosphonium (TPP^+) and Thiocyanate (SCN^-) Ions by *Saccharomyces cerevisiae* NCYC 431	80

Page No.

Measurement of Intracellular pH Values of Organisms 81

Measurement of Membrane Potential ($\Delta\psi$) Values of Organisms 83

Calculation of Δp 83

ISOLATION OF PLASMA MEMBRANES 83

Isolation Using Sucrose Density-Gradient Centrifugation 83

Isolation Using Cationic Silica Microbeads 84

ANALYSIS OF PLASMA MEMBRANES 85

Electron Microscopy 85

Estimation of Protein Content 86

Assay of ATPase Activity 87

MEASUREMENT OF VELOCITY OF GLYCINE ACCUMULATION 88

Assay Method 88

Measurement of the Effectof Ethanol on Accumulation of Glycine by the GAP 89

LIPID ANALYSES 89

VIABILITY MEASUREMENTS 91

MATERIALS 92

RESULTS 93

GROWTH OF SACCHAROMYCES CEREVISIAE NCYC 431 UNDER SELF-INDUCED ANAEROBIC CONDITIONS 93

EFFECT OF ETHANOL ON THE VIABILITY OF POULATIONS OF SACCHAROMYCES CEREVISIAE NCYC 431 93

DISSIPATIVE EFFECT OF ETHANOL ON THE PROTON-MOTIVE FORCE ACROSS THE PLASMA MEMBRANE OF SACCHAROMYCES CEREVISIAE NCYC 431 96

Page No.

Effect of Culture Age and Ethanol on Values for ΔpH, Δψ and Δp 102

KINETIC CHARACTERISTICS OF, AND EFFECT OF ETHANOL ON, THE PLASMA-MEMBRANE ATPASE OF SACCHAROMYCES CEREVISIAE NCYC 431 115

Comparison of Methods for Plasma-Membrane Isolation 115

Properties of the Plasma-Membrane ATPase 116

PHOSPHOLIPID AND FATTY-ACYL COMPOSITION OF PLASMA MEMBRANES ISOLATED FROM SACCHAROMYCES CEREVISIAE NCYC 431 128

EFFECT OF ETHANOL ON GLYCINE ACCUMULATION BY SACCHAROMYCES CEREVISIAE NCYC 431 134

DISCUSSION 147

EFFECT OF ETHANOL ON THE PROTON-MOTIVE FORCE ACROSS THE PLASMA MEMBRANE OF SACCHAROMYCES CEREVISIAE NCYC 431 149

THE PLASMA-MEMBRANE ATPASE OF SACCHAROMYCES CEREVISIAE 156

Isolation of Plasma Membranes from Saccharomyces cerevisiae NCYC 431 156

Characteristics of, and Effect of Ethanol on, the Plasma-Membrane ATPase of Saccharomyces cerevisiae NCYC 431 159

Can Alterations in Plasma-Membrane Phospholipid Composition Explain Culture Age-Dependent Variations in the Properties of the Plasma-Membrane ATPase of Saccharomyces cerevisiae NCYC 431 163

TRANSPORT OF GLYCINE BY SACCHAROMYCES CEREVISIAE 171

Characteristics of Glycine Transport in Saccharomyces cerevisiae NCYC 431 172

Effect of Ethanol on Glycine Accumulation by Saccharomyces cerevisiae NCYC 431 175

REFERENCES 179

SUMMARY

Populations of Saccharomyces cerevisiae NCYC 431, harvested after 16 h incubation under self-induced anaerobic conditions, were more tolerant to the inhibitory effect of ethanol on viability than organisms harvested from 8 h cultures. Ethanol increased the rate of passive influx of protons into de-energized organisms at a rate which was greater with organisms from 8 h compared with 16 h cultures. Rates of passive influx of protons into spheroplasts were significantly greater than into intact organisms, although culture age did not affect rates of ethanol-induced influx of protons into spheroplasts. Ethanol retarded both the initial net rate of proton efflux and the final extent of acidification produced by suspensions of energized organisms, both effects being more pronounced with organisms from 8 h as compared with 16 h cultures. The magnitude of the proton-motive force (Δp) was decreased by ethanol in both energized and de-energized organisms. Although culture age did not affect the extent of ethanol-induced decrease in Δp in de-energized organisms, in energized organisms harvested from 8 h cultures, ethanol produced a significantly greater decrease in Δp as compared with organisms from 16 h cultures. If the ability of ethanol to decrease Δp is important in its inhibitory effect on growth, it seems that some phenomenon other than proton uncoupling is involved.

ATPase activity in plasma membranes from Saccharomyces cerevisiae NCYC 431 grown for 8 h or 16 h was inhibited by orthovanadate, N,N'-dicyclohexylcarbodiimide (DCCD) and diethylstilbestrol but was unaffected by oligomycin. The pH optimum of the enzyme in

membranes from organisms from 8 h cultures was around 6.5 and that in membranes from organisms from 16 h cultures near 6.0. The K_m[ATP] value of the enzyme was virtually unaffected by the age of culture from which organisms were harvested, although the V_{max} value of the enzyme in membranes from organisms from 8 h cultures was higher than that for organisms from 16 h cultures. Ethanol non-competitively inhibited ATPase activity in membranes, the inhibition constant for the enzyme from organisms from 8 h cultures was lower than that from organisms from 16 h cultures.

Glycine accumulation by the general amino-acid permease (GAP) was also non-competitively inhibited by ethanol. Inhibition constants were virtually the same for glycine uptake by de-energized organisms from 8 h and 16 h cultures, but under energized conditions the value was greater for organisms from 16 h rather than 8 h cultures. These data suggest that inhibition of plasma-membrane ATPase activity by ethanol, via the effect this would have on the ability of organisms to generate a Δp, explains, at least in part, inhibition of glycine accumulation by this alkanol.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor Anthony H. Rose for his help and advice throughout the duration of this project. I am also indebted to a number of people for their assistance with specific methodological problems, principally, Dr. Jill Calderbank for her advice on the more mathematical aspects of transport kinetics and Mrs Felicity J. Veazey for her help with the electron microscopy. Especial thanks are also due to Dr. Bruce S. Jacobson for his generous provision of cationically-charged silica microbeads and invaluable guidance on the techniques associated with their use in plasma-membrane purification. Finally, I would like to express my gratitude to the University of Bath for the award of a Research Bursary and thank Mrs Judith Harbutt for typing this thesis.

INTRODUCTION

One of the most important and characteristic features of biological systems is their ability to adapt to environmental change. This adaptive capability is particularly pronounced in micro-organisms and has enabled them to thrive under conditions of considerable biochemical and physiological stress. Extremes of environmental temperature and pH value, for example, impose obvious burdens which organisms must strive to overcome. However, physiological stress can also be generated as a consequence of an organism's own metabolic processes. Many micro-organisms release potentially toxic end-products of metabolism into their environment, the best documented example of which is production of ethanol by Saccharomyces cerevisiae. The primary reason for interest in this organism and its metabolism has not been its suitability as a model for studying mechanisms of cellular adaptation to environmental change, but rather as a consequence of its commercial importance. Exploitation of the ability of Sacch. cerevisiae to ferment sugars to ethanol, via the Embden-Meyerhof-Parnas glycolytic pathway, hence producing potable alcoholic beverages, constitutes one of Man's oldest and most profitable industries.

Nonetheless, a long recognised problem with yeast fermentations is that ethanol production ceases well before the exhaustion of utilisable substrates. Over half a century ago, the reason for this premature termination of fermentations was shown to be inhibition of metabolism by ethanol (Richards, 1928). The inhibitory effect of ethanol on Sacch. cerevisiae has since been shown to be unconnected

with its role as an end-product of yeast metabolism, being instead a non-specific effect dependent on its properties as a member of a class of amphiphilic compounds (Troyer, 1955). Numerous studies have been conducted in an attempt to elucidate both the mechanism by which ethanol inhibits growth and fermentation in Sacch. cerevisiae and to discover the physiological adaptations which enable this organism to tolerate concentrations of ethanol lethal to the majority of other micro-organisms. A considerable volume of literature has also been published on the effects of ethanol on micro-organisms other than Sacch. cerevisiae providing a valuable insight into the complex nature of interactions between ethanol and biological structures and showing the multiplicity of effects of this compound on metabolic function.

In addition to microbiologically-orientated studies, the effects of ethanol on biological processes and the mechanisms of acquiring tolerance to this alkanol have received considerable attention from pharmacologists. Alcohol abuse constitutes a major drug problem and, consequently, many investigations have been undertaken on the short-term physiological consequences of alcohol intake in higher animals, as well as investigations into the more long-term clinical effects of alcohol dependence.

Accumulated evidence both from work on micro-organisms (Ingram and Buttke, 1984) and higher animals (Michaelis and Michaelis, 1983) has shown that both the inhibitory and anaesthetic potency of alcohols is closely correlated with the ability of these compounds to interact with cellular membranes, particularly with the lipid

component of membranes. Purely from physicochemical considerations, the importance of membranes as targets for alcohol action would seem likely, since both alcohols and the lipid constituents of membranes are amphipathic molecules containing hydrophilic and hydrophobic moieties. The partitioning of relatively short-chain alcohols into the lipid regions of membranes would therefore be expected to cause disruptions of the physical and chemical interactions that maintain the level of structural organisation necessary for membrane function. As far as Sacch. cerevisiae is concerned, the plasma membrane appears to constitute one of the primary sites for the inhibitory action of ethanol. Before considering the experimental evidence to support this statement, however, an understanding of the structural properties and functional importance of the plasma membrane is essential.

THE YEAST PLASMA MEMBRANE

The plasma membrane of Sacch. cerevisiae has several important functions. Firstly, it is the structure responsible for protecting the organism from changes in the external medium, thereby enabling the establishment of a relatively constant internal environment. Secondly, by controlling entry and exit of solutes and metabolites to and from the cell, the plasma membrane allows selective interaction with the outside. Finally, it is involved in synthesis and packaging of cell-wall and periplasmic components. The multifunctional nature of the plasma membrane clearly therefore invokes the need for a high degree of structural organisation.

Composition of the Plasma Membrane of *Saccharomyces cerevisiae*

Although the precise composition of plasma membranes varies according to their source, they generally contain, in terms of dry weight, approximately 40% lipid and 60% protein held together by non-covalent interactions. Some carbohydrate is also usually present covalently linked to lipid or protein and, in the hydrated state, approximately 20% water which is tightly bound and essential for maintenance of structural integrity (Harrison and Lunt, 1980).

Relatively little has been reported on the plasma membrane composition of *Sacch. cerevisiae*. Moreover, published information on isolated plasma membranes is somewhat contradictory, probably due to artefactual problems associated with membrane purification. Boulton (1965) analysed the gross composition of membranes obtained by controlled lysis of spheroplasts followed by differential centrifugation. Plasma membranes isolated by this method contained roughly equal amounts of lipid and protein (45 - 50%, by weight) with carbohydrate making up the remaining 5 - 10%. The results of subsequent investigations employing sucrose density-gradient centrifugation as an additional membrane purification step, were in agreement with this initial analysis (Longley *et al.*, 1968; Schibeci *et al.*, 1973).

Of the two main constituents of the yeast plasma membrane only the composition of the lipid component has been resolved in any detail. Cellular lipids can be divided into two main classes, namely, polar lipids which in eukaryotic micro-organisms are principally the amphipathic glycerophospholipids, glycolipids and free sterols, and neutral lipids comprising triacylglycerols and sterol esters.

There are considerable discrepancies in the published literature concerning the relative contribution of various lipid classes to the overall lipid composition of plasma membranes obtained from Sacch. cerevisiae. Kramer et al. (1978) reported that the phospholipid content of yeast plasma membranes was very low, comprising only 5 - 6% of the total lipid. However, Kaneko et al. (1976) found that phospholipids constituted over 50% of the total cellular lipid of Sacch.cerevisiae intimating, if only by inference, a high plasma-membrane phospholipid content. Enzymic digestion of phospholipids and, or, contamination of plasma-membrane preparations with intracellular lipid material could explain the findings of Kramer and his co-workers. Degradation of phospholipids by non-specific lipases or phospholipases seems a likely possibility since both Kramer et al. (1978) and Schneider et al. (1979), in a similar study on Candida tropicalis, reported an abnormally high content of free fatty acids in their plasma-membrane preparations. Taking thermodynamic and structural factors into consideration it seems probable that improved purification techniques will show that phospholipids and free sterols constitute the overwhelming majority of plasma-membrane lipids in Sacch. cerevisiae, as indeed is the case in plasma membranes obtained from other eukaryotic organisms (Harrison and Lunt, 1980).

Glycerophospholipid is a general term applied to any lipid containing phosphoric acid as a mono- or di-ester, in which a hydrophilic head-group is linked via a glycerol residue to a hydrophobic tail consisting of two long chain fatty-acyl residues esterified to hydroxyl groups of the glycerol moiety. Choline, ethanolamine, inositol and serine constitute the principal head-groups found in

plasma-membrane phospholipids from Sacch. cerevisiae. Reported values for the phospholipid composition of yeast plasma membranes vary considerably, although it is generally accepted that phosphatidylcholine (25 - 40%) and phosphatidylethanolamine (25 - 35%) predominate, with variable, but smaller, proportions of phosphatidylinositol and phosphatidylserine (Longley et al., 1968; Kramer et al., 1978). The chain-length and degree of unsaturation of the hydrocarbon chain of phospholipids also varies although, in plasma membranes isolated from aerobically grown Sacch. cerevisiae, $C_{16:1}$ and $C_{18:1}$ residues account for between 70 - 80% of the total (Longley et al., 1968; Kaneko et al., 1976). There is some evidence, both in Sacch. cerevisiae and other organisms, that unsaturated fatty-acyl residues are not evenly distributed amongst phospholipid classes. Longley et al. (1968) reported that, in the plasma membrane of Sacch. cerevisiae NCYC 366, phosphatidylserine and phosphatidylinositol contained the greatest proportion of saturated residues, whilst Breckenridge et al. (1972) showed that, in certain other eukaryotic plasma membranes, ethanolamine phospholipids were especially rich in unsaturated fatty-acyl residues. This inequality in the degree of fatty-acyl unsaturation amongst phospholipid classes could have a physiological role by allowing a more thermodynamically favourable packing of phospholipid molecules in membranes.

Sterols are steroid alcohols, derivatives of the saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene. They contain a hydroxyl group at C-3 which represents the polar moiety of the molecule and a branched aliphatic side-chain of eight to ten carbons at C-17, which, along with the steroid skeleton, constitutes the hydrophobic part of the molecule. Ergosterol is the major sterol

component of yeast plasma membranes (Nurminen et al., 1975) although the presence of a number of other sterols has been reported, most commonly 24(28)-dehydroxyergosterol (Longley et al., 1968).

Despite the abundance of protein in the plasma membrane of Sacch. cerevisiae and the undisputed functional importance of this component, relatively few attempts have been made to characterise the protein composition of the yeast cell membrane. Perhaps the most significant contribution to analysing plasma-membrane proteins of Sacch. cerevisiae has been made by Santos and his colleagues (Santos et al., 1978; 1982a). To assist plasma-membrane purification, spheroplasts, prior to lysis in hypotonic buffer, were stabilised by addition of concanavalin A. After isolation of plasma membranes by sucrose density-gradient centrifugation, proteins were analysed by one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Santos et al. (1978) detected at least 25 polypeptide and 12 glycoprotein bands in plasma membranes from Sacch. cerevisiae with molecular weights between 10,000 and 300,000, although high molecular-weight species predominated. A similar diversity of polypeptide constituents was found in plasma membranes obtained from C. tropicalis, where SDS-PAGE analysis of purified plasma membrane vesicles revealed the presence of 17-19 polypeptide bands in addition to a prominent high molecular-weight glycoprotein band (Schneider et al., 1979). A glycoprotein with a molecular weight of 28,000 appears to be the principal component of the plasma membrane of Sacch. cerevisiae (Santos et al., 1982a). However, although Rank and Robertson (1983) could detect the presence of this glycoprotein in whole cells and spheroplasts, they were unable to find it

in concanavalin A-stabilised plasma membrane preparations.

Structure of the Plasma Membrane

The first realistic attempt to solve the problem of membrane structure was made by Danielli and Davson (1935). Their model envisaged a phospholipid bilayer held together by van der Waal forces amongst the fatty-acyl tails of the lipid molecules, sandwiched between two layers of protein electrostatically bound to the lipid head-groups. The Danielli-Davson model, reinforced and extended somewhat (Robertson, 1964), remained as the basis of our understanding of membrane structure until the mid 1960s. It was at this time that membranes were discovered to be prominent sites of dynamic metabolic and biosynthetic events, with proteins being predominantly responsible for these phenomena. This realisation of the dynamic nature of membranes led to the postulation of a number of alternatives to the rather inflexible Danielli-Davson model, including the lipoprotein sub-unit model (Lucy and Glauert, 1964), the mosaic model (Lenard and Singer, 1966) and the fluid mosaic model (Singer and Nicolson, 1972), all of which emphasised the importance of membrane proteins rather than lipids. It is now generally accepted that the fluid mosaic model constitutes the most satisfactory and widely applicable explanation of membrane structure.

Singer and Nicolson (1972) envisaged the membrane as a 2-dimensional solution of amphipathic, globular proteins dispersed in a fluid lipid matrix. These proteins were termed intrinsic, in order to differentiate them from polar, extrinsic protein species loosely attached to the lipid head-groups by ionic interactions. The two

essential features of this model which set it apart from previous attempts to rationalise membrane structure are, firstly, that membranes can exhibit an asymmetric distribution of proteins and lipids, and secondly, the idea of membrane lipids being in a predominantly fluid state. This fluidity of the membrane permits, at least in theory, considerable lateral and rotational movement of lipids and proteins. In addition to giving due consideration to the dynamic nature of membranes, the fluid mosaic model marked an improvement in the visualisation of membrane organisation in several other ways. Firstly, this model is highly favourable from a thermodynamic point of view since the presence of intrinsic proteins enables both hydrophilic and hydrophobic interactions between membrane components to be maximised. Secondly, the traversing of the membrane by proteins provides a means by which selective exchange of hydrophilic compounds can occur, an extremely important consideration when dealing with metabolically active membranes like the plasma membrane.

A flaw in the fluid mosaic model is that it leaves the impression that membrane lipids function only to provide an environment of the proper fluidity for intrinsic proteins. Boggs (1980) argued that the variability of lipids found in membranes is not consistent with this simple function.She suggested that specific lipid-lipid interactions, via intermolecular hydrogen bonding, could be important in influencing intrinsic protein conformation.

Nonetheless, the principal function of membrane lipid is to regulate intra-membrane viscosity. Membrane fluidity is a somewhat vague concept involving the intramolecular mobility of individual

phospholipid molecules, their lateral mobility and interactions between phospholipids on a molecular level. The fluid mosaic model envisaged the entire lipid matrix as being in a fluid condition such that all lipids are above their transition temperatures. The transition temperature of a phospholipid is the point at which a sharp rise in heat absorption occurs and the mobility of the hydrocarbon chains abruptly increases. This causes a transition from the crystalline gel phase to the highly mobile liquid-crystalline phase. Phase transition temperatures of individual phospholipids depend both on the composition of the head-group, and on the length and degree of unsaturation of the hydrocarbon chains(Michaelson et al., 1974). Membranes contain a considerable diversity of phospholipids and consequently are likely to show quite complex phase behaviour, involving the co-existence at any given temperature of both gel and liquid-crystalline phases. Indeed, experimental evidence supports the idea that phospholipid membrane bilayers are not universally fluid but contain distinct domains of lipid in predominantly gel or liquid-crystalline form (Karnovsky et al., 1982). Esfahani et al. (1981) indicated that the distribution of intrinsic proteins could be at least partially responsible for the formation of such domains by influencing lipid-lipid interactions and hence perturbing the idealised fluid bilayer. Work on isolated yeast plasma-membrane vesicles (Rank et al., 1978) also illustrated the role intrinsic proteins have in regulating membrane fluidity. Plasma-membrane vesicles isolated from Sacch. cerevisiae could be separated into high viscosity (A^-) and low viscosity (A^+) fractions. Analysis of protein composition revealed that A^- vesicles contained a low molecular-weight glycoprotein not found in the A^+ fraction. Rank and his colleagues suggested that a hydrophobic region of this glyco-

protein probably spans the membrane, serving as a focus for a highly viscous lipid domain. This evidence again indicates the non-ideal nature of *in vivo* lipid bilayers, the distribution of intrinsic proteins being particularly important in determining the location of areas of greater or lesser fluidity.

The Proton Motive Force across the Yeast Plasma Membrane

One of the most important functions of all membranes, especially plasma membranes, is to regulate the nature and extent of trans-membrane passage of solutes. The hydrophobic interior of the lipid bilayer provides a barrier to the transfer of hydrophilic species present in the aqueous environment on either side of the membrane. Markedly polar entities, such as inorganic anions and cations are virtually unable to cross membranes by free diffusion through the lipid phase and therefore must depend on the assistance of a protein or combination of proteins if they are to traverse the plasma membrane. Using selective ion-transport systems, and relying on the inherent ion-impermeability of membranes, organisms have developed mechanisms for establishing physiologically useful trans-membrane ion gradients. Harnessing the potential energy contained within these electrochemical gradients enables the operation of thermodynamically unfavourable membrane-located processes.

As far as *Sacch. cerevisiae* and indeed all other fungi are concerned, the establishment and maintenance of an electrochemical gradient of protons across the plasma membrane, usually expressed as the proton-motive force (Δp), is of fundamental physiological importance. Both the biochemical mechanism responsible for the creation of the Δp,

namely a proton-translocating ATPase located in the plasma membrane and the means by which the Δp is used to energize solute uptake will be described later. In this section, I describe firstly what a proton-motive force is, and secondly discuss the experimental evidence for the existence of a Δp across the plasma membrane of Sacch. cerevisiae.

The principle of coupling energy-containing proton or other cationic gradients to energy-requiring membrane-located processes constitutes a cornerstone of our understanding of bio-energetics. Originally, the concept of ion cycling through membranes, encapsulated in the chemiosmotic hypothesis of Mitchell (1961), was developed to explain how the passage of electrons down the respiratory chain contained in the inner mitochondrial membrane results in the formation of ATP from ADP. In simplified terms, Mitchell postulated that the function of the electron carriers of the respiratory chain was to serve as a specific ion transport system, pumping protons from the mitochondrial matrix across the inner mitochondrial membrane and thus generating a gradient of H^+ ions across the membrane, which is impermeable to inorganic ions. Once established, this electrochemical gradient of protons or Δp could, according to Mitchell, cause formation of ATP from ADP by a vectorial reaction involving the $F_1 - F_o$ ATPase complex of the inner mitochondrial membrane. Mitchell (1961) proposed that transport of two protons from the external medium, through the ATPase complex, into the mitochondrial matrix caused formation of a molecule of ATP.

Mitchell (1963) subsequently expanded the original chemi-osmotic hypothesis by suggesting that asymmetrical distributions of protons or other inorganic ions, produced by primary transport systems in

membranes could be used to drive uptake of other solutes through secondary transport systems. By coupling the backflow of ions to a solute-transport system of the symport type, Mitchell envisaged that accumulation of solutes might be achieved. Energization of secondary active transport is the primary function of the plasma-membrane Δp of Sacch. cerevisiae.

The electrochemical nature of the Δp is crucial and cannot be overstressed.An asymmetrical distribution of any chemical species across a membrane impermeable to that species will result in the generation of a chemical concentration gradient. A concentration gradient of H^+ ions can simply be represented as the differences in pH value (ΔpH) across a membrane. However, since protons carry a positive charge,an unequal distribution of this ion will result in the establishment of an electrical as well as a chemical gradient. This electrical difference across the membrane is termed the membrane potential ($\Delta\psi$). The overall proton-motive force is therefore comprised of two theoretically distinguishable and experimentally determinable components, namely ΔpH and $\Delta\psi$. To assess the relative contribution of these two parameters to the overall magnitude of the Δp, it is convenient to express them in the same terms. Thus all measurements are converted to units of charge, usually millivolts, by using the Nernst equation which relates the concentration of an ion to the voltage resulting from its concentration gradient.This equation is usually expressed as follows:

$$E = \frac{RT}{F} \log \frac{[I]_o}{[I]_i}$$

where E = electrical gradient (mV)

R = universal gas constant

F = Faraday's constant (96.5 coulombs mol^{-1})

T = temperature (°K)

$[I]_i$ = intracellular concentration of protons

$[I]_o$ = extracellular concentration of protons

The existence of a ΔpH and a $\Delta\psi$ across the yeast plasma membrane has been conclusively demonstrated and from these measurements, quantitative estimates have been made of the magnitude of the Δp in Sacch. cerevisiae. Assessment of the size of the pH gradient across the plasma membrane presents a major practical problem, namely, determining the intracellular pH value of yeast cells. The first concerted attempt to measure cellular pH value in Sacch. cerevisiae was made by Conway and Downey (1950) using two different methods, both of which have since been used extensively. Firstly, they measured intracellular pH value directly by immersing glass electrodes into thick suspensions of disrupted organisms. Although this method appeared to be successful, insofar as Conway and Downey (1950) obtained reproducible values for internal pH, added to which it has been used subsequently (Ryan and Ryan, 1972; Riemersma and Alsbach, 1974), this technique has largely been superseded by less disruptive and potentially more accurate methods.

The second approach used by Conway and Downey (1950) for determining intracellular pH values of Sacch. cerevisiae was a non-invasive chemical method, namely, the distribution of a weak acid across the plasma membrane. This technique is based on the impermeability of the plasma membrane to ions, as a consequence of which only undissociated acid molecules enter the cell. Assuming that the internal pH value

of organisms is higher than the pH value of the external medium, intracellular accumulation of the anionic form of the acid will occur and, if the pK value of the acid is known, a mean intracellular pH value can be calculated from the measured distribution. This method is particularly suitable for estimating intracellular pH values in Sacch. cerevisiae where, under most physiological conditions, extracellular pH values are low, thereby ensuring little or no external dissociation of the acid. In addition to acetic and carbonic acid, both of which were used by Conway and Downey (1950), a number of other weak acids have been employed in measuring internal pH values of yeast cells including, propionic acid (Seaston et al., 1976), bromophenol blue (Sigler et al., 1981), caproic acid (Borst-Pauwels and Dobbelmann, 1972) and 2,4-dinitrophenol (Kotyk, 1962). Of these, propionic acid is perhaps the most reliable since the others are either metabolized, actively extruded, or not take up at all by certain yeast strains (Borst-Pauwels, 1981).

More recently, attempts have been made to improve on these by now classical methods of measuring intracellular pH values. A highly promising technique for internal pH value determination and one which is receiving increasing attention is ^{31}P-nuclear magnetic resonance (NMR). This method, which utilises the fact that the ^{31}P-NMR chemical shift of orthophosphate is quantitatively related to the cellular pH value, has been used to measure the intracellular pH value of yeast cells (Salhany et al., 1975; Navon et al., 1979). Slavik (1982) proposed that the use of fluorescent dyes might provide a more convenient, less expensive, but equally accurate alternative to ^{31}P-NMR for determining internal pH values. As well as being an extremely rapid method, since no equilibration time is required

temporal resolutions of less than one second can be achieved (Slavik, 1982; Vigne et al., 1984), this technique used in conjunction with fluorescence microscropy has shown the existence of discrete pH domains within the yeast cell (Slavik, 1983; Slavik and Kotyk, 1984). A long recognised drawback of the weak-acid method is that it only gives a mean internal pH value and cannot take account of how the considerable level of membrane organisation and intracellular compartmentalisation present in eukaryotic organisms might affect regulation of cellular pH value.

Direct comparisons between the various approaches used to measure internal pH values are not really possible because different experimental conditions are employed in each individual investigation. Nonetheless, ^{31}P-NMR generally gives higher pH values than the weak-acid method under corresponding conditions (Ballarin-Denti et al., 1984; Höfer et al., 1985). Although absolute values differ, certain general trends in internal pH regulation by yeast cells are discernible. All yeast species so far studied, including Sacch. cerevisiae, strive to maintain an intracellular pH value of near to neutrality. Measured values of cellular pH in Sacch. cerevisiae vary from around pH 5.5 up to pH 7.5.

Two major factors determine the intracellular pH value, namely, the metabolic state of the cell and the pH value of the surrounding medium. Addition of a metabolisable substrate to a suspension of resting yeast cells results in a substantial increase in the internal pH value of organisms, of the order of 0.5 to 1.0 pH units (Conway and Downey, 1950; Riemersma and Alsbach, 1974; Navon et al., 1979; Ballarin-Denti

et al., 1984). This increase in cellular pH value is presumably caused by energization of a proton pump in the plasma membrane, the activity of which therefore regulates, to some extent, both the intracellular pH value and the magnitude of ΔpH. Slavik and Kotyk (1984) reported that although addition of glucose to suspensions of Sacch. cerevisiae resulted in extracellular acidification, a concomitant increase in the intracellular pH value could not be detected. Interestingly, these workers also demonstrated the existence of an intracellular pH gradient in this organism, in that the cellular pH value decreased from the relatively alkaline interior (pH 7.0 - 7.5) to the comparatively acidic periphery (pH 5.5 - 6.0) of the cell. More importantly Slavik and Kotyk (1984) found no distinct break in this pH gradient at the plasma membrane. This finding questions the validity of using bulk internal pH values and, particularly, estimates of ΔpH derived from them as measures of plasma-membrane energization.

Most reports on intracellular pH value have also indicated the existence of a relationship between cellular pH value and the pH value of the extracellular medium. As external pH value increases, so corresponding although smaller increases occur in internal pH value. This phenomenon has been described in Sacch. cerevisiae (Ryan and Ryan, 1972; Slavik, 1982), Endomyces magnusii (Slavik, 1983), and somewhat more convincingly in Rhodotorula glutinis (Höfer and Misra, 1978). Other reports have, however, claimed that the intracellular pH value of Sacch. cerevisiae is not influenced by environmental pH (Salhany et al., 1975; Navon et al., 1979; Ballarin-Denti et al., 1984). Perhaps significantly, all of these investigations utilised ^{31}P-NMR spectroscopy to monitor internal pH values.

In addition to cellular metabolism and extracellular pH, the ionic composition of the medium and particularly the potassium content appears to influence the intracellular pH value of Sacch. cerevisiae. The presence of millimolar concentrations of K^+ in the extracellular phase of suspensions of Sacch. cerevisiae caused an exaggeration in the intracellular alkalinisation induced by glucose addition (Riemersma and Alsbach, 1974), a finding which led these workers to propose a passive role for K^+ in facilitating proton translocation. Ryan and Ryan (1972) showed that K^+ influx into Sacch. cerevisiae was strongly pH-dependent. If the intracellular pH value was artificially decreased by acid loading, both K^+ influx and H^+ efflux increased. Ryan and Ryan (1972) suggested that decreases in internal pH value stimulate organisms to expel protons via an H^+/K^+ antiport system.

Although measuring the intracellular pH value of yeast cells presents some difficulties, these have largely been overcome and remarkably few inconsistencies exist in the published literature. Disappointingly, this consensus does not extend to the determination of membrane potentials in these organisms. The most direct method of measuring $\Delta\psi$ is by the insertion of micro-electrodes into individual organisms.This technique has been used to great effect in hyphal fungi,in Neurospora crassa $\Delta\psi$ values as high as -200 to -300 mV were recorded by Slayman (1965a, b). However, the comparatively small size of most yeast cells precludes the use of micro-electrodes and only a few, largely abortive, attempts have been made to determine $\Delta\psi$ in Sacch. cerevisiae by this means (Borst-Pauwels, 1981). The giant yeast Endomyces magnusii with an internal volume approximately

100 times greater than that of Sacch. cerevisiae is perhaps better suited to $\Delta\psi$ determination by the micro-electrode method. Vacata et al. (1981), nevertheless, recorded a steady state $\Delta\psi$ value of only -35 mV in E. magnusii, a markedly lower value than that found in N. crassa under similar experimental conditions. This suggests that physical penetration of the micro-electrode precipitates a rapid depolarization of the yeast plasma membrane and, consequently, quantitative estimates of $\Delta\psi$ obtained with micro-electrodes must be viewed with some suspicion.

As with intracellular pH determination, the overwhelming majority of studies have employed non-invasive chemical methods to quantify $\Delta\psi$ in Sacch. cerevisiae. In particular, the equilibrium distributions of a number of lipophilic cations across the yeast plasma membrane have been used to compute $\Delta\psi$ values. The characteristics of tetraphenylphosphonium (TPP^+), trimethylphenylphosphonium ($TPMP^+$) and dimethyldibenzylammonium (DDA^+) accumulation have been examined in some detail, especially in the obligately aerobic yeast Rh. glutinis. Hauer and Höfer (1978) showed that both TPP^+ and $TPMP^+$ fulfilled the majority of requirements necessary for an indicator of $\Delta\psi$. Both ions were accumulated by Rh. glutinis against concentration gradients, accumulation being dependent on the existence of a negative potential difference across the plasma membrane. Uptake of TPP^+ and $TPMP^+$ was prevented by agents known to depolarize membranes and nystatin, a compound which selectively increases plasma-membrane permeability, caused a total efflux of accumulated lipophilic cations from Rh. glutinis. Hauer and Höfer (1978) therefore concluded that an electrical potential difference across the plasma membrane, with little or no

contribution from mitochondrial membrane potentials, is responsible for lipophilic cation accumulation. The suitability of TPP^+ as an indicator of $\Delta\psi$ in Rh. glutinis was substantiated by Höfer and Künemund (1984) who demonstrated that TPP^+ accumulation increased, in a quantitatively predictable manner, when organisms were subjected to osmotic stress, a treatment known to cause hyperpolarization of the plasma membrane.

Despite the success of measuring $\Delta\psi$ with lipophilic cations in Rh.glutinis, the use of these compounds in Sacch. cerevisiae remains somewhat controversial. Certain lipophilic cations appear to utilise specific solute-transport systems to cross the plasma membrane of Sacch. cerevisiae. Barts et al. (1980)showed that uptake of DDA^+ and $TPMP^+$ was catalysed by the thiamine carrier of this organism, and consequently these ions are obviously unsuitable for assessing $\Delta\psi$. However, TPP^+ appears to enter Sacch. cerevisiae by free diffusion, and the equilibrium distribution of this ion has been used to indicate the magnitude of $\Delta\psi$. Membrane potential values measured by TPP^+ distribution have varied between -50 and -130 mV depending on the physiological state of the organisms studied (Barts et al., 1980; Eilam, 1984). Nonetheless, considerable doubts remain about the validity of such measurements, and there are a number of methodological discrepancies amongst the published reports on TPP^+ uptake by Sacch. cerevisiae.

One particular inconsistency is the length of time required for equilibration of TPP^+ across the plasma membrane of Sacch. cerevisiae. Reported estimates vary from 10 - 20 min (de la Peña et al., 1982; Eilam, 1984) to several hours (Boxman et al., 1984).Eraso et al.,

(1984) observed a biphasic pattern of TPP^+ uptake in Sacch. cerevisiae, involving the establishment of two transient equilibria. This finding led Eraso and his colleagues to conclude that accumulation of TPP^+ was a complex process dependent on several factors, not all of them related to the degree of polarization of the plasma membrane.

Binding of TPP^+, both intracellular and extracellular, also provides a potential source of inaccuracy in quantitatively measuring $\Delta\psi$. Eilam (1984) showed that membrane depolarization caused a loss of most, but not all, intracellularly accumulated TPP^+, and Boxman et al., (1980) demonstrated that apparent TPP^+ accumulation occurred even in permeabilised organisms, indicative of the presence of intracellular TPP^+-binding sites in Sacch. cerevisiae. It is apparent, therefore, that a correction factor to account for TPP^+ binding must be incorporated into any calculation seeking to relate accumulation of this ion to $\Delta\psi$. Despite the problematical nature of using TPP^+ accumulation to measure $\Delta\psi$ in Sacch. cerevisiae, no convincing alternative has yet been proposed. Kovác̆ and Varecka(1981) attempted to use the fluorescent dye 3,3'-dipropylthiodicarbocyanine to evaluate $\Delta\psi$. Although this technique appeared to provide qualitatively useful data, no quantitative estimate of $\Delta\psi$ could be made, since measured values were composites of both plasma membrane and mitochondrial membrane potentials.

The existence of a proton-motive force across the yeast plasma membrane is, despite experimental difficulties, a widely accepted fact. Organisms appear to expend considerable amounts of metabolic energy in an attempt to maintain a constant Δp value, measured estimates

of which vary from -130 to -220 mV (Borst-Pauwels, 1981; Vacata et al., 1981; de la Peña et al., 1982; Höfer et al., 1985). The relative contribution of ΔpH and $\Delta\psi$ to the overall magnitude of Δp is strongly pH-dependent. In Rh. glutinis at an environmental pH value of 4.0, Δp consisted almost entirely of the ΔpH component with a negligible contribution from $\Delta\psi$, while at an external pH value of 7.0 the reverse situation obtained, with $\Delta\psi$ predominating (Hauer et al., 1981). Indeed, at external pH values of less than 4.0, Höfer and Künemund (1984) measured accumulation of thiocyanate ions by Rh. glutinis indicating the presence of a positive electrical potentialacross the plasma membrane. The effect of environmental pH value on the magnitude and ultimately the polarity of $\Delta\psi$ is, according to Höfer et al. (1983), due to an increase in proton diffusion into organisms as the pH gradient across the plasma membrane increases. A similar relationship between $\Delta\psi$ magnitude and environmental pH value has been demonstrated in Sacch. cerevisiae (de la Peña et al., 1982; Boxman et al., 1984) although the reversibility of $\Delta\psi$ polarity in this organism has yet to be proven.

The Plasma-Membrane ATPase of Saccharomyces cerevisiae

The presence of a proton-translocating ATPase in the fungal plasma membrane responsible for the generation of a Δp is now well established (Goffeau and Slayman, 1981). Plasma membrane-bound proton-translocating ATPases have also been described in other groups of micro-organisms, namely, the slime-moulds (Pögge von Strandmann et al., 1984) and cyanobacteria (Scherer and Böger, 1983; Nitschmann and Peschek, 1983). Evidence for the existence and function of a plasma-membrane ATPase in Sacch. cerevisiae comes

from two separate though interrelated sources, firstly, biochemical analysis of plasma-membrane composition and, secondly, electrophysiological studies on the ion-translocating properties of intact cells, isolated plasma-membrane vesicles and proteoliposomes.

Crucial to any meaningful examination of the characteristics of a membrane-bound enzyme such as the plasma-membrane ATPase is the ability to obtain pure membrane preparations from the organism under investigation. Until comparatively recently, most methods of plasma-membrane isolation from fungi involved producing a crude homogenate of cellular constituents, either by mechanical disruption of whole cells or hypotonic lysis of spheroplasts, and then fractionating the homogenate by differential and density-gradient centrifugation. In general, using spheroplasts to obtain plasma membranes is preferable, since rupturing of these structures requires comparatively mild disruptive action. By contrast, commencing the process of plasma-membrane purification with whole cells necessitates the use of extremely harsh treatments in order to cause physical damage to the cell wall and this may result in unwanted functional inactivation of organisms.

Nonetheless, an 'ATPase-like' activity in the plasma membrane of Sacch. cerevisiae was first observed in fractions obtained by differential and density-gradient centrifugation of whole cell homogenates(Matile et al., 1967). Enzymic analysis of fractions shown by freeze-etch electron microscopy to contain plasma-membrane fragments revealed the presence of only one enzyme, a Mg^{2+}-dependent ATPase. The association of this enzyme with the plasma membrane

was further illustrated by the fact that ATPase activity was 35 - 40 times greater in the purified plasma-membrane fraction than in cell-free extracts. Importantly, Matile and his coworkers demonstrated that this ATPase activity was not inhibited by oligomycin, a compound known to inhibit the $F_1 - F_o$ ATPase of the yeast mitochondrion (Schatz, 1965). Thus, Matile et al. (1967) had conclusively established the existence of a second ATPase enzyme in Sacch. cerevisiae. Although this study is now only of historical interest, it does highlight the two main problems that had to be overcome in order to achieve a thorough understanding of the plasma-membrane ATPase. The first was the apparent absence of enzymic markers for the yeast plasma membrane. Nurminen et al. (1970) conducted a detailed enzymic and compositional analysis of plasma membranes isolated from Sacch. cerevisiae by density-gradient centrifugation, but failed to find any enzymic activity, other than the oligomycin-insensitive ATPase, exclusively located in the plasma-membrane fraction. Indeed, ATPase activity is still the most reliable indicator of plasma-membrane purity with perhaps electron microscopy offering the only reliable ancillary aid to plasma-membrane identification. The second important point raised by the study of Matile et al. (1967) is the need to eliminate mitochondrial contamination from plasma-membrane preparations, so as to avoid confusion between the properties of the mitochondrial and plasma-membrane ATPases. The need for plasma-membrane purity has become even more apparent with the discovery of an ATPase enzyme in the vacuolar membrane of Sacch. cerevisiae.

The majority of influential studies on the plasma-membrane ATPase of yeast (Ahlers et al., 1978; Delhez et al., 1977; Willsky,

1979) or Neurospora crassa (Bowman et al., 1981) have used plasma membranes isolated from lysates prepared by gentle homogenisation of spheroplasts in hypotonic media, the cell wall having been removed by enzymic digestion. Although in some investigations differential centrifugation only has been used to fractionate spheroplast lysates (Ahlers et al., 1978), most workers have employed an initial short, low-speed differential centrifugation step to eliminate intracellular organelles, followed by a high-speed, sucrose density-gradient centrifugation procedure to isolate plasma-membrane fragments. Over the past fifteen years, increasingly elaborate and involved centrifugation procedures have resulted in a steady improvement in plasma-membrane purification with a corresponding increase in the specific activity of the plasma-membrane ATPase. Nonetheless, published values for plasma-membrane ATPase activity differ considerably with pronounced discrepancies between individual species of micro-organism. Published values include 0.4 - 1.8 μmol Pi (mg protein)$^{-1}$min^{-1} in Sacch. cerevisiae (Ahlers et al., 1978; Serrano, 1978; Willsky, 1979), 5.6 - 25.7 μmol Pi (mg protein)$^{-1}$min^{-1} in Schizosaccharomyces pombe (Delhez et al., 1977; Dufour and Goffeau, 1978, 1980), 0.4 - 8.0 μmol Pi (mg protein^{-1})min^{-1} in N. crassa (Scarborough, 1977; Bowman et al., 1981), 4.2 μmol Pi (mg protein)$^{-1}$ min^{-1} in C. tropicalis (Blasco et al., 1981) and 1.6 μmol Pi (mg protein)$^{-1}$ min^{-1} in Metschnikowia reukaufii (Aldermann and Höfer, 1984). Whether these differences are truly inherent ones, or simply artefacts, produced by variations in membrane preparation, remains open to question.

Recently, an alternative method of isolating plasma membranes from micro-organisms has been developed using colloidal suspensions

of cationically-charged silica microbeads (10 - 50 nm diam). Originally used for obtaining plasma membranes from the slime-mould Dictyostelium discoideum (Chaney and Jacobson, 1983), this technique was subsequently shown to be highly suitable for isolation of plasma membranes from spheroplasts of Sacch. cerevisiae (Schmidt et al., 1983). Membranes contain, at their hydrophilic exteriors, a large number of negatively charged groups. By mixing suspensions of whole cells, if a wall-less organism like D. discoideum is used, or spheroplasts, and cationically-charged microbeads, the external face of the plasma membrane becomes coated with beads. This silica coat preferentially increases the density of the plasma membrane relative to intracellular organelles, and provides a support for the membrane, preserving its structural integrity and shielding it from extracellular chemical and enzymic attack. The enhancement of plasma-membrane density means that, after cell or spheroplast rupture, only short, low-speed centrifugation of suspensions is required to sediment selectively the silica-coated plasma membranes. Schmidt et al. (1983) showed that plasma-membrane preparations obtained from Sacch. cerevisiae by this method were extremely pure, being essentially free from intracellular contamination and, most importantly, possessed levels of plasma-membrane ATPase activity at least equal to those obtained using established membrane purification techniques.

The silica microbead method has two main advantages over procedures involving sucrose density-gradient centrifugation. The first is speed of membrane isolation. A total preparation time of only three hours constitutes an important improvement on the

time-consuming preparation and running of sucrose-density gradients, since rapidity of membrane isolation reduces the possibility of inactivation of the plasma-membrane ATPase prior to assay. The second is preservation of plasma-membrane structure. Rather than the membrane fragmentation and vesicularisation observed in fractions from sucrose density-gradients, large open sheets of plasma membrane can be seen in preparations obtained using silica microbeads. Consequently, by structurally supporting plasma membranes with silica, any deleterious alterations in the environment of the ATPase enzyme produced during plasma-membrane purification will be minimised.

The three ATPase enzymes synthesised by Sacch. cerevisiae can be distinguished biochemically, principally, by the use of inhibitors, measurement of the ratio of ATP hydrolysis to that of other nucleoside triphosphates and determination of pH optima.

The optimum pH value for plasma-membrane ATPase activity is below neutrality. This is a notable distinguishing feature of this enzyme since the mitochondrial ATPase of Sacch. cerevisiae has a pH optimum of 8.5 (Peters and Borst-Pauwels,1979). However, the recently identified vacuolar ATPase of this organism also has a pH optimum of around 6.5 to 7.0 (Kakinuma et al., 1981; Okorokov et al., 1982). Published values for the optimal pH of the plasma-membrane ATPase of Sacch. cerevisiae vary somewhat from 5.5 - 6.0 (Willsky, 1979; Borst-Pauwels and Peters, 1981) to 6.5 - 7.0 (Sigler and Kotyk, 1976; Serrano, 1978). A very broad pH optimum of 5.5 - 6.75 was reported for the plasma-membrane ATPase of a respiratory-deficient mutant of Sacch. cerevisiae (McDonough et al., 1980).

Similar pH values to those found as optimal for ATPase activity in Sacch. cerevisiae also appear necessary for maximal rates of ATP hydrolysis by this enzyme in C. tropicalis (Schneider et al., 1979; Blasco et al., 1981). Schizosacch. pombe (Delhez et al., 1977) and N. crassa (Bowman and Slayman, 1979).

The specificity of the plasma-membrane ATPase for ATP as a substrate is pronounced and distinctive. Reports on Sacch. cerevisiae (Peters and Borst-Pauwels, 1979), Schizosacch. pombe (Dufour and Goffeau, 1980) and N. crassa (Bowman et al., 1981) showed that rates of CTP, GTP or ITP hydrolysis amounted to less than 5% of the ATP hydrolytic activity of purified plasma-membrane preparations. By contrast, vacuolar membranes of Sacch. cerevisiae contain considerable amounts of CTPase and GTPase activity (Kakinuma et al., 1981; Okorokov and Lichko, 1983) and the mitochondrial ATPase of this organism can effectively hydrolyse GTP and ITP (Takeshige et al., 1976).

Considerable efforts have been made to discover specific inhibitors for all three ATPases known to be synthesised by Sacch. cerevisiae. A number of compounds have been shown to inhibit selectively the mitochondrial ATPase including oligomycin (Schatz, 1965), azide (Serrano, 1978), venturocidin (Delhez et al., 1977) and triethyltin (Delhez et al., 1977). At micromolar concentrations, these compounds produce a pronounced decrease in mitochondrial ATPase activity without having any significant effect on the plasma membrane-bound enzyme (Serrano, 1978). Okorokov et al. (1982) showed that the vacuolar ATPase of Sacch. cerevisiae was also insensitive to mitochondrial ATPase inhibitors.

Two compounds have been employed as specific inhibitors of the plasma-membrane ATPase, namely, orthovanadate and diethylstilbestrol. Orthovandate has been shown to inhibit noticeably the plasma-membrane ATPase of Sacch. cerevisiae at concentrations of less than 10 μM (Willsky, 1979; Borst-Pauwels and Peters, 1981) without affecting the mitochondrial enzyme. A similar selectivity of orthovanadate action has been reported in N. crassa (Bowman and Slayman, 1979), Schizosacch. pombe (Dufour and Goffeau, 1980) and C. tropicalis (Blasco et al., 1981). Diethylstilbestrol also inhibits the plasma-membrane ATPase, although the action of this compound is less well characterised and probably not as specific as orthovanadate (Blasco et al., 1981; Serrano, 1983). Significantly, while the mitochondrial ATPase is insensitive to both orthovanadate and diethylstilbestrol the vacuolar enzyme shows a differential response to these compounds, being strongly inhibited by diethylstilbestrol but unaffected by orthovanadate (Kakinuma et al., 1981; Okorokov et al., 1982). In addition to specific inhibitors, a number of toxic, but relatively non-specific compounds have been used in investigations on the plasma-membrane ATPase, including N,N'-dicyclocarbodiimide (DCCD), Dio-9, NaF and quercetin (Serrano, 1978; Dufour and Goffeau, 1978).

One problem with all the inhibitors employed so far is that they show considerably less efficacy against the plasma-membrane ATPase in intact cells, as compared with their effect on enzyme activity in purified plasma-membrane preparations. Eilam et al. (1984) successfully blocked active proton secretion by Sacch. cerevisiae with DCCD, diethylstilbestrol and orthovanadate. However, millimolar rather than micromolar concentrations of these inhibitors were needed

to produce this effect, and at such high concentrations all toxic compounds are likely to have multiple sites of action. Goffeau and Slayman (1981), in their review on the plasma-membrane ATPase, stressed the need for a specific inhibitor of this enzyme which is effective in whole cells by emphasizing the part such an inhibitor, namely ouabain, has played in elucidating the biochemical mechanism and physiological function of the (Na^+-K^+)-ATPase found in plasma membranes of higher animals.

The activity of the plasma-membrane ATPase is strongly influenced by the ionic composition of the assay medium. This enzyme shows an absolute requirement for Mg^{2+} (Ahlers et al., 1978; Serrano, 1978), the optimal Mg^{2+} : ATP ratio being 1 : 1 in Sacch. cerevisiae (Willsky, 1979; Ota, 1985) and 1.5 : 1 in Schizosacch. pombe (Delhez et al., 1977). Indeed, Mg^{2+} is the physiological cofactor of the ATPase, and rather than ATP, the Mg-ATP complex is believed to be the true substrate for this enzyme. Monovalent cations have been examined for possible stimulatory or inhibitory effects on ATPase activity. Borst-Pauwels and Peters (1979) showed that both K^+ and Na^+ stimulated ATPase activity in isolated plasma membranes from Sacch. cerevisiae. Dufour and Goffeau (1980), however, were unable to demonstrate any significant effect of K^+ on ATPase activity of plasma-membrane preparations from Schizosacch pombe, and they showed that millimolar concentrations of Na^+ caused a 40% decrease in enzyme activity.

In an interesting paper, Ahlers (1984) re-examined the effect of ions on plasma-membrane ATPase activity. He found that plasma-membrane ATPase activity could be stimulated by addition

of various salts to the incubation medium, but this effect appeared to be caused primarily by the anionic moiety of the salt. Cations, apart from Mg^{2+}, were found to have little or no effect on the rate of ATP hydrolysis. More detailed kinetic analysis revealed that an anionically-induced enhancement of the affinity of ATPase molecules for Mg^{2+} was primarily responsible for the stimulatory effect of inorganic and organic salts.

Serrano (1983) reported that incubation of Sacch. cerevisiae with glucose prior to purification of plasma membranes by differential and density-gradient centrifugation resulted in a 10-fold increase in ATPase activity. Sychrová and Kotyk (1985) found that glucose-mediated ATPase activation occurred under aerobic and anaerobic conditions, and in a respiration-deficient mutant. These investigators suggested, therefore, that the glycolytic part of sugar oxidation provides a 'factor' necessary for converting the plasma-membrane ATPase, presumably by covalent modification, into its 'high-energy' form.

A distinguishing feature of any enzyme is its affinity for the preferred substrate, in this instance Mg-ATP. In kinetic investigations, this property of enzymes is quantitatively represented by the Michaelis constant (K_m). Although K_m values for the plasma-membrane ATPase of Sacch. cerevisiae as small as 0.05 mM (Ahlers et al., 1978) and 0.1 mM (Serrano, 1978) have been reported, most investigations have indicated a somewhat lower affinity of this enzyme for its substrate, for example 0.7 - 1.7 mM (Willsky, 1979; Borst-Pauwels and Peters, 1981). This last value is in broad agreement

with K_m values measured for this enzyme in other organisms, namely 1.8 - 3.3 mM in Schizosacch. pombe (Delhez et al., 1977; Dufour and Goffeau, 1980), 0.76 mM in C. tropicalis (Blasco et al., 1981) and 2.0 mM in N. crassa (Bowman and Slayman, 1977).

The ability of the plasma-membrane ATPase of Sacch. cerevisiae to act as an electrogenic proton pump has been demonstrated in a number of electrophysiological studies. The idea of an ion exchange system in the plasma membrane was first proposed by Conway and his coworkers in a series of publications in the late 1940's and early 1950's. These investigators observed that, during yeast fermentations, a massive efflux of protons occurred and that this efflux was accompanied by a simultaneous influx of K^+ ions (Conway and O'Malley, 1946; Conway and Brady, 1947; Conway and Brady, 1950). Conway et al. (1950) suggested that this ionic exchange might be facilitated by the activity of a heavy metal-containing catalyst, possibly a cytochrome, located in the plasma membrane. However, the failure of subsequent studies to find cytochromes or other redox systems in the yeast plasma membrane meant an alternative explanation for the mechanism of ion translocation was necessary.

The now established concept of ion translocation across the fungal plasma membrane occurring via an active electrogenic pump was first proposed by Slayman (1965a, b) following measurements of membrane potentials in N. crassa. Slayman (1965a) showed that a large negative potential of 200 - 300 mV existed across the plasma membrane of this organism under metabolically active conditions, far too large to be established by passive diffusion of ions.

Furthermore, a rapid depolarization of the plasma membrane was produced if hyphae were treated with metabolic inhibitors (Slayman, 1965b), strongly indicating the presence of a metabolically-coupled ion pump in the plasma membrane. Subsequent investigations (Slayman, 1970; Slayman et al., 1973) demonstrated that protons were translocated by the plasma-membrane ion pump and that ATP hydrolysis provided the energy necessary to drive proton translocation against a concentration gradient. All of this evidence suggested, albeit indirectly, that the plasma-membrane ATPase synthesised by N. crassa was responsible for proton translocation. Work by Peña and his colleagues showed that an ATP-dependent proton pump also operated in the plasma membrane of Sacch. cerevisiae (Peña et al., 1969, 1972), responsible for catalysing H^+ and K^+ exchange at the expense of ATP.

Somewhat more recently, direct evidence has been obtained which unequivocally demonstrates the proton-translocating function of the plasma-membrane ATPase of Sacch. cerevisiae. Although some investigators have examined the effect of specific ATPase inhibitors on the ability of intact organisms to translocate protons (Serrano, 1980; Eilam et al., 1984), demonstrations of an inhibitory effect of DCCD, diethylstilbestrol or orthovanadate on proton efflux do not constitute irrefutible proof of an involvement of the plasma-membrane ATPase in this process. Borst-Pauwels et al. (1983) showed that ATPase inhibitors, at the concentrations used in the above studies, caused a non-specific increase in plasma-membrane permeability of Sacch. cerevisiae. Consequently, decreases in measured rates of proton efflux may be produced as a consequence of increased passive

influx of protons into organisms rather than by a direct effect of inhibitors on the plasma-membrane ATPase. Far more convincing evidence for the proton-pumping ability of the plasma-membrane ATPase has come from studies either on plasma-membrane vesicles or reconstituted proteoliposomes containing the purified ATPase enzyme .

The plasma-membrane ATPase has been successfully purified from Sacch. cerevisiae (Malpartida and Serrano, 1980, 1981a, b); Schizosacch. pombe (Dufour and Goffeau,1978, 1980; Villalobo et al., 1981) and N. crassa (Bowman et al., 1981; Scarborough and Addison, 1984). Precise purification methods differ, but in general involve treatment of plasma membranes with various detergents to extract intrinsic proteins followed by density-gradient centrifugation (Goffeau and Slayman, 1981). Analysis of the purified plasma-membrane ATPase by SDS-PAGE has revealed that in all organisms the enzyme is comprised of a single polypeptide of 100,000 - 105,000 molecular weight (Dufour and Goffeau,1978; Malpartida and Serrano, 1980; Bowman et al., 1981). By contrast, the mitochondrial ATPase of Sacch. cerevisiae is a much larger protein with a molecular weight of 400,000 and is composed of 10 distinct polypeptide subunits (Takeshige et al., 1976). Recently, the vacuolar ATPase of Sacch. cerevisiae has also been shown to possess several polypeptide subunits (Lichko and Okorokov, 1985). Differences in polypeptide arrangement of ATPases may have significant evolutionary and mechanistic implications. Both the $[Na^+-K^+]$-ATPase of animal cell membranes (Peterson and Hokin, 1981) and the $[Ca^{2+}]$-ATPase of sarcoplasmic reticulum (Rizzolo et al., 1976) contain major

polypeptides of 100,000 molecular weight, suggesting that this is a characteristic feature of ion-translocating ATPases. The vacuolar ATPase of Sacch. cerevisiae appears, therefore, to be something of an enigma since, although it catalyses proton translocation (Okorokov and Lichko, 1983; Okorokov et al., 1985), it is vanadate-insensitive (Okorokov et al., 1982) and composed of discrete, low molecular-weight subunits (Lichko and Okorokov, 1985).

There is still some debate over the existence of in vivo interactions between plasma-membrane ATPase polypeptides. Both Willsky (1979) working on Sacch. cerevisiae and Bowman et al. (1985) studying N. crassa showed that the catalytic form of the plasma-membrane ATPase was a dimer, consisting of two 100,000 molecular weight polypeptides. Goormaghtigh et al. (1986) demonstrated, however, that both the monomeric and dimeric form of this enzyme catalysed proton translocation in N. crassa.

A report by Scarborough (1976) was the first to implicate directly the plasma-membrane ATPase in the establishment of a Δp. Addition of Mg^{2+} and ATP to purified plasma-membrane vesicles from N. crassa resulted in the formation of an electrical potential across the vesicular membrane . This potential, once established, could be dissipated by addition of proton uncouplers. Translocation of protons was subsequently shown to be responsible for the generation of this vesicular membrane potential (Scarborough, 1980), demonstrating that the plasma-membrane ATPase can catalyse proton translocation and thereby generate a transmembrane electrical potential. An identical conclusion was reached by workers who studied the

function of the plasma-membrane ATPase by reconstituting the purified enzyme in pure phospholipid bilayers to form sealed proteoliposomes. Reconstitituted proteoliposomes containing plasma-membrane ATPase molecules purified from Sacch. cerevisiae (Malpartida and Serrano, 1981a, b), Schizosacch. pombe (Foury et al., 1981; Villalobo et al., 1981; Villalobo, 1984) and N. crassa (Scarborough and Addison, 1984) are all capable of electrogenically translocating protons and establishing membrane potentials. Malpartida and Serrano (1981a) measured a stoichiometry of 1 : 1 between ATP hydrolysis and proton expulsion in proteoliposomes containing plasma-membrane ATPase purified from Sacch. cerevisiae, while Dufour et al. (1982) proposed an ATP : H^+ stoichiometry of 1 : 2 in Schizosacch. pombe.

Reconstitution experiments have also helped to resolve the role of K^+ in proton translocation by the plasma-membrane ATPase. The electrogenic pumping of H^+ ions across the plasma membrane will rapidly result in formation of a membrane potential. However, under certain physiological conditions, Sacch. cerevisiae can establish a significant pH gradient across the plasma membrane, a phenomenon that requires sustained proton pumping. Evidence from reconstitution studies (Villalobo et al., 1981; Dufour et al., 1982) indicates that the membrane potential exerts a powerful regulatory influence on ATPase activity. Uncoupler-mediated dissipation of the $\Delta\psi$ across proteoliposomal membranes has a marked stimulatory effect on the rate of ATP hydrolysis. Consequently, in order for sustained proton translocation to occur, the magnitude of the $\Delta\psi$ must be restricted by a charge compensatory mechanism (de la Peña et al.,1982). In view of the well established stoichiometric relationship between H^+ efflux

and K^+ influx in Sacch. cerevisiae, K^+ might be the ion used, under certain conditions, to compensate for the electrical imbalance produced by proton ejection. Malpartida and Serrano (1981b), in support of this hypothesis, demonstrated that the plasma-membrane ATPase of Sacch. cerevisiae can catalyse both electrogenic H^+ transport and electroneutral H^+/K^+ exchange, although these workers did not indicate whether K^+ uptake occurred directly via the ATPase.

Villalobo (1984) reported that the plasma membrane ATPase of Schizosacch. pombe does indeed function as a K^+ transport system. Villalobo suggested that the plasma-membrane ATPase operates as an electrogenic proton pump until a threshold value of $\Delta\psi$ is attained, triggering the opening of a K^+ gate in the enzyme and thus allowing electroneutral K^+/H^+ exchange to occur.

Although the primary function of the plasma-membrane ATPase of Sacch. cerevisiae is unquestionably the maintenance of a Δp of appropriate magnitude across the plasma membrane, the fact that this enzyme can catalyse proton translocation naturally implicates it in the wider physiological role of intracellular pH regulation. Sanders et al. (1981) demonstrated that plasma-membrane ATPase activity in N. crassa was correlated with intracellular pH value; artificially induced decreases of the internal pH value of this organism resulted in a stimulation of proton pumping. However, as indicated previously, the sustained proton efflux required to increase internal pH could only be achieved by maintaining the plasma membrane in a prolonged state of depolarization. Sanders and his colleagues suggested that an alteration in the permeability of the plasma membrane to organic

anions could allow enhanced anion efflux to occur, dissipating $\Delta\psi$ and hence stimulating proton pumping. It is tempting to link this hypothesis with the finding that addition of organic anions to plasma-membrane preparations from Sacch. cerevisiae stimulates ATPase activity (Ahlers, 1984). Possibly, therefore, anionic efflux can increase proton ejection both by directly stimulating the plasma-membrane ATPase and by having a dissipative effect on the $\Delta\psi$. Sanders and Slayman (1982) showed that, although proton ejection via the ATPase was involved in cellular pH value regulation, the rate of generation of protons was of more fundamental significance, at least in N. crassa. In all probability, pH homeostasis in Sacch. cerevisiae is ensured by the establishment of a dynamic equilibrium between proton generation by intracellular metabolic processes and proton expulsion via the plasma-membrane ATPase.

Solute Transport in Saccharomyces cerevisiae

An important function of the plasma membrane of Sacch. cerevisiae is to restrict and regulate the passage of solutes into and out of the cell. To achieve the required degree of selective permeability specific solute-transport systems are necessary, the activity of which may be linked to energy-yielding metabolic processes. Four main mechanisms of achieving transfer of solutes across membranes have been characterised, namely, simple diffusion, facilitated diffusion, active transport and group translocation. Simple diffusion is a purely physical process in which the rate of transfer of a solute is dependent only on its concentration gradient and, therefore, uptake obeys Fick's law of diffusion:

$$J = -D \frac{dc}{dx}$$

where J is the rate of movement or flux, D is the diffusion coefficient and dc/dx is the concentration gradient. A permeability barrier, such as the plasma membrane,will impose considerable restrictions on the number of molecular species capable of entering the cell by free diffusion. The likelihood of a solute being able to cross the lipid bilayer can be estimated from its octanol:water partition coefficient. The more soluble a species is in octanol, the greater the probability is that its transport occurs by diffusion. The yeast plasma membrane is freely permeable to water, some gases , free ammonia, and to a greater or lesser extent, according to their degree of hydrophobicity, amphipathic molecules. However, a number of biochemically important solutes such as inorganic ions, amino acids and carbohydrates are effectively excluded by the lipid bilayer of the plasma membrane (Eddy, 1982).

Accumulation of these molecules does occur, however, and at considerably higher rates than can be explained by simple diffusion. Facilitated diffusion, active transport and group translocation enable the uptake of compounds with low octanol:water partition coefficients by employing membrane-located carriers. Carrier molecules are intrinsic proteins that span the lipid bilayer of the plasma membrane. They show many of the properties of enzymes, being saturable, temperature dependent and substrate specific. Thus, carrier-mediated solute transport rather than obeying Fick's law, complies with Michaelis-Menten kinetics and consequently can be described by the Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_T + [S]}$$

where v is the velocity of solute transport at a given solute concentration, S, V_{max} is the maximum velocity of solute transport and K_T is the half-saturation constant.

Of the carrier-mediated solute transport processes so far studied in Sacch. cerevisiae, only accumulation of monosaccharide sugars does not involve active transport, uptake of these compounds occurring by facilitated diffusion (Eddy, 1982). Facilitated diffusion is really only a variation of simple diffusion, the difference being the involvement of a carrier protein. The feature of facilitated diffusion that distinguishes it from other carrier-mediated transport processes is that facilitated diffusion systems operate independently of metabolic energy. Consequently, they cannot operate against concentration gradients and once an equilibrium distribution of any given solute is established across the plasma membrane, net uptake of that solute ceases. Group translocation, by contrast, is a metabolically dependent process capable of achieving concentrative uptake of solutes. As distinct from 'true' active transport, however, an enzyme-mediated chemical modification of the transported solute is an integral part of all group translocation systems.

Active transport is similar to facilitated diffusion insofar as a carrier protein is involved and saturation kinetics can be observed. The crucial difference is that active transport systems are coupled to cellular metabolism, enabling the input of energy necessary to carry out solute uptake against a concentration gradient. Therefore, as distinct from other carrier-mediated processes, active transport results in concentrative intracellular accumulation of

chemically unaltered solute molecules. Energy-dependent transport systems can be sub-divided into two main classes. Carriers catalysing primary active transport are coupled directly to exergonic chemical reactions, usually the hydrolysis of ATP. An example of such a system is the proton-translocating ATPase of the yeast plasma membrane. Secondary active transport, on the other hand, depends for its operation on coupling transport proteins to energy-containing electrochemical gradients previously established by primary active transport systems. Active uptake of solutes by Sacch. cerevisiae is mediated by such transport systems, the energy necessary to drive concentrative uptake being provided by the Δp generated via the proton-translocating ATPase.

Saccharomyces cerevisiae takes up several groups of solutes via Δp-energized active transport systems; these include amino acids, disaccharide sugars and inorganic ions. The mechanism of amino-acid uptake in this organism has been particularly thoroughly investigated. In general, uptake of amino acids is unidirectional, occurs against concentration gradients and is prevented by metabolic inhibitors, all features which indicate the active nature of amino-acid accumulation by Sacch. cerevisiae. Amino-acid transport systems can be sub-divided into two groups. The first group of transport systems are those that are specific for one, or a small number of specific amino acids. The second category of carriers, of which there is only one representative in Sacch. cerevisiae, namely, the general amino-acid permease (GAP), can catalyse uptake of a wide range of amino acids. The characteristics of the principal amino-acid permeases so far detected in Sacch. cerevisiae are summarised in Table 1.

Table 1. The Well Characterised Amino-Acid Permeases of Saccharomyces cerevisiae

	Primary substrates	Apparent K_T value (μM)	Reference
Lysine permease	L-lysine	25	Grenson (1966)
Arginine permease	L-arginine	10	Grenson et al. (1966)
	L-lysine	200	Larrimore and Roon (1978)
Histidine permease	L-histidine	20	Crabeel and Grenson (1970)
Methionine permease	L-methionine	12	Gits and Grenson (1967)
Dicarboxylate permease	L-glutamic acid	17	Darte and Grenson (1975)
	L-aspartic acid	70-180	Kotyk et al. (1971)
Leucine permease	L-leucine	50	Ramos et al. (1980)
Proline* permease	L-proline	25	Lasko and Brandriss (1981)
General amino-acid permease	All amino acids and proline	variable	Grenson et al.(1970)

* Imino acid

For the purpose of this thesis, further discussion of amino-acid transport will be limited to a consideration of the GAP. The GAP of Sacch. cerevisiae is a high-capacity, broad-specificity transport system subject to nitrogen metabolite repression (Grenson et al., 1970) and consequently is not expressed,to any appreciable extent, in organisms grown in the presence of ammonium ions. The physiological function of the GAP appears to be that of an amino-acid scavenger, operating under conditions of nitrogen starvation. The GAP can effect uptake of all amino acids including D-amino acids (Rytka, 1975), a feature which has been used to select mutants lacking GAP activity, as well as proline (Lasko and Brandriss, 1981). For a number of amino acids, including glycine, tryptophan, tyrosine, alanine and phenylalanine, the GAP is believed to constitute their principal route of entry into Sacch. cerevisiae (Greasham and Moat, 1973; Cooper, 1982). The mechanism of GAP regulation is complex. However, modulation of the activity of this transport system is known to involve both ammonia inhibition and catabolic nitrogen repression. In a kinetic study, Grenson et al. (1970) showed that ammonium ions directly inhibited GAP activity but did not affect its synthesis. Genetic analyses (Grenson and Hou, 1972; Roon et al., 1975) indicated that ammonium-mediated inhibition of the GAP resulted from uptake of ammonium ions and their subsequent incorporation into α-amino groups of certain amino acids, particularly glutamic acid. Indeed, this amino acid seems to be intimately involved in repression and derepression of GAP activity. The GAP consists of four distinct polypeptides. Three of these components, with molecular weights of 53,000, 45,000 and 30,000 respectively, exist in a plasma membrane-bound multi-protein complex. The fourth polypeptide, a loosely-bound periplasmic protein with a molecular weight of 14,000, is responsible for binding amino

acids (Woodward and Kornberg, 1980) and its presence is essential for the uptake process.

The process of glycine accumulation by the GAP was chosen by Eddy and his coworkers (Eddy et al., 1970a, 1970b; Eddy and Nowacki, 1971) to investigate the mechanism of energy-coupling during amino-acid transport by Sacch. cerevisiae (Sacch. carlsbergensis). Eddy et al. (1970a) depleted yeast suspensions of ATP by adding antimycin and 2-deoxy-D-glucose, and showed that de-energized organisms could still accumulate glycine against a concentration gradient indicating that glycine uptake by the GAP occurs via a secondary active transport mechanism. Eddy et al. (1970b) demonstrated that the rate of glycine uptake under energy-depleted conditions was strongly dependent on extracellular pH; decreasing the pH value of the medium resulted in a marked increase in glycine transport. Increasing external K^+ concentrations, however, appeared to produce an inhibitory effect on glycine uptake. Eddy and his colleagues suggested that protons act as a cosubstrate of the glycine-transport system with the transmembrane pH gradient providing the energy necessary for concentrative uptake of this solute. Furthermore, it was envisaged that, under de-energized conditions, K^+ efflux from organisms compensates for the influx of positive charges produced by the electrogenic co-transport of glycine and protons. Under energy-replete conditions, electrical neutrality is maintained by an outward flow of H^+ ions through the plasma-membrane proton pump.

A glycine:proton stoichiometry of 1:2 was measured in energy-depleted Sacch. cerevisiae (Eddy and Nowacki, 1971) and an expulsion

of 2 K^+ ions for each glycine molecule transported was also observed. Eddy and his coworkers had consequently shown that amino-acid uptake could occur independently of ATP hydrolysis by demonstrating that glycine accumulation in Sacch. cerevisiae is facilitated by an electrogenic proton-symport mechanism, coupling between an electrochemical proton gradient and a solute-transport system occurring in the way predicted by Mitchell (1963).

Subsequent studies have provided confirmatory evidence for the operation of a proton symport in amino-acid uptake. Seaston et al. (1976) found that the extent of glycine accumulation by Sacch. cerevisiae was decreased following addition of the proton uncoupler 2,4-DNP to suspensions. Presumably, 2,4-DNP allows non-specific proton conduction through the plasma membrane to occur, short-circuiting the proton gradient necessary for glycine transport. A recent study by Ballarin-Denti et al. (1984) showed that, rather than the overall pH difference across the plasma membrane, it is the intracellular pH value that regulates the extent of glycine accumulation by Sacch. cerevisiae, extracellular pH value having only a weak effect on glycine influx. A similar proton-symport mechanism to that described in Sacch. cerevisiae was shown by Sanders et al. (1983) to be responsible for amino-acid uptake by the general amino-acid transport system of N. crassa, a constant H^+:amino acid stoichiometry of 2:1 being measured by current-voltage analysis. The operation of specific amino-acid permeases in Sacch. cerevisiae also involves a proton-symport mechanism. Seaston et al. (1973) established 1:1 proton: amino acid stoichiometries for accumulation of L-methionine, L-proline and L-arginine via their specific permeases, while a 2-3:1 proton to

L-glutamate ratio was reported by Cockburn et al. (1975).

Saccharomyces cerevisiae catalyses uptake of certain disaccharide sugars by a proton-symport mechanism. Maltose (Seaston et al., 1973; Serrano, 1977), α-methyl-D-glucoside (Brocklehurst et al., 1978), trehalose (Kotyk and Michaljaničová, 1979) and sucrose (Santos et al., 1982b)are accumulated via multiple, inducible and largely uncharacterised transport systems all of which are concentrative and metabolically dependent. A disaccharide:proton stoichiometry of 1:1 has been established (Serrano, 1977; Brocklehurst et al., 1978; Loureiro-Dias and Peinado, 1984). This evidence and the finding that maltose uptake is inhibited both by proton uncouplers and extracellular K^+ (Serrano, 1977) strongly suggests that disaccharide uptake in Sacch. cerevisiae occurs by a proton-symport mechanism similar to the one responsible for amino-acid accumulation by this organism.

The kinetics of monovalent cation uptake in Sacch. cerevisiae are rather complex. Potassium, as I have indicated, is involved in regulation of the $\Delta\psi$ and this might influence the mechanism of accumulation of this ion (Barts and Borst-Pauwels, 1983). The existence of a K^+ transport system was first demonstrated by Conway and Duggan (1958) who showed that this system also catalysed Rb^+, Na^+, Cs^+, Li^+ and NH_4^+ uptake, although all of these ions showed lower affinities for the carrier than K^+. Subsequent studies, both in Sacch. cerevisiae (Armstrong and Rothstein, 1964; Borst-Pauwels et al., 1971) and N. crassa (Slayman and Slayman, 1970) demonstrated that K^+ uptake deviated from Michaelis-Menten kinetics in a manner dependent on the extracellular pH value. Borst-Pauwels et al. (1971) invoked a model

for K^+ transport involving three binding sites, by proposing the existence of a modifier and activation site in addition to the substrate site. Depending on their respective concentrations, the activation and modifier sites may be occupied by K^+ or H^+ ions. Although these two sites are not directly involved in ion translocation, the nature of the ion bound to them influences the affinity of the substrate site for K^+ and therefore affects the kinetics of uptake. Accumulation of K^+ is generally believed to be energized by Δp and probably involves a K^+/H^+ symport mechanism (Peña, 1975; Boxman et al., 1984). However, the plasma-membrane ATPase may, as was indicated previously, be directly involved in K^+ uptake by functioning as a K^+/H^+ antiport system (Villalobo, 1984). In addition to the K^+ carrier, Sacch. cerevisiae also possesses a second monovalent cation-transport system responsible for metabolically dependent, high-affinity NH_4^+ uptake (Roon et al., 1975; Bogonez et al., 1983).

Divalent cations are transported into Sacch. cerevisiae via a relatively non-specific carrier capable of catalysing Mg^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+} and Ca^{2+} uptake (Norris and Kelly, 1977). Transport of divalent cations is an energy-dependent process (Fuhrmann, 1973; Okorokov et al., 1977) and appears to be linked to ATPase activity (Fuhrmann, 1974). Although no direct evidence has yet been presented for a divalent cation/proton symport in Sacch. cerevisiae, the existence of such a carrier has been established in N. crassa (Stroobant and Scarborough, 1979). Eilam (1983, 1984) suggested that Ca^{2+} uptake in Sacch. cerevisiae is driven by the $\Delta\psi$ since hyperpolarization of the plasma membrane stimulated Ca^{2+} influx.

Phosphate uptake into Sacch. cerevisiae is a complicated process involving at least three transport systems (Borst-Pauwels, 1981). One of these is an inducible high-affinity H^+/phosphate cotransport system with an $H^+:PO_4^{3-}$ stoichiometry of 3:1 (Cockburn et al., 1975). Little is known about the energization of either of the other two phosphate carriers except that uptake is active (Borst-Pauwels and Jager, 1969) and seems to depend on the $\Delta\psi$ (Roomans and Borst-Pauwels, 1977). Sulphate is transported by Sacch. cerevisiae via a single, metabolically-dependent transport system (Horák et al., 1981). Roomans et al. (1979) showed that sulphate uptake involved a proton symport and these workers established an $H^+:SO_4^{2-}$ stoichiometry of 3:1 in Sacch. cerevisiae.

Energization of solute transport by the Δp is consequently a well established phenomenon. Indeed, uptake of most solutes by Sacch. cerevisiae appears to involve a direct proton-symport mechanism. Future studies may implicate the Δp in energization of other plasma membrane-associated processes since studies on bacteria have shown that the Δp is involved both in the incorporation of proteins into the plasma membrane (Enequist et al., 1981) and in the secretion of proteins into the periplasm (Daniels et al., 1981).

ETHANOL AND MEMBRANES

Effects of Ethanol on Micro-Organisms other than Saccharomyces cerevisiae

As in most areas of microbial biochemistry and physiology, Escherichia coli has been the most extensively studied prokaryote with respect to the physiological effects of ethanol and other

alcohols. Ethanol causes a dose-dependent inhibition of growth in E. coli, 0.7 M being sufficient to cause a 50% decrease in the growth rate of E. coli K-12 (Fried and Novick, 1973). The toxic effect of aliphatic alcohols on bacterial growth can be directly related to their chain length and hydrophobicity (Harold, 1970) and, in E. coli, the growth-inhibitory effect of alkanols approximately doubles with each additional carbon up to octanol (Ingram and Buttke, 1984). Longer chain alkanols (C_{10} and greater) are comparatively ineffective as inhibitors.

A series of publications by Ingram and his coworkers demonstrated that the ability of ethanol to affect plasma-membrane structure and function is of crucial importance in the inhibitory effect of this compound on E. coli. The capacity of ethanol for perturbing plasma-membrane lipid organisation was shown to be of particular significance. Ingram (1976) revealed that addition of alkanols of different chain lengths to cultures of E. coli K-12 produced differential effects on the fatty-acyl composition of cellular phospholipids. The presence of alkanols of chain length C_1 - C_4 caused an increase in the proportion of vaccenyl ($C_{18:1}$) residues in phospholipids, at the expense of palmitoyl ($C_{16:0}$) residues. Alkanols of C_5 - C_{10} chain length, however, induced the opposite response. Ingram (1976) suggested that these changes in phospholipid fatty-acyl composition represented an adaptive membrane alteration to compensate for destabilising interactions of alkanols with membrane lipids. Principally, according to Ingram, these modifications are necessary to maintain an environment of appropriate fluidity for the activity of intrinsic membrane proteins. Addition of short-chain alkanols was believed to cause an overall decrease in membrane fluidity, principally by insertion of alkanol molecules into

gaps in the hydrophobic interior of lipid bilayers. Such insertion could result in a decrease in the mobility of phospholipid fatty-acyl side chains and hence precipitate a decrease in membrane fluidity. Adaptation to growth in the presence of short-chain alkanols, therefore, necessitates increasing the proportion of unsaturated fatty-acyl residues in plasma-membrane phospholipids in order to counteract this alkanol-induced decrease in fluidity. Longer-chain alkanols are too large to fill gaps in the hydrophobic core of lipid bilayers without creating additional ones. Hence, they increase membrane fluidity, and their addition to cultures of E. coli results in an increase in the palmitoyl residue content of membrane phospholipids in an attempt to restore optimal fluidity.

More recently, adaptive changes in fatty-acyl composition have been shown to be of somewhat less than primary importance in determining how alkanols alter membrane fluidity in E. coli. The existence of a differential response of E. coli to the presence of alkanols of different chain length was questioned by Sullivan et al. (1979). Sullivan and her coworkers found that alkanols of chain length C_4 - C_8 all caused an increase in the degree of plasma-membrane fatty-acyl unsaturation in E. coli. Consequently, they suggested that perhaps all alkanols cause an increase in membrane fluidity, the extent of this fluidizing effect being related to the hydrophobicity of individual alkanols.

In a more recent study from Ingram's laboratory, Dombek and Ingram (1984) used a variety of fluorescent lipid probes to examine the fluidity gradient across the width of E. coli plasma membranes.

The addition of ethanol or hexanol resulted in increased rather than decreased overall membrane fluidity. These data conflicted with the previous findings of Ingram (1976). Ethanol produced a relatively small increase in lipid mobility, and this effect was confined to the exterior of the plasma membrane. Vollherbst-Schneck et al. (1984) showed a similar fluidizing effect of ethanol on isolated lipid arrays from E. coli K-12. Hexanol, however, affected the mobility of fluorescent probes more deeply located in the plasma membrane. Taking these results into consideration, Dombek and Ingram (1984) proposed a general model for the interaction of alkanols with lipid bilayers. They suggested that the hydroxyl groups of alkanols are localised near the surface of bilayers, interacting via hydrogen bonding with polar surface groups. The hydrocarbon chains of alkanols were envisaged as penetrating towards the centre of the lipid bilayer, disrupting hydrophobic interactions necessary for maintaining the required level of membrane rigidity. The combined result of these effects would be an increase in membrane fluidity. In addition, Dombek and Ingram (1984) showed that growing E. coli in the presence of sub-lethal concentrations of ethanol resulted in decreased membrane fluidity. Consequently, increases in fatty-acyl unsaturation are probably not important in adaptation to ethanol. Dombek and Ingram (1984) suggested that a decrease in the lipid:protein ratio of the plasma membrane might be involved in the acquisition of resistance to ethanol.

It appears, therefore, that ethanol has a disordering effect on the lipid bilayer of the plasma membrane in E. coli. The consequences of alkanol-induced increases in plasma-membrane fluidity

appear to be considerable. Ethanol caused an increase in the release of nucleotides from E. coli, the extent of this effect being less pronounced in organisms adapted by growth in the presence of ethanol (Eaton et al., 1982). Eaton and his coworkers concluded that increased metabolite leakage, facilitated by an ethanol-induced decrease in the integrity of the plasma membrane in E. coli, is of primary importance in bacterial inactivation by this alkanol. Membrane-located enzymes and solute-transport systems of E. coli have also been shown to be affected by ethanol. Interestingly, plasma-membrane bound enzymes, such as the ATPase, NADH oxidase and D-lactate oxidase, are relatively resistant to inactivation by ethanol (Eaton et al., 1982). However, the lactose permease (Fried and Novick, 1973; Ingram et al., 1980) and certain specific amino-acid permeases (Eaton et al., 1982) of this organism are considerably more sensitive to ethanol. Transport of lactose and amino acids in E. coli is active and occurs via an ion cotransport mechanism. Consequently, the ability of ethanol to increase ionic leakage across the plasma membrane has been invoked as the reason for the increased sensitivity of solute-transport systems, as compared with membrane-bound enzymes, to alkanol inactivation (Ingram and Buttke, 1984). The existence of alkanol-enhanced membrane leakage has also been demonstrated in Pseudomonas aeruginosa. Addition of alkanols to suspensions of this organism resulted in a marked increase in cellular permeability to both H^+ and K^+ ions (Bernheim, 1978).

Studies on bacteria other than E. coli have shown similar effects of ethanol on membrane structure and function. Supplementing cultures of Mycobacterium smegmatis with ethanol caused temperature-dependent

alterations in the fatty-acyl composition of cellular phospholipids (Taneja and Khuller,1980). Presumably, such modifications are designed to counteract changes in membrane fluidity induced by ethanol addition.

Of perhaps greater significance are studies on mechanisms of adaptation in bacteria whose natural environment contains appreciable concentrations of ethanol or other alkanols. The most ethanol-tolerant of all organisms are the hiochii bacteria commonly found as contaminants of saké. Lactobacillus heterohiochii and L. homohiochii are able to thrive in 20% (v/v) ethanol. Growth of L. heterohiochii is actually stimulated by incorporation of low concentrations of ethanol in culture media (Demain et al., 1961). The unusual lipid composition of L. heterohiochii, in particular the presence of extremely long-chain fatty-acyl residues (C_{20} - C_{30}; Uchida, 1975), may be responsible for the remarkable ethanol tolerance of this organism. The presence of such long chain fatty-acyl residues in membrane phospholipids will strengthen hydrophobic interactions in the interior of the lipid bilayer, minimising the disruptive effect of ethanol. Phospholipids of L. heterohiochii contain a high proportion of vaccenyl residues, as indeed do the lipids of another ethanol-tolerance species, namely, Zymomonas mobilis (Carey and Ingram, 1983). This suggests that enrichment of membranes with $C_{18:1}$ residues may, after all, be involved in adaptation to ethanol. Zymomonas mobilis is an obligate ethanol producer and considerable accumulations of ethanol occur during fermentations by this bacterium (Swings and de Ley, 1977). The ability of ethanol to increase metabolite leakage, as shown by Eaton et al. (1982) in E. coli, has also been demonstrated in Z. mobilis. Addition

of ethanol to suspensions of Z. mobilis caused a non-selective increase in plasma-membrane permeability (Osman and Ingram, 1985), resulting in the loss of inorganic ions and nucleotides from organisms. By supplying exogenous Mg^{2+}, some restoration of fermentative activity could be achieved even in the presence of high concentrations of ethanol (4.4 M). This evidence suggests very strongly that membrane leakage and not enzyme denaturation is responsible, at least in part, for the inhibitory effect of ethanol on growth and fermentation in Z. mobilis.

Certain clostridia are also of interest in terms of alkanol production and tolerance. Addition of 0.15 M butanol to growing cultures of the butanol producer Clostridium acetylbutylicum resulted in a 50% decrease in growth rate (Moreira et al., 1981). This concentration of butanol was also sufficient to halve the rate of glucose uptake by Cl. acetylbutylicum. Moreira and his colleagues postulated that the inhibitory effect of ethanol on glucose transport, and possibly uptake of other solutes, is primarily responsible for the decrease in growth rate. Cultures of Cl. acetylbutylicum appear to respond to incorporation of low concentrations of butanol during growth by increasing the proportion of saturated fatty-acyl residues in cellular phospholipids (Vollherbst-Schneck et al.,1984). Two other clostridia, namely, Cl. thermocellum and Cl. thermohydrosulphuricum have been studied with a view to using them to produce ethanol on an industrial scale. Herrero and Gomez (1980) and Herrero et al. (1982) isolated ethanol-resistant strains of Cl. thermocellum capable of growth in considerably higher concentrations of ethanol than the parent strain. Increases in fatty-acyl unsaturation were implicated in the increased ethanol

tolerance of these mutants. Cl. thermohydrosulphuricum is inherently more resistant than Cl. thermocellum to the growth-inhibitory effects of ethanol and other organic solvents. Lovitt et al. (1984) suggested that this general solvent tolerance may be a result of the unique membrane lipid composition of this organism. Cl. thermohydrosulphuricum, but not Cl. thermocellum, synthesises a C_{30} dicarboxylic acid believed to exist in vivo as a tetra-ester of glycerol. The presence of such a long-chain component, should it be located in a membrane, would increase the rigidity of membrane lipids, perhaps preventing the fluidizing effects of ethanol and other organic solvents.

With the exception of Sacch. cerevisiae, little is known about the effects of ethanol on eukaryotic micro-organisms. Growth of Tetrahymena pyriformis, a free-living protozoan, in the presence of ethanol elicited an alteration in phospholipid composition (Nandini-Kishore et al., 1979). Increased levels of $C_{18:2}$ fatty-acyl residues were observed in membrane phospholipids at the expense of both $C_{16:1}$ and $C_{16:2}$ residues. Nandini-Kishore and his colleagues suggested that, since ethanol preferentially increases fluidity in the peripheral regions of membranes, increasing the degree of phospholipid fatty-acyl unsaturation might equalise the fluidizing effect of ethanol by enabling greater lipid mobility in the interior of the bilayer. This explanation of how an increase in fatty-acyl unsaturation could minimise the deleterious effects of ethanol on membrane organisation might explain why plasma membranes of ethanol-tolerant bacteria are enriched in $C_{18:1}$ fatty-acyl residues (Uchida, 1975; Carey and Ingram, 1983).

Effects of Ethanol on Saccharomyces cerevisiae

As a consequence of the industrial importance of Sacch. cerevisiae as well as its position as one of the most ethanol-tolerant organisms known, a considerable number of studies have been undertaken in an attempt to discover the physiological basis of this yeast's tolerance to ethanol. In general, factors which influence the effect of ethanol on prokaryotic micro-organisms are equally important in Sacch. cerevisiae. Both the inhibitory effects of ethanol on Sacch. cerevisiae and the ability of this organism to tolerate high concentrations of ethanol are dependent on the lipid composition of the plasma membrane (Ingram and Buttke, 1984). Although ethanol has been reported to affect numerous metabolic and biosynthetic processes in Sacch. cerevisiae, functions associated with the plasma membrane are believed to constitute the principal targets for the inhibitory action of this compound.

Nevertheless, the underlying mechanisms of ethanol tolerance in Sacch. cerevisiae are still incompletely understood. The absence of an unequivocal solution to this problem is almost entirely attributable to the fact that the organism itself produces ethanol. Ethanol production also occurs in fermentative bacteria, but studies on these organisms have yet to reveal how this function affects the parameters of ethanol tolerance.

Production of ethanol by Sacch. cerevisiae has an inhibitory effect on both the growth rate and fermentative activity of the yeast. Brown et al. (1981) reported a K_i value of 0.97 M for inhibition of fermentation by ethanol, but only 0.44 M ethanol was required to lower the growth rate of this particular strain of

Sacch. cerevisiae by 50%. This ability to determine distinct inhibition constants for the effects of ethanol on growth and fermentation implies the existence of different targets for the action of ethanol on these two complicated physiological processes. Brown and his coworkers also found that a saké yeast strain was able to retain fermentative activity in the presence of higher concentrations of ethanol compared with a haploid laboratory strain, but could not sustain growth at elevated concentrations of ethanol. This supports the concept of a multiplicity of ethanol-sensitive sites in Sacch. cerevisiae since saké strains will be selected for their ability to ferment rather than proliferate in substantial concentrations of ethanol. This study illustrates the complexity of ethanol tolerance in Sacch. cerevisiae since it shows that, in all probability, no single ethanol-sensitive process exists whose inactivation can account for the inhibitory effect of ethanol on growth, fermentation and the maintenance of viability by Sacch. cerevisiae. Not all investigators, however, have agreed on the separability of inhibitory effects of ethanol on Sacch. cerevisiae. Both Luong (1985) and Aguilera and Benitez (1985) could not kinetically differentiate between the inhibitory action of ethanol on growth and fermentation. As with many physiological properties of Sacch. cerevisiae, it appears that strain differences and variations in the conditions used to culture organisms influence the kinetics of ethanol inhibition, further complicating attempts to resolve mechanisms of ethanol tolerance.

The potential complexity of inhibitory effects of ethanol on Sacch. cerevisiae is undoubtedly due, at least in part, to the relatively complicated physiology of this organism when compared with

prokaryotes. Since Sacch. cerevisiae is a eukaryote it possesses a considerably higher level of intracellular organisation than prokaryotic micro-organisms such as E. coli. The presence of functional mitochondria, for example, appears to influence the effect of ethanol on growth and fermentation. Aguilera and Benitez (1985) showed that the presence of respiratory processes increased the ethanol tolerance of a laboratory strain of Sacch. cerevisiae. The factor responsible for this improvement in tolerance appeared not to be respiration per se, but rather alterations in cellular composition and physiology induced by respiration. The inner mitochondrial membrane may, under certain circumstances, constitute an important target site for ethanol. Leão and van Uden (1982a) demonstrated that alkanols enhanced thermal death in Sacch. cerevisiae and thermosensitive sites in this organism are probably located in the inner mitochondrial membrane. however, Sá -Correia and van Uden (1986) showed that, although ethanol addition at normal growth temperatures resulted in a loss of viability in populations of Sacch. cerevisiae, this was caused by a separate mechanism from thermal inactivation, with the sensitive sites being primarily located in the plasma membrane.

Metabolic production of ethanol by Sacch. cerevisiae raises the possibility of intracellular accumulation of this compound. Extracellular ethanol concentrations of 1 - 2 M are inhibitory to growth and fermentation in even the most tolerant strains of Sacch. cerevisiae (Rose, 1980). What is not so certain,however, is whether ethanol is accumulated by this organism such that higher intracellular concentrations of ethanol are attained during the course of laboratory, and more importantly, industrial fermentations.

Determination of intracellular concentrations of ethanol in organisms poses considerable experimental difficulties, and this probably explains the discrepancies in reported values for this concentration. The first evidence for ethanol accumulation by Sacch. cerevisiae came from studies on so called "rapid fermentations" by Nagodawithana and Steinkraus (1976). To achieve an increased rate of ethanol production, media with a high sugar content were inoculated with large numbers of organisms and the fermentation carried out at 30°C. Although this resulted in an increased fermentation rate, 9.5% (v/v) ethanol being produced in only 3 h, the viability of populations decreased substantially during the course of fermentations. By lowering the inoculum size and decreasing the fermentation temperature to 15°C, the incubation time necessary to produce the same concentration of ethanol was doubled but the proportion of viable organisms in cultures remained high. Nagodawithana and Steinkraus (1976) suggested that intracellular accumulation of ethanol during rapid fermentations was responsible for increased cellular damage and hence loss of viability. These workers proposed that, under rapid fermentation conditions, the rate of ethanol production exceeds the rate of efflux of ethanol and, consequently, internal accumulation of ethanol occurs. Lowering the rate of ethanol generation by decreasing growth temperature and inoculum size, enables diffusion of ethanol across the plasma membrane to keep pace with ethanol production and prevents accumulation of ethanol to toxic levels. Intracellular ethanol accumulation was subsequently shown not to be confined only to high-density fermentations of Sacch. cerevisiae (Navarro and Durand, 1978; Beavan et al., 1982). To enable high intracellular: extracellular ethanol ratios to be established, under certain conditions

as high as 10:1 (Beavan et al., 1982), either the ethanol permeability of the yeast plasma membrane is unaccountably low or Sacch. cerevisiae is capable of intracellularly binding substantial amounts of ethanol. Novak et al. (1981) showed that endogenously produced ethanol was more toxic to growth of Sacch. cerevisiae than exogenously added ethanol. These workers suggested that this was due to the inability of ethanol to cross the plasma membrane and therefore low plasma-membrane permeability might enable accumulation of intracellular ethanol to take place.

However, recent studies have provided conclusive evidence that ethanol accumulation does not occur in Sacch. cerevisiae. Loureiro and Ferreira (1983) showed that the plasma membrane of this organism is highly permeable to ethanol. Consequently, any technique for determining intracellular ethanol concentrations that involves washing organisms is liable to be inaccurate, casting doubt on the measurements of intracellular ethanol levels performed by Nagodawithana and Steinkraus (1976). Loureiro and Ferreira (1983) also found no evidence for ethanol binding sites in Sacch. cerevisiae. It seems that, regardless of culture conditions, the rate of ethanol efflux from organisms always exceeds the rate of ethanol production. Guijarro and Lagunas (1984) found that, under all experimental conditions tested, intracellular ethanol concentrations were remarkably similar to concentrations of ethanol in the culture filtrate. Dasari et al. (1986) were unable to demonstrate substantial differences between high and low-density fermentations in terms of the loss of viability of organisms. They concluded that, rather than ethanol being responsible for increased viability losses in rapid fermentations, as

reported by Nagodawithana and Steinkraus (1976), this effect was probably produced as a consequence of imbalances in cellular osmolarity incurred as cultures were diluted prior to the determination of viability.

It is becoming increasingly apparent, therefore, that Sacch. cerevisiae does not accumulate ethanol to any appreciable extent. Consequently, any metabolic process which is important in relation to the inhibitory effect of ethanol on growth, fermentation and viability must be sensitive to ethanol concentrations similar to those found extracellularly in yeast fermentations.

The lipid content and composition of Sacch. cerevisiae is unquestionably a determining factor in the ability of this organism to tolerate ethanol. Perturbation of membrane lipid organisation is involved in the inhibitory effects of ethanol on all biological processes including growth of, and glycolytic fermentation by, Sacch. cerevisiae. Gray (1941, 1948) was the first to link variations in the ability of yeasts to tolerate ethanol with the lipid content of organisms. Strains of Saccharomyces with a low content of lipid were generally more resistant to the inhibitory effects of ethanol on growth and fermentation, and Gray (1948) suggested that organisms with high cellular lipid contents are more permeable to ethanol and therefore more susceptible to the inhibitory effects of this alkanol. The relationship between ethanol action and lipid content of Sacch. cerevisiae was further substantiated by Gray and Sova (1956) who demonstrated that all alkanols produced similar damaging effects on this organism. However, the magnitude of alkanol-induced inhibition

increased as the hydrocarbon chain length of alkanol molecules increased. Consequently, Gray and Sova (1956) concluded that ethanol has a non-specific inhibitory effect on Sacch. cerevisiae caused by disruption of hydrophobic, probably lipid, 'target' sites. It is, therefore, not surprising that alterations in lipid content are considered to be important in the acquisition of ethanol tolerance, although precisely how experimentally induced changes in lipid composition influence the ability of Sacch. cerevisiae to resist ethanol inactivation remains unclear.

The importance of lipid composition in ethanol tolerance has perhaps been most convincingly demonstrated in studies where growing cultures of Sacch. cerevisiae were supplemented with individual lipids. Such lipid supplementation appears to occur naturally as an integral part of the saké fermentation. Production of saké is a complicated process involving the inoculation of rice extracts, known as koji, with both Sacch. cerevisiae and Aspergillus oryzae, followed by a prolonged low-temperature fermentation. In a laboratory study, Hayashida et al. (1974) demonstrated that 20% (v/v) ethanol could be produced by sake yeasts in a chemically defined medium, but only if they were supplied with a proteolipid mixture derived from the cell envelope of A. oryzae. Supplementation of Sacch. cerevisiae cultures with this proteolipid enhanced cell growth, fermentative activity and increased the ability of organisms to retain viability in the presence of ethanol. Hayashida and Ohta (1980) showed that the lipid moiety of the proteolipid from A. oryzae was composed almost entirely of phosphatidylcholine, containing a preponderance of $C_{18:1}$ fatty-acyl residues, and ergosterol ester. Supplementation of yeast

cultures with phosphatidylcholine from A. oryzae increased the ability of organisms to grow and ferment in high concentrations of ethanol, while addition of ergosterol ester aided retention of viability. This evidence illustrates the complexity of lipid effects on ethanol tolerance in Sacch. cerevisiae,as well as further substantiating the idea that different sites of action are responsible for the effect of ethanol on growth, fermentation and viability.

Lipid supplementation of Sacch. cerevisiae cultures was also shown by Thomas et al. (1978) to influence the ethanol tolerance of this organism. By exploiting the requirement of Sacch. cerevisiae, under anaerobic conditions, for exogenously supplied sterol and unsaturated fatty acid, Thomas and her colleagues showed that organisms whose plasma membranes were enriched in linoleyl ($C_{18:2}$) residues, as opposed to oleyl ($C_{18:1}$) residues, were more resistant to the effect of ethanol on viability. The nature of sterol supplementation was also shown to be important in the retention of viability in the presence of ethanol. Addition of ergosterol to Sacch. cerevisiae cultures appeared to be more effective than supplementation with cholesterol in conferring tolerance to ethanol. Thomas et al. (1978) invoked two separate mechanisms to explain the effects of fatty-acyl unsaturation and sterol enrichment on ethanol tolerance. Increased fatty-acyl unsaturation of membrane lipids, it was suggested, produces a more fluid membrane better able to accommodate ethanol without undue perturbation of structural integrity. The protective effect of ergosterol enrichment was thought to be due to the ability of this sterol to form more stable complexes than cholesterol with membrane phospholipids, resulting in localised increases in membrane rigidity

and hence decreasing the ability of ethanol to partition into these regions of membranes.

Not all studies on the effect of lipid supplementation on ethanol tolerance have concurred with the findings of Thomas et al. (1978). Indeed, enrichment of membrane phospholipids with $C_{18:2}$ fatty-acyl residues, as opposed to $C_{18:1}$ residues, appeared to have a detrimental effect on the ability of certain strains of Sacch. cerevisiae to tolerate ethanol (S.M.F. de Morais, personal communication). Walker-Caprioglio et al. (1985) found that supplementation of cultures of Sacch. cerevisiae with various fatty acids and sterols had no significant effect on the sensitivity of organisms to ethanol, although it must be said that no evidence was presented to show that, under the experimental conditions used, supplementation influenced the lipid composition of cellular membranes.

As yet, there is no definitive answer to the question of how changes in membrane lipid composition affect the interaction of ethanol with Sacch. cerevisiae. Nonetheless, it is clear that the partitioning of ethanol into the lipid phase of yeast plasma membranes results in an increase in lipid mobility (Curtain et al., 1984; Walker-Caprioglio et al., 1985). Consequently, physiological adaptation to ethanol, whether this involves increasing or decreasing overall membrane fluidity, must have the effect of minimising the disruptive influence of ethanol on membrane structure, thereby limiting the inhibitory effect of ethanol on membrane-located processes.

Despite some difficulty in differentiating between primary inhibitory effects of ethanol and secondary effects produced as a consequence of alkanol interaction with more sensitive primary target sites, there is now overwhelming evidence that ethanol-induced inhibition of plasma membrane-located processes is of vital importance in the overall inactivating effect of this compound on Sacch. cerevisiae.

Since ethanol is known to have a distinct inhibitory effect on fermentation, a number of studies have been carried out to determine whether ethanol can inhibit glycolytic enzyme activity in Sacch. cerevisiae. Although it is not a widely held view, certain enzymes of the Embden-Meyerhof-Parnas pathway may be membrane-bound (Green et al., 1965) and consequently an inhibitory effect of ethanol would seem, at least theoretically, to be a likely possibility. Indeed, Nagodawithana et al. (1977) demonstrated that hexokinase activity determined the rate of glycolytic throughput in Sacch. cerevisiae, and suggested that inhibition of this enzyme was a determining factor in loss of fermentative activity produced by ethanol. A similar conclusion was reached by Navarro (1980), although Millar et al. (1982) showed that glyceraldehyde phosphate dehydrogenase was the glycolytic enzyme most sensitive to ethanol inactivation. Significantly, in all three studies, substantial concentrations of ethanol, between 5 and 15% (v/v), were necessary to produce even moderate inhibitory effects on enzyme activity. Millar et al. (1982) concluded that intracellular accumulation of ethanol is an essential prerequisite if glycolytic enzyme denaturation is responsible for the inhibitory effect of ethanol on fermentation. Since it now appears that

intracellular accumulation does not occur and glycolytic enzymes have recently been shown to retain their activity through the duration of fermentations (Larue et al., 1984), it seems unlikely that inhibition of fermentation in Sacch. cerevisiae results from enzyme denaturation.

Ethanol has a pronounced and well characterized inhibitory effect on solute uptake in Sacch. cerevisiae. Accumulation of amino acids (Thomas and Rose, 1979; Leão and van Uden, 1984a), ammonium ions (Leão and van Uden, 1983), maltose (Loureiro-Dias and Peinado, 1982) and glucose (Thomas and Rose, 1979; Leão and van Uden, 1982b) is non-competitively inhibited by ethanol at concentrations similar to those found extracellularly in yeast fermentations. Leão and van Uden (1982b, 1983, 1984a) and Loureiro-Dias and Peinado (1982) showed that the inhibitory effect of alkanols on solute uptake was directly proportional to their lipid-buffer partition coefficients. This importance of alkanol solubility in hydrophobic environments led Leão and van Uden to suggest that perturbations of plasma-membrane lipid organisation, leading to deleterious conformational changes in the membrane-bound transport proteins, is the cause of ethanol-induced inhibition of solute transport in Sacch. cerevisiae. By demonstrating a relationship between plasma-membrane phospholipid composition and ethanol inhibition of solute accumulation, the results of Thomas and Rose (1979) also showed that interactions of ethanol with membrane lipids affect rates of solute uptake.

With the notable exception of glucose, all facilitated transport systems are active and depend on the existence of a proton-motive force across the plasma membrane for their operation

(Borst-Pauwels, 1981; Eddy, 1982). Leão and van Uden (1984b) showed that alkanols enhanced the permeability of the plasma membrane of Sacch. cerevisiae to protons in a manner resembling that of an uncoupling agent. Furthermore, the extent of this effect was a function of the lipid-buffer partition coefficient of alkanols. Thus, ethanol-induced alterations in plasma-membrane permeability, produced as a consequence of alkanol-lipid interactions, could be responsible for the effect of ethanol on solute uptake. Indeed, Jiménez and van Uden (1985) were able to demonstrate a correlation between the inhibitory effect of ethanol on growth of Sacch. cerevisiae and the enhancement of proton diffusion across the plasma membrane produced by this alkanol. The existence of a direct relationship between plasma-membrane permeability to protons and growth rate suggests that the ability of ethanol to de-energize the plasma membrane and hence prevent uptake of metabolically important solutes is a vital factor in the overall toxic effect of this compound on Sacch. cerevisiae.

Effects of Ethanol on Animal and Model Membranes

The available information on interactions between ethanol and animal or model membranes is extensive and a number of substantial reviews (Seeman, 1972; Janoff and Miller, 1982; Michaelis and Michaelis, 1983) have been published on the subject, giving a more comprehensive survey of the published literature than is required here.

The relationship between the anaesthetic effect of alcohols on animal tissues and the solubility of these compounds in the lipid

phase of membranes is well established (Seeman, 1972). Several studies have been conducted with the aim of determining the nature of ethanol-lipid interactions and the consequences of these interactions on lipid organisation. These investigations have been carried out both on model lipid bilayers and on membranes isolated from animal tissue. Jain and Wu (1977) studied the effect of a large number of lipid-soluble compounds on the phase transition behaviour of synthetic phospholipid vesicles. The results of Jain and Wu suggested that the type of effect produced by lipid-soluble compounds on membrane organisation was strongly dependent on the localisation of these agents within the lipid bilayer. Short-chain alkanols (C_1 - C_4) modified phospholipid transition profiles in a way which suggested that they were localised in the C_1 - C_8 region of fatty-acyl side chains of phospholipids, near the exterior surface of the lipid bilayer. Longer chain alkanols (C_5 - C_{10}), however, appeared to be associated with the C_9 - C_{16} regions of phospholipid fatty-acyl residues indicating that these compounds can penetrate the interior of the bilayer. The work of Jain and Wu (1977) appears to support, if only indirectly, the evidence of Ingram (1976) for differential effects of short-chain and long-chain alkanols on the membrane phospholipid composition of E. coli. Lenaz et al. (1976) had previously demonstrated that addition of butanol to isolated beef-heart mitochondrial membranes resulted in an increase in the mobility of phospholipids, this effect being most pronounced near the membrane surface. This also suggests that short-chain alkanols are primarily located in the relatively hydrophilic surface region of membranes. However, as pointed out by Michaelis and Michaelis (1983), even if short-chain alkanols are primarily located in the more polar areas of membranes, disruption of phospholipid head-group distances, hydrogen

bonding and electrostatic interactions could influence fatty-acyl mobility in the interior, as well as the exterior, part of the bilayer.

Studies on the acquisition of ethanol tolerance by animals fed on alcohol-containing diets have demonstrated that alterations in membrane-lipid configuration are important in adaptation to ethanol. Unfortunately, as in micro-organisms, precisely which modifications in membrane-lipid composition are the vital ones in terms of resistance to ethanol remains unclear. The first demonstration of membrane-related tolerance to ethanol in higher animals was provided by Chin and Goldstein (1977a, b). These investigators showed that, after induction of tolerance to ethanol in mice, exposure of isolated erythrocyte membranes to ethanol produced significantly smaller increases in phospholipid fatty-acyl mobility than occurred in membranes from non-adapted mice. Similar results were obtained by Johnson et al. (1979) studying the fluidizing effect of ethanol on brain synaptosomal membranes of control and ethanol-treated mice. However, Johnson and his co-workers showed that, if cholesterol was removed from ethanol-tolerant synaptosomes, the ability of their membranes to resist the fluidizing effect of ethanol was lost. Controlled supplementation with cholesterol in order to produce identical phospholipid: cholesterol ratios in both control and ethanol-resistant membranes resulted in the re-establishment of differences between the two groups in terms of their response to ethanol. The results of Johnson et al. (1979) highlighted an important consideration in determining the effect of lipid composition on ethanol tolerance. The presence of cholesterol was important for expression of tolerance to ethanol, but membrane cholesterol content per se did not appear

to be a significant factor. Rather, it seemed that the ability of this molecule to interact with other membrane components resulted in a decreased effect of ethanol on lipid structure. Consequently, as opposed to changes in the overall membrane content of various lipid classes,precise, localised alterations in lipid-lipid interactions may lead to the modifications necessary to prevent ethanol-induced fluidization.

Chin and Goldstein (1984), however, demonstrated that simple cholesterol enrichment of membranes was sufficient to lower the disordering effect of ethanol. Interestingly, they suggested that, since sterols are unevenly distributed in normal biological membranes, perhaps the major disruptive effects of ethanol occur in areas which are poor in sterol and therefore rich in protein. Thus, improvement in membrane tolerance to ethanol might involve a strengthening of protein-lipid as well as lipid-lipid interactions. Waring et al. (1981) found that ethanol-fed rats had an altered mitochondrial membrane fatty-acyl composition as compared with control animals. Increased levels of saturated ($C_{16:0}$ and $C_{18:0}$) and mono-unsaturated fatty-acyl residues ($C_{18:1}$), and lower amounts of $C_{18:2}$ fatty-acyl residues were envisaged as causing the decreased ethanol-induced phospholipid mobility observed in membranes from ethanol-treated animals. Rather surprisingly, in the absence of ethanol, membranes from ethanol-treated and control animals showed similar levels of phospholipid mobility, making the cause and effect relationship between changes in fatty-acyl composition and membrane fluidity proposed by Waring et al. (1981) seem somewhat questionable. Head-group structure does not appear to influence the interaction of

ethanol with phospholipids, since Taraschi et al. (1985) showed that ethanol increased the mobility of fatty-acyl side chains of phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid to an almost identical extent.

Regardless of precisely which alterations in lipid conformation affect the interaction of ethanol with membranes, there is clear evidence that limiting the extent of ethanol-induced membrane fluidization is involved in the acquisition of resistance to the wider physiological effects of ethanol. Johnson et al. (1980) showed that rats which were tolerant to the anaesthetic effect of ethanol also resisted pentobarbital-induced anaesthesia. Since tolerant organisms were able to prevent the fluidizing effect of both compounds on membrane lipids, Johnson and his colleagues suggested that lipid disordering results, either directly or indirectly, in the onset of anaesthesia.

The ability of ethanol to perturb the organisation of animal membranes has been shown to have important effects on membrane-related biochemical processes in much the same way as occurs in micro-organisms. Particular attention has been paid to the inhibition of membrane-associated enzymes and transport proteins, although more recently the importance of deleterious effects of ethanol on membrane permeability has been demonstrated. The effect of ethanol on the activity of the plasma membrane-bound $[Na^+-K^+]$-ATPase has been extensively examined. Grisham and Barnett (1972) showed that disadvantageous alterations in the immediate lipid environment of ATPase molecules was the primary cause of inhibition of this enzyme by ethanol, addition of 4.5 M ethanol caused a 50% reduction

in ATPase activity (Grisham and Barnett, 1973). By successfully correlating the effectiveness of alkanol inhibition of the $[Na^+-K^+]$-ATPase with hydrocarbon chain length and hence hydrophobicity, Grisham and Barnett (1973) further established lipids as targets for the effect of alkanols on membrane-bound enzymes. Lin (1980), however, suggested that inhibition of $[Na^+-K^+]$-ATPase activity by ethanol was due to a direct effect on a hydrophobic protein component of this enzyme rather than an indirect effect on the lipid environment.

Gordon et al. (1980) showed that benzyl alcohol had a concentration-dependent effect on $[Na^+-K^+]$-ATPase activity in isolated rat-liver plasma membranes. The presence of low concentrations of this alcohol stimulated enzyme activity while higher concentrations were inhibitory. It was suggested that activation of ATPase activity is due to an alcohol-induced increase in bulk lipid fluidity. Inhibition of activity at higher concentrations, according to Gordon et al. (1980), is produced as a result of disruptions in the boundary lipid layer believed to surround intrinsic proteins. These results suggest that, although bulk lipid fluidity influences membrane-bound enzyme activity, the relative importance of lipid-protein interactions in regulating the conformation of individual intrinsic proteins probably determines the response of any given enzyme to disruptions in fluidity. The significance of effects of alcohols on lipid-protein interactions was also demonstrated by Lenaz et al. (1976). These workers showed that addition of butanol to beef-heart mitochondrial membranes caused a more pronounced effect on membrane fluidity than if model phospholipid membranes were treated with identical concentrations of this alkanol. Consequently, disruption of lipid-protein interactions appeared

to be of considerable importance in determining the extent of butanol-induced fluidization of membranes.

Ethanol has also been shown to affect the operation of plasma membrane-located solute-transport systems in higher animals. Israel et al. (1969a, b) showed that uptake of various amino acids by brush border cells of the human small intestine was inhibited by low concentrations of ethanol. Importantly, although the uptake of actively transported amino acids was completely inhibited by 2% (v/v) ethanol, accumulation of D-phenylalanine,which occurs by facilitated diffusion, was unaffected by this concentration of ethanol (Israel, 1969b).Active transport of solutes across the plasma membrane of higher animals is achieved by coupling uptake to an electrochemical Na^+ gradient established by the activity of the $[Na^+-K^+]$-ATPase (Wilson, 1978). The work of Israel (1969b) suggests that inhibition of solute transport might occur as a consequence of an ethanol-mediated increase in the permeability of the plasma membrane to Na^+ ions and/or by an inhibitory effect of ethanol on the $[Na^+-K^+]$-ATPase. The former possibility was strengthened by the finding of Pang et al. (1979) that butanol caused a concentration-dependent efflux of Rb^+ from preloaded phospholipid vesicles. Perturbation of membrane lipid structure, indicated by alterations in lipid probe mobility, was shown to be responsible for this increase in cation permeability.

A correlation between the effects of ethanol on plasma-membrane permeability and solute transport in higher animals was conclusively demonstrated by Hunter et al. (1983). These workers showed that a

dissipative effect of ethanol on the Na^+ gradient across the plasma membrane of rat intestinal brush-border cells was responsible for the inhibitory effect of this alkanol on D-glucose transport. Two separate pieces of evidence led Hunter and his colleagues to reach this conclusion. Firstly, ethanol did not inhibit D-glucose uptake under conditions where a Na^+ electrochemical gradient was absent, and secondly, spin-label studies showed that ethanol had a significant effect on plasma-membrane fluidity and permeability without producing qualitative changes in membrane proteins. Subsequent investigations (Elgavish and Elgavish, 1985; O'Neill et al., 1986) demonstrated that the ability of ethanol to collapse Na^+ electrochemical gradients was responsible for the inhibitory effect of this compound on a number of other solute-transport systems.

The overwhelming conclusion from studies on micro-organisms, higher animals and model membranes is that the inhibitory effects of ethanol on biochemical and physiological processes result from a common mechanism of action. This mechanism centres on the ability of ethanol to partition into the lipid bilayer of membranes, perturbing their structural organisation and hence adversely affecting membrane-associated functions. The plasma membrane of Sacch. cerevisiae appears to be of crucial importance in the inhibitory effect of ethanol on this organism. Consequently, the study reported in this thesis was undertaken in an attempt to determine the effects of ethanol on certain functional properties of the plasma membrane of Sacch. cerevisiae, namely, the generation and maintenance of a Δp and if an ethanol-induced dissipation of Δp is important in the ability of this alkanol to inhibit solute transport. By

determining the phospholipid composition of isolated membranes it was hoped that alterations in lipid arrangement, and perhaps membrane fluidity, occurring during growth could be correlated with growth phase-related variations in the inhibitory effect of ethanol on plasma-membrane functions.

METHODS

ORGANISM

The organism used throughout this study was Saccharomyces cerevisiae NCYC 431, which was maintained at 4°C on slopes of MYGP medium (Wickerham, 1951) containing per litre of water:

Malt extract (lab m)	3 g
Yeast extract (lab m)	3 g
Glucose	10 g
Peptone (lab m)	0.5 g
Agar (lab m no. 2)	20 g

A mutant of Sacch. cerevisiae NCYC 431, lacking the GAP and designated CC1, was maintained at 4°C on slopes of a defined medium which contained per litre of water:

Glucose	20 g
KH_2PO_4	4.5 g
L-Proline	1 g
$MgSO_4.7H_2O$	25 mg
$CaCl_2.2H_2O$	25 mg
D-Methionine	20 mg
meso-Inositol	10 mg
Calcium D-panthothenate	1 mg
Pyridoxine HCl	1 mg
Thiamin	1 mg
Biotin	20 µg
Agar (lab m no. 2)	20 g

EXPERIMENTAL CULTURES

Organisms were cultivated under self-induced anaerobic conditions as described by Beavan et al. (1982) in a medium which contained

per litre of water:

Glucose	200 g
KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	3 g
$CaCl_2.2H_2O$	250 mg
$MgSO_4.7H_2O$	250 mg
Yeast extract (lab m)	4 g

The pH value of the medium was adjusted to 4.5, after which one-litre portions of media in two litre round flat-bottomed flasks fitted with fermentation locks, were sterilised by autoclaving at 10 lb in^{-2} (6.89×10^4 Pa) for one minute. Batches of sterile media were inoculated with 5 mg dry wt. equivalent of organisms from an overnight starter culture and incubated in a constant temperature (30°C) room with stirring (100 rev min^{-1}) on a flat-bed stirrer.

Growth was followed by measuring optical density at 600 nm, optical density values ($O.D._{600}$) being related to mg dry wt ml^{-1} by a calibration curve. Organisms were harvested from cultures by centrifugation (3,500 x g; 5 min; 4°C) after approximately 8 h (0.28 mg dry wt ml^{-1}) or 16 h (2.4 mg dry wt ml^{-1}) incubation. All centrifugation steps were carried out in a Sorvall RC5B refrigerated Superspeed Centrifuge, (Du Pont Company, Wilmington, Delaware, U.S.A.) unless otherwise stated.

Mutants of Sacch. cerevisiae NCYC 431 lacking the GAP were prepared by the method of Gregory et al. (1982). Portions (1 ml) of a stationary phase starter culture of organisms were mixed with 4 ml **ethylmethanesulphonate** and incubated for 1 h, prior to being

plated on to the defined medium described previously. After incubation for 3 d at 30°C, single colonies were streaked on to plates of medium containing per litre of water:

Glucose	20 g
Yeast extract (lab m)	10 g
Peptone (lab m)	20 g
Agar (lab m no. 2)	20 g

After a further 3 d incubation at 30°C, colonies were streaked on to plates of defined medium containing either L-proline (1 g l^{-1}) or $(NH_4)_2SO_4$ (1 gl^{-1}) and D-methionine (20 mgl^{-1}). Mutants which grew on both types of media were assumed to lack GAP activity.

PREPARATION OF SPHEROPLASTS

Harvested organisms, after first washing in water, were washed once in buffered sorbitol (20 mM-Tris containing 10 mM-$MgCl_2$, 1.2 M-sorbitol and where indicated in the text, 0.1 M-sodium metabisulphite, adjusted to pH 7.2) and resuspended in the same buffer to 10 mg dry wt ml^{-1}. After supplementation with Zymolyase 60,000 (0.1 ml (6 mg dry wt organisms)$^{-1}$) or Zymolyase 100,000 (0.1 ml (10 mg dry wt organisms)$^{-1}$), the suspension was incubated at 30°C with reciprocal shaking (120 r.p.m.). After incubation for 1 h, a check that formation of spheroplasts was complete was made by diluting 0.1 ml portions of the suspension into 2.9 ml of either 1.2 M-sorbitol or water and measuring the optical density at 600 nm.

DETERMINATION OF THE MAGNITUDE OF Δp

Measurements of Rates of Proton Flux

Harvested organisms, after being washed twice in distilled water, were suspended in water to 2.5 - 5.0 mg dry wt ml^{-1}. To measure

rates of passive influx of protons, an aliquot (50 ml) of this suspension was placed in a 100 ml round-bottomed flask fitted with two ports (1.5 cm diam.), in addition to a Suba-seal port and a short gas-entry port (0.5 cm diam.). The flask was maintained in a water bath at 30°C and the suspension stirred with a magnetic flea. Changes in pH value in the suspension were measured by two combination pH electrodes (type CMWL; Russell pH Ltd., Auchtermuchty, Fife, Scotland) inserted through the ports. One was connected to a digital read-out pH meter (model 9409; Philips, Cambridge, England) and the other through a second meter (model 7050; EIL, Chertsey, Surrey, England) to a single-pen, high-sensitivity recorder (model PM 8251; Philips, Cambridge, England). Organisms were de-energized by addition of 2-deoxy-D-glucose (25 mm) and, after 5 min equilibration time, the pH value of the suspension was lowered to 4.0 by addition of 0.1 M-HCl. Proton influx was then followed by measuring the rise in pH value of the suspension for a period of approx. 5 min. To measure the effect of ethanol on proton influx, ethanol (95%, v/v) was added to the suspension, after the pH value had been lowered to 4.0, to give final concentrations of 0.5 M, 1.0 M, 1.5 M or 2.0 M, and changes in extracellular pH value followed over a 5 min period. Rates of passive influx of protons are quoted as nequiv H^+ $(mg\ dry\ wt)^{-1} min^{-1}$.

After preparation, spheroplasts of Sacch. cerevisiae NCYC 431 were harvested by centrifugation (1,500 x g; 3 min), washed twice in 1.2 M-sorbitol and then suspended in this solution to 2.5 - 5.0 mg dry wt organisms $equiv.ml^{-1}$. Rates of proton influx into spheroplasts were measured as described for intact organisms, except that the

suspension was supplemented with ethanol to a maximum concentration of 1.0 M; supplementation with higher concentrations caused spheroplast lysis. After a proton influx rate had been measured on a suspension, the number of spheroplasts present was determined using a haemocytometer. Rates of passive influx of protons into spheroplasts are quoted as nequiv H^+ (mg dry wt equiv.)$^{-1}$ min^{-1}. With organisms from 8 h cultures, 1 mg dry wt was equivalent to 3.0 x 10^7 organisms and with those from 16 h cultures 3.9 x 10^7 organisms (Beavan, 1982).

Glucose-stimulated proton efflux from energized Sacch. cerevisiae NCYC 431 was followed as already described for proton influx, except that 2-deoxy-D-glucose was not included and, after adjusting the suspension to pH 4.0, glucose (20 mM) was added before proton efflux was measured over a 5 min period. The effect of ethanol on glucose-induced proton efflux was measured by supplementing the suspension with 95% (v/v) ethanol after adjusting its pH value to 4.0, to give final concentrations of 0.5, 1.0, 1.5 or 2.0 M, before addition of glucose (20 mM) and then following proton flux over a 5 min period.

Accumulation of propionic acid, tetraphenylphosphonium (TPP^+) and thiocyanate (SCN^-) ions by Saccharomyces cerevisiae NCYC 431

Suspensions (10 ml; 2.5 - 5.0 mg dry wt ml^{-1}) of energized or de-energized organisms were prepared as already described for measuring rates of proton flux. After the pH value had been decreased to 4.0, they were incubated for a further 5 min in the absence, or where indicated, presence of ethanol. Samples (4.5 ml) were then removed and added to 0.5 ml of either [2-^{14}C] propionic

acid (sodium salt; 0.1 mM; 1 μCi μmol^{-1}), T $[^3H]PP^+$ (bromine salt; 100 μM; 10 μCi μmol^{-1}) or $S^{14}CN^-$ (potassium salt; 50 μM; 50 μCi μmol^{-1}). At predetermined time intervals, portions (0.5 ml) of these suspensions were removed, rapidly filtered through prewashed (10 ml; 1 mM-propionate, 10 mM-TPP^+ or 5 mM-SCN^-; 4°C) membrane filters (0.45 μm pore size; 25 mm diam., Millipore), and washed (4 x 1-2 ml) with a non-radioactive, ice-cold solution of the appropriate salt at the concentration included in the cell suspension. The filters and organisms were transferred to scintillation vials containing 7.5 ml of Cocktail T (B.D. H.) or Optiphase Safe (Fisons) and the radioactivity of the contents measured in a liquid scintillation spectrometer (LKB Rackbeta, model 1217). To determine the extent of TPP^+ binding to the yeast wall and plasma membrane, 2,4-DNP (1 mM; Eilam, 1984) was added at the same time as radioactive TPP^+ and the incubation and filtration procedures carried out as already described.

Measurement of Intracellular pH Values of Organisms

Intracellular pH values of energized and de-energized organisms were calculated by determining the equilibrium distribution of a weak acid, namely propionic acid, across the plasma membrane (Conway and Downey, 1950; Seaston et al., 1976). To allow equilibration of propionate across the plasma membrane, portions (1.8 ml) of a suspension of organisms, prepared as described for measuring propionate accumulation, were incubated with [2-^{14}C] propionic acid (0.1 mM; 1 μCi μmol^{-1}) for 3 min at 30°C during which time the extracellular pH value of the suspension was monitored. Upon equilibration, triplicate samples (0.3 ml) were rapidly removed and

filtered through membrane filters (0.45 μm pore size; 25 mm diam., Millipore). Organisms on filters were washed with 4 x 1 ml 0.1 mM-propionate (4°C) and the radioactivity of filters and organisms determined as described previously. When measuring the effect of ethanol on the intracellular pH value of organisms, ethanol (95%, v/v) was added to the suspension after the pH value had been lowered to 4.0, to give a final ethanol concentration of 0.5, 1.0, 1.5 or 2.0 M. In experiments with energized organisms, glucose (20 mM) was incorporated in the suspension 30 s after addition of ethanol. Intracellular pH values were calculated from the expression derived by Waddell and Butler (1959):

$$pH_i = pK_i + \log [R(10^{(pH_e - pK_e)} + 1) - 1]$$

where $$R = \frac{TA_i}{TA_e} \cdot \frac{V_e}{V_i},$$

and pH_i and pH_e respectively the internal and external pH values, TA_i and TA_e the internal and external total amounts of propionic acid, V_i and V_e the intracellular and extracellular water volumes, and pK_i and pK_e the dissociation constants for propionic acid in the internal and external environments. Intracellular volumes were calculated from the dry wt of organisms used assuming that, for organisms from 8 h cultures, the cell volume was 1.93 μl (mg dry wt)$^{-1}$ and for organisms from 16 h cultures 1.61 μl (mg dry wt)$^{-1}$ (Beavan et al., 1982). The internal and external dissociation constants for propionic acid were calculated from the Davies' simplified version of the Debye-Hückel equations (Davies, 1962), assuming that the ionic strength within organisms was in the region 0.15 - 0.25 (Conway and Downey, 1950). Values for pK_i and pK_e were calculated to be 4.86 and 4.75, respectively.

Measurement of Membrane Potential ($\Delta\psi$) Values of Organisms

Values for $\Delta\psi$ were determined from the equilibrium distribution of T[^{3}H]PP$^+$ across the yeast plasma membrane (Hauer and Höfer, 1978). To achieve equilibration of TPP$^+$, the protocol already described for measuring intracellular pH values was repeated, except that portions (1.8 ml) of suspensions were supplemented with TPP$^+$ (0.2 ml; 100 µM; 10 µCi µmol^{-1}) and then allowed to equilibrate for 25 min. After filtration, organisms were washed with 4 x 2 ml 10 µM-TPP$^+$ (4°C) and the radioactivity of organisms and filters measured. $\Delta\psi$ in millivolts was calculated using the equation:

$$\Delta\psi = -2.3 \frac{RT}{F} \cdot \log \frac{[TPP^+]_{inside}}{[TPP^+]_{outside}}$$

Intracellular TPP$^+$ concentrations were obtained by deducting the value for non-specific TPP$^+$ binding, measured by incorporating 2,4-DNP in suspensions, from the total amount of TPP$^+$ absorbed.

Calculation of Δp

Values for Δp were calculated by using the equation:

$$\Delta p = -2.3 \frac{RT}{F} \Delta pH + \Delta\psi$$

ISOLATION OF PLASMA MEMBRANES

Isolation Using Sucrose Density-Gradient Centrifugation

Spheroplasts, prepared as described previously, were harvested by centrifugation (1,500 x g; 3 min), washed twice in buffered sorbitol and resuspended in buffered mannitol (50 mM-Tris containing 0.3 M-mannitol; pH 7.2; 10 ml (200 mg dry wt organisms equiv.)$^{-1}$). The suspension was homogenised (10 strokes of a Teflon-glass hand

homogeniser) and incubated on ice for 20 min. To obtain plasma membranes, a 10 ml aliquot of spheroplast lysate was layered on top of a discontinuous sucrose-density gradient consisting of 10 ml 60% (w/v) sucrose, 10 ml 55% (w/v) sucrose and 30 ml 45% (w/v) sucrose. After centrifugation (50,000 x g; 150 min) in an MSE Prepsin 75 Ultracentrifuge (MSE Scientific Instruments, Crawley, Sussex, England), visible bands were removed with a Pasteur pipette, diluted in solubilisation buffer (10 mM-Tris containing 1 mM-ATP and 1 mM-EDTA; pH 7.0), the resultant suspension centrifuged (25,000 x g; 25 min) and the pellet retained. Prior to assaying the ATPase activity of fractions, the enzyme was solubilised by treatment with lysophosphatidylcholine (Dufour and Goffeau, 1978). The pellet obtained previously was resuspended in solubilisation buffer containing lysophosphatidylcholine (2 mg ml^{-1}), sonicated (2 x 30 sec; 3 mm probe; drive setting 7) using an MSE 100W ultrasonicator and incubated for 20 min at 4°C. Following this incubation period, the sample was centrifuged (25,000 x g; 10 min) and the supernatant retained for assay.

Isolation Using Cationic Silica Microbeads

The selective attachment of cationically-charged silica microbeads to yeast spheroplasts was used as an alternative method of plasma membrane isolation, as described by Schmidt et al. (1983). Spheroplasts were prepared and harvested as already described, washed three times in coating buffer (1.2 M-sorbitol containing 25 mM-sodium acetate and 0.1 M-KCl; pH 6.0), the population counted and then suspended in coating buffer to 1.5 x 10^8 spheroplasts ml^{-1}. Suspensions of spheroplasts and silica microbeads (3%, w/v, in coating

buffer) were mixed in the ratio 2:1, incubated for 3 min at 4°C, after which the coated spheroplasts were centrifuged (500 x g; 4 min), and washed once in coating buffer before being suspended in the same buffer to a concentration of 10^8 spheroplasts ml^{-1}. This suspension was diluted with an equal volume of polyacrylic acid (M_r 90,000; 2 mg ml^{-1} in coating buffer; pH 6.0), centrifuged (500 x g; 4 min) and the pellet washed once with coating buffer. Spheroplasts were then resuspended in lysis buffer (5 mM-Tris-HCl containing 1 mM-EGTA; pH 8.0) to 10^8 spheroplasts ml^{-1} and the suspension vortexed using a Whirlimix (Fisons) for 5 min or until >95% lysis of spheroplasts was achieved. The lysate was centrifuged (1,000 x g; 5 min), the resultant plasma-membrane pellet washed three times in lysis buffer and then resuspended in a suitable volume of assay buffer (100 mM-Mes-Tris containing 80 mM KCl; pH 6.5 unless otherwise stated).

ANALYSIS OF PLASMA MEMBRANES

Electron Microscopy

The purity of plasma-membrane preparations was examined by transmission electron microscopy using a modification of the method of Henschke et al. (1983). Samples were washed with an equal volume of 50 mM-sodium cacodylate, the pellet suspended in 50 mM-sodium cacodylate containing 2% (w/v) osmium tetroxide and incubated at 4°C for 60 min with occasional agitation. After centrifugation, the pellet was washed three times with 50 mM-sodium cacodylate, and then suspended in 2% (w/v) agar (lab m MC2). The resulting slurry was extruded from a Pasteur pipette and the agar, once set, cut into small sections (2 mm^2). Samples were then dehydrated by sequentially

passing them through the following acetone (v/v) solutions: 15% (1 x 10 min), 50% (1 x 10 min), 90% (1 x 10 min) and 100% (3 x 15 min). The preparations were transferred into Taab resin (Taab Laboratories, Reading, Berks, England), rotated for 24 h, placed in Taab capsules and additional resin added before they were polymerized for 72 h at 60°C. Ultrathin sections were cut on a Reichert OMU3 ultramicrotome (Reichert-Jung, Slough, England) and stained for 10 min in ethanol (70%, v/v) saturated with uranyl acetate followed by 15 min in lead citrate (Reynolds, 1963). Sections were examined in a JEOL 100CX transmission electron microscope (JEOL U.K., Colindale, London, England).

Estimation of Protein Content

The protein content of solubilised plasma-membrane preparations was assayed using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, West Germany), a method based on the dye-binding technique of Bradford (1976). Samples (0.1 ml) of plasma membranes isolated using silica microbeads were solubilised by boiling for 5 min after addition of an equal volume of 0.1 M-NaOH. The suspension was then neutralised by addition of 0.1 M-HCl (0.1 ml) and 0.2 ml acetic acid-sodium acetate buffer (0.2 M; pH 5.0). Aliquots (0.1 ml) of the suspension, diluted if necessary, were mixed with Bio-Rad dye reagent (5 ml; 20%, v/v, in distilled water), the solution vortexed using a Whirlimix (10 s) and the colour allowed to develop for 5 min at room temperature. The absorbance of solutions was then measured at 595 nm, absorbance values (A_{595}) being related to protein content by a calibration curve prepared using bovine serum albumin as a standard.

Assay of ATPase Activity

Plasma-membrane ATPase activity was assayed by following release of Pi from ATP. The reaction mixture consisted of 1 ml 100 mM-Mes-Tris buffer containing 80 mM-KCl, 6 mM-$MgCl_2$ and plasma-membrane preparation, pH 6.5. Where indicated, sodium orthovanadate (100 µM) was included specifically to inhibit plasma-membrane ATPase activity. The reaction was started by adding 6 mM-Na-ATP and the mixture incubated for 10 - 40 min at 30°C. The amount of Pi liberated was determined as described by Serrano (1978). The reaction was stopped by addition of 2 ml acidified molybdate solution (2.0%, v/v, conc. H_2SO_4 containing 0.5%, w/v, ammonium molybdate and 0.5%, w/v, SDS). Ascorbic acid (0.02 ml; 10%, w/v) was added and the colour allowed to develop over 5 min at 30°C. The absorbance of the solution was measured at 750 nm and the value related to Pi concentration by a standard curve. ATPase activities are quoted as µmol Pi liberated (mg protein)$^{-1}$ min^{-1}.

The effect of four specific inhibitors was examined on the ATPase activity of purified plasma membranes, sodium orthovanadate, DCCD, diethylstilbestrol (25 - 300 µM) and oligomycin (25 - 300 µg ml^{-1}). In each case, the plasma-membrane preparation (25 µg protein) was incubated at 30°C for 10 min with the desired inhibitor prior to ATPase activity being assayed as described previously.

To examine the effect of ethanol on ATPase activity, the alkanol (95%, v/v) was included in suspensions of plasma membrane in assay buffer to give final concentrations of 0.5, 1.0, 1.5 or 2.0 M. After 5 min incubation at 30°C, the reaction was started by

addition of a suitable concentration of ATP, and the liberation of Pi followed as already indicated. In all reaction mixtures the Mg^{2+}:ATP ratio was maintained at unity (Willsky, 1979).

MEASUREMENT OF VELOCITY OF GLYCINE ACCUMULATION

Assay Method

Organisms from 8 h or 16 h cultures of wild-type Sacch. cerevisiae NCYC 431 or the GAP-less mutant (CC1) were harvested, washed twice with citric acid/trisodium citrate buffer (20 mM; pH 4.0), suspended at 5 mg dry wt ml^{-1} in the same buffer and stored in an ice-water mixture.

The velocity of glycine accumulation by these organisms was determined by a modification of the method of Calderbank et al. (1984). The suspension (5 ml) used consisted of citrate buffer (20 mM; pH 4.0), glucose (100 mM), organisms (2.5 mg dry wt ml^{-1}) and [U-^{14}C] glycine (10^{-6} - 10^{-3} M; 0.1 - 1.0 µCi μmol^{-1}). Initially, the suspension, without glycine, was placed in a Universal bottle maintained in a water bath at 30°C, and the contents allowed to equilibrate for 5 min whilst being stirred with a magnetic flea. The experiment was started by adding glycine (tracer and carrier) to the bottle, after which portions (0.75 ml) were removed at predetermined times, rapidly filtered through membrane filters (0.45 µm pore size; 25 mm diam., Millipore) and the filter and organisms immediately washed with ice-cold citrate buffer (5 ml; pH 4.0) containing glycine at the concentration included in the suspension. Filters were then transferred to scintillation vials containing Optiphase Safe (7.5 ml) scintillant and radioactivity of organisms on filters measured as described previously. Velocities

of accumulation of glycine were determined from the linear sections of plots of the time-course of accumulation for up to 60 s, and are expressed as pmol glycine accumulated (mg dry wt)$^{-1}$ sec^{-1}.

Measurement of the Effect of Ethanol on Accumulation of Glycine by the GAP

Organisms were harvested, washed and stored as described in the previous section. Suspensions (5 ml) used to measure the effect of ethanol on glycine accumulation by the GAP consisted of citrate buffer (20 mM; pH 4.0) and organisms (2.5 mg dry wt ml^{-1}). In experiments with de-energized organisms, 2-deoxy-D-glucose (25 mM) was also incorporated in suspensions. After 5 min equilibration at 30°C, ethanol (95%, v/v), and glucose (100 mM) in experiments with energized organisms, was added to a final concentration of 0.5, 1.0, 1.5 or 2.0 M and, after a further 5 min incubation, the experiment was started by addition of [U-^{14}C] glycine (50 µM - 100 µM; 0.66 µCi µmol^{-1}). At 15 s intervals, up to 60 s, samples (0.75 ml) were removed and filtered, organisms washed and the radioactivity of organisms and filters determined as previously described.

LIPID ANALYSES

Isolated plasma membranes, from 500 mg dry wt organisms, were prepared using cationically-charged silica microbeads as already described. The plasma-membrane pellet was taken up in 15 - 20 ml methanol, to which chloroform was added to give a chloroform: methanol ratio of 2:1 (v/v). Non-specific lipase activity was inhibited by inclusion of p-chloromercuribenzoate (1 mM; Schousboe, 1976), and the suspension was stirred on a flatbed stirrer for 2 h

at room temperature. Extracts were washed with 0.25 vol. 0.88% (w/v) KCl and allowed to separate overnight at -20°C. The lower phase was removed, taken to dryness on a rotary evaporator and the residue immediately dissolved in a minimum of chloroform. Phospholipids were separated from other lipid classes by thin-layer chromatography (TLC) on plates of silica gel G (Whatman, 0.25 mm thick). Samples were streaked onto plates using a 50 µl Terumo Micro Syringe (Terumo Corporation, Tokyo, Japan) and the separation was achieved by using a solvent system of petroleum ether (60° - 80°C):diethyl ether:acetic acid (70:30:1, by vol.). Bands of lipid classes were visualized by spraying with 2',7'-dichlorofluorescein (0.2%, w/v, in ethanol) and then viewing plates under ultraviolet radiation at a wavelength of 254 nm. Individual lipid classes were identified by reference to simultaneously run standards of L-α-phosphatidylcholine (for phospholipids), cholesterol palmitate (sterol esters), ergosterol (free sterols), stearic acid (free fatty acids) and tripalmitin (triglycerides), and marked with a pencil. Bands containing phospholipid classes were scraped off and suspended in 10 ml petroleum ether (60° - 80°C):methanol (1:1, by vol.). The suspension was vigorously shaken, 5 ml of NaCl (30%, w/v) added, the mixture allowed to separate at room temperature and the upper layer removed and retained. A further 5 ml petroleum ether (60° - 80°C) was added and the extraction procedure repeated. The combined extracts were taken to dryness under nitrogen gas, the residue taken up in 0.3 ml chloroform,and individual phospholipid classes separated by TLC using a solvent system of chloroform:methanol:acetic acid (65:25:8, by vol.; Kramer et al., 1978). Individual phospholipid classes were visualized by spraying plates with molybdenum blue reagent (0.65%,

v/v, molybdenum oxide in 4.2 M-H_2SO_4; Dittmer and Lester, 1964) and identified by reference to standards of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine. The relative proportions of the major phospholipid classes were determined using a scanning densitometer (wavelength 626 nm, aperture width 0.1 - 0.3 mm; Chromoscan 3, Joyce Loebl, Gateshead, Tyne and Wear, England).

To determine the fatty-acyl composition of phospholipid classes, bands were visualised and identified as described previously and then scraped off. Fatty-acyl methyl esters were prepared by heating (80°C; 60 min) the silica gel with 5 ml methanol containing 14% (w/v) BF_3 in a Reactivial (Pierce Chemical Co., Chester, England). After cooling, the solution was extracted twice with a petroleum ether (60° - 80°C):methanol:30% (w/v) NaCl mixture (1:1:1, by vol.). Fatty-acid methyl esters were analysed by gas-liquid chromatography on a glass column (1.5 m) containing 10% S2330 supported on 100 - 120 mesh Chromosorb W-AW at 165°C, using a Pye Unicam GCD gas chromatograph. The injection port was at 210°C, the detector temperature was 190°C and the carrier gas (N_2) flow rate was 60 ml min^{-1}. Percentage fatty-acyl compositions were calculated using a Pye Unicam CDP1 integrator.

VIABILITY MEASUREMENTS

Organisms were harvested, washed and resuspended in distilled water (2.5 - 5.0 mg dry wt ml^{-1}) as previously described for measuring rates of proton flux. Portions (10 ml) of suspensions were incubated

at 30°C, with stirring, for 5 min prior to the addition of ethanol (95%, v/v) to give final concentrations of 1.0 M, 2.0 M or 3.0 M. Viability of yeast populations was measured by staining with methylene blue (Fink and Kühles, 1933). At predetermined time intervals, aliquots of suspensions (0.5 ml) were removed and after appropriate dilution,mixed with equal volumes of methylene blue solution (0.01%, w/v, methylene blue in 2%, w/v, sodium citrate). After 5 min incubation at room temperature, wet preparations were prepared on haemocytometer slides, and the numbers of live and dead cells established microscopically in a population of at least 500 organisms.

MATERIALS

All chemicals used were AnalaR grade or of the highest purity available commercially. Adenosine 5'-triphosphate (disodium salt), 2',7'-dichlorofluorescein, boron trifluoride, N,N'-dicyclohexylcarbodiimide, diethylstilbestrol, p-chloromercuribenzoate, molybdenum blue reagent, oligomycin and all lipid standards were purchases from Sigma Chemical Co. Ltd., Poole, Dorset, England. Sodium ortho-vanadate was obtained from BDH Chemicals Ltd., Poole, England. Tetraphenylphosphonium bromide and polyacrylic acid came from Aldrich Chemical Co. Ltd., Gillingham, England. Zymolyase was provided by ICN Biomedicals Ltd., High Wycombe, Bucks, England. All radioactively labelled compounds were obtained from Amersham International, Amersham, England. Cationically-charged silica microbeads were a generous gift from Dr. Bruce Jacobson, Dept. of Biochemistry, University of Massachusetts, Amherst, Mass., U.S.A. Gas-liquid chromatography columns were purchased from Pye Unicam, Cambridge, England and the packing material was supplied by Chromatography Services ltd., Hoylake, Merseyside, England.

RESULTS

GROWTH OF SACCHAROMYCES CEREVISIAE NCYC 431 UNDER SELF-INDUCED ANAEROBIC CONDITIONS

Saccharomyces cerevisiae NCYC 431 grew under self-induced anaerobic conditions with a specific growth rate of 0.43 h^{-1} in a medium containing 200 g glucose per litre (Fig. 1). Both the measured growth rate and the final growth yield (5.40 mg dry wt ml^{-1}) were in agreement with previous reports on the cultivation of this organism under self-induced anaerobic conditions (Beavan, 1982). A mutant of Sacch. cerevisiae NCYC 431, CC1, which lacked the GAP, showed identical growth characteristics to the wild-type strain.

EFFECT OF ETHANOL ON THE VIABILITY OF POPULATIONS OF SACCHAROMYCES CEREVISIAE NCYC 431

Ethanol, up to 3.0 M, caused a concentration-dependent decrease in the viability of populations of Sacch. cerevisiae NCYC 431. This effect of ethanol on viability was more pronounced in suspensions of organisms from 8 h cultures as compared with 16 h cultures (Figs. 2 and 3).

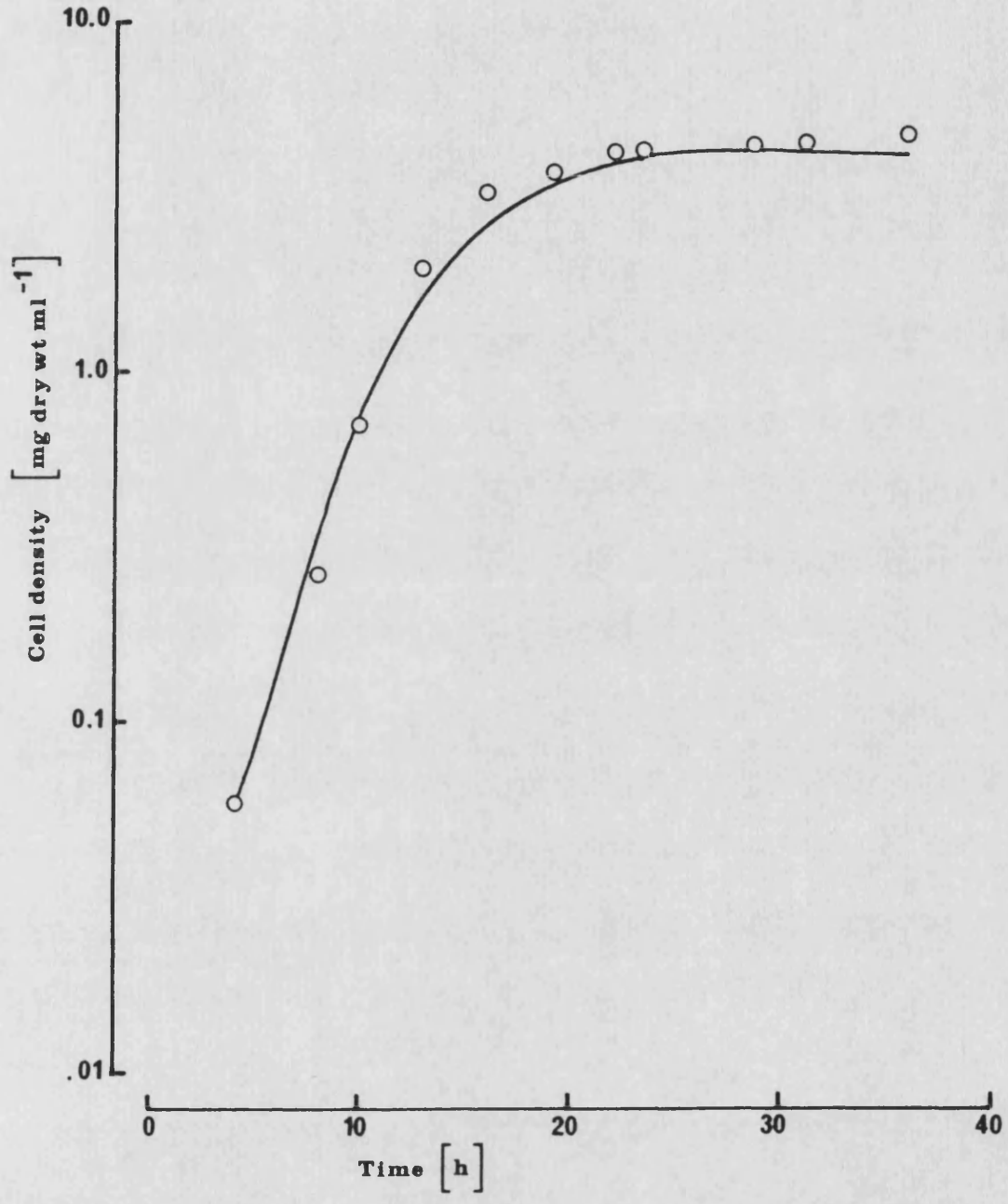
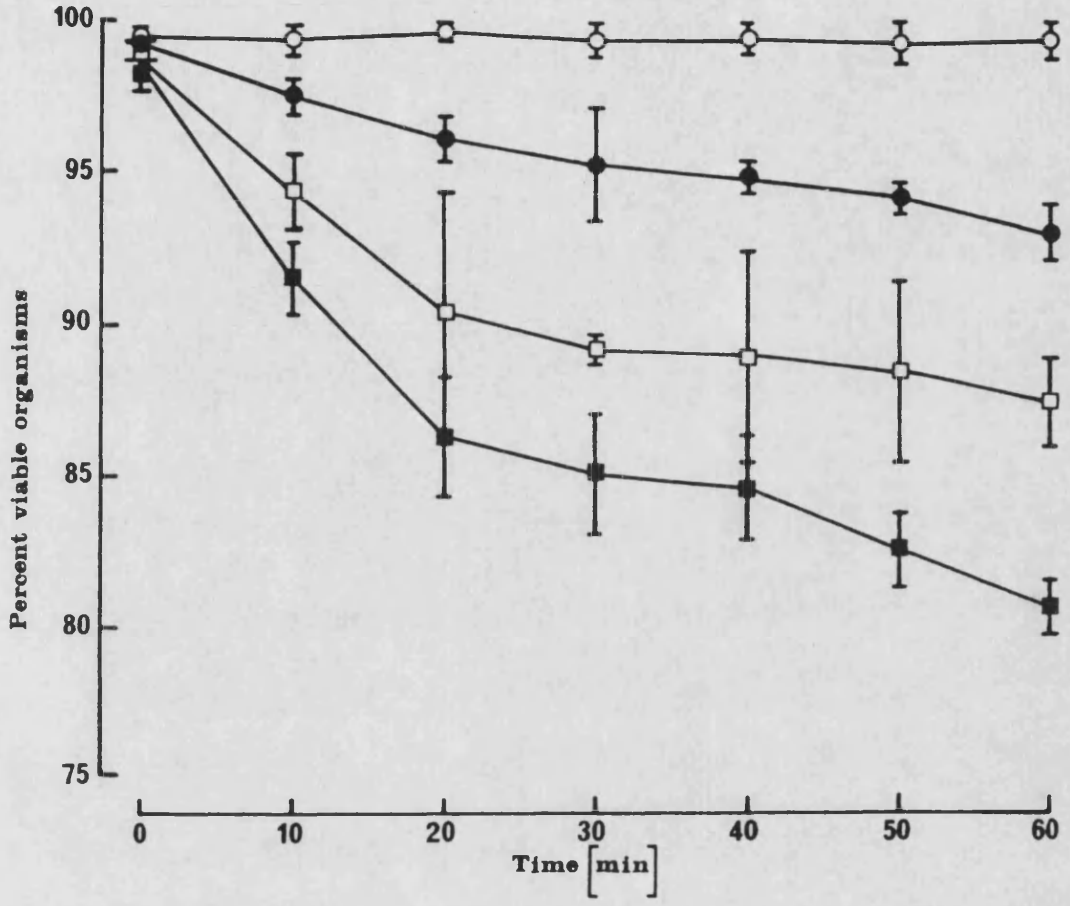
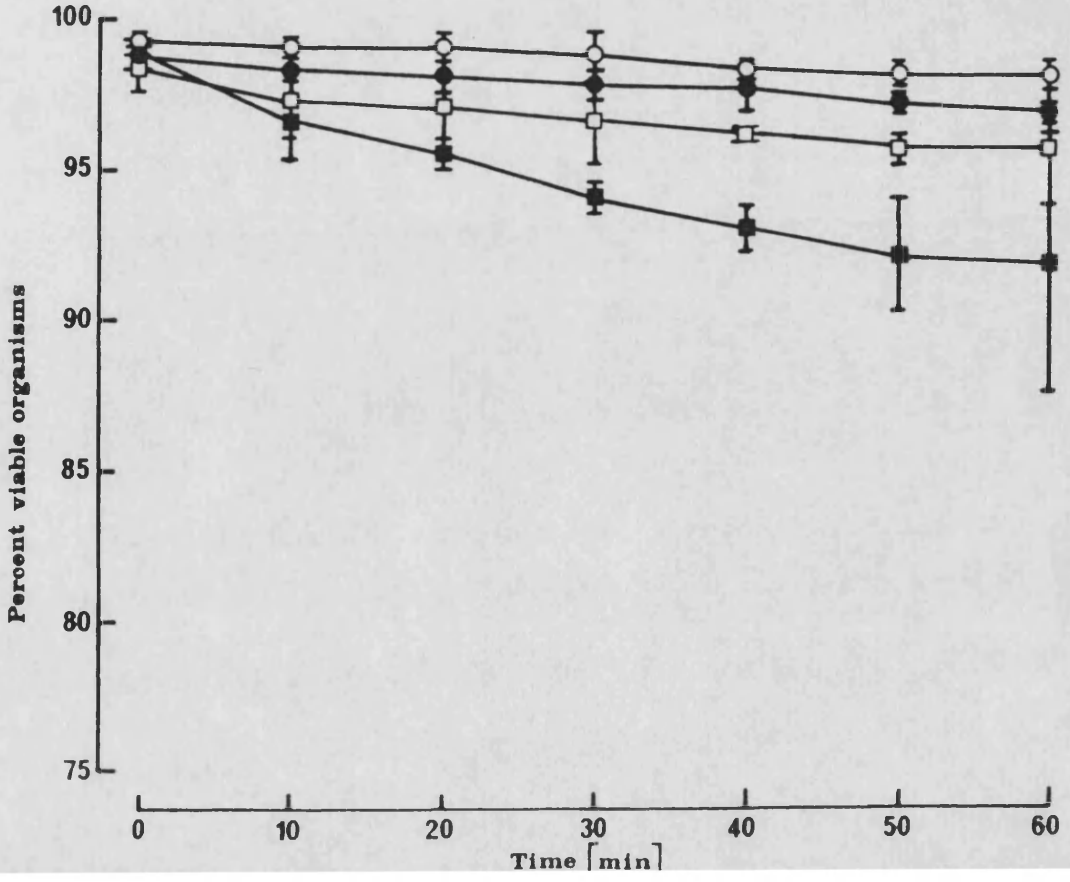


Figure 1. Time-course of growth of Saccharomyces cerevisiae NCYC 431 cultured at 30°C under self-induced anaerobic conditions in a medium containing 20% (w/v) glucose.

Figure 2. Effect of ethanol on the viability of populations of Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures. (●) indicates the response of suspensions supplemented with 1.0 M, (□) with 2.0 M and (■) with 3.0 M-ethanol. (○) indicates the response of unsupplemented suspensions. Vertical bars indicate standard errors.

Figure 3. Effect of ethanol on the viability of populations of Saccharomyces cerevisiae NCYC 431 harvested from 16 h cultures. (●) indicates the response of suspensions supplemented with 1.0 M, (□) with 2.0 M and (■) with 3.0 M-ethanol. (○)indicates the response of unsupplemented suspensions. Vertical bars indicate standard errors.


100
95
90
85
80
75
Percent viable organisms
0
10
20
30
40
50
60
Time [min]


100
95
90
85
80
75
Percent viable organisms
0
10
20
30
40
50
60
Time [min]

DISSIPATIVE EFFECT OF ETHANOL ON THE PROTON-MOTIVE FORCE ACROSS THE PLASMA MEMBRANE OF SACCHAROMYCES CEREVISIAE NCYC 431

Ethanol, up to 2.0 M, accelerated passive influx of protons into de-energized organisms, as reported by Leão and van Uden (1984b). However, ethanol-induced acceleration of proton influx was greater with organisms from 8 h cultures compared with 16 h cultures (Fig. 4). With organisms from 8 h cultures, the ethanol-induced increase in rate of proton influx was 11.6 nequiv. H^+ (mg dry wt organisms)$^{-1}$min^{-1} (ethanol conc.; M)$^{-1}$, while the organisms from 16 h cultures the increase in rate was 6.8 nequiv. H^+ (mg dry wt organisms)$^{-1}$min^{-1} (ethanol conc.; M)$^{-1}$. Proton influx was considerably accelerated when spheroplasts were used instead of intact organisms, but the influx rate was only slightly greater with spheroplasts from organisms from 8 h compared with 16 h cultures (Fig. 5). The ethanol-induced increase in the rate of passive influx of protons into spheroplasts from organisms from 8 h cultures was 412.0 nequiv. H^+ (mg dry wt organisms equiv.)$^{-1}$min^{-1} (ethanol concn.; M)$^{-1}$, while that with spheroplasts from organisms from 16 h cultures was 365.8 nequiv. H^+ (mg dry wt organisms equiv.)$^{-1}$ min^{-1} (ethanol concn.; M)$^{-1}$.

The extent of extracellular acidification of suspensions of energized organisms was decreased in the presence of ethanol, less so with organisms from 16 h compared with 8 h cultures (Figs. 6 and 7). Ethanol-induced decrease in the initial rate of proton efflux, calculated from the linear portions of the acidification curves (measured over the period approximately 0.5 - 2.0 min), was greater with organisms from 8 h compared with 16 h cultures (Fig. 8). Comparable measurements were not possible with spheroplasts since their incubation in glucose-containing buffer resulted in lysis.

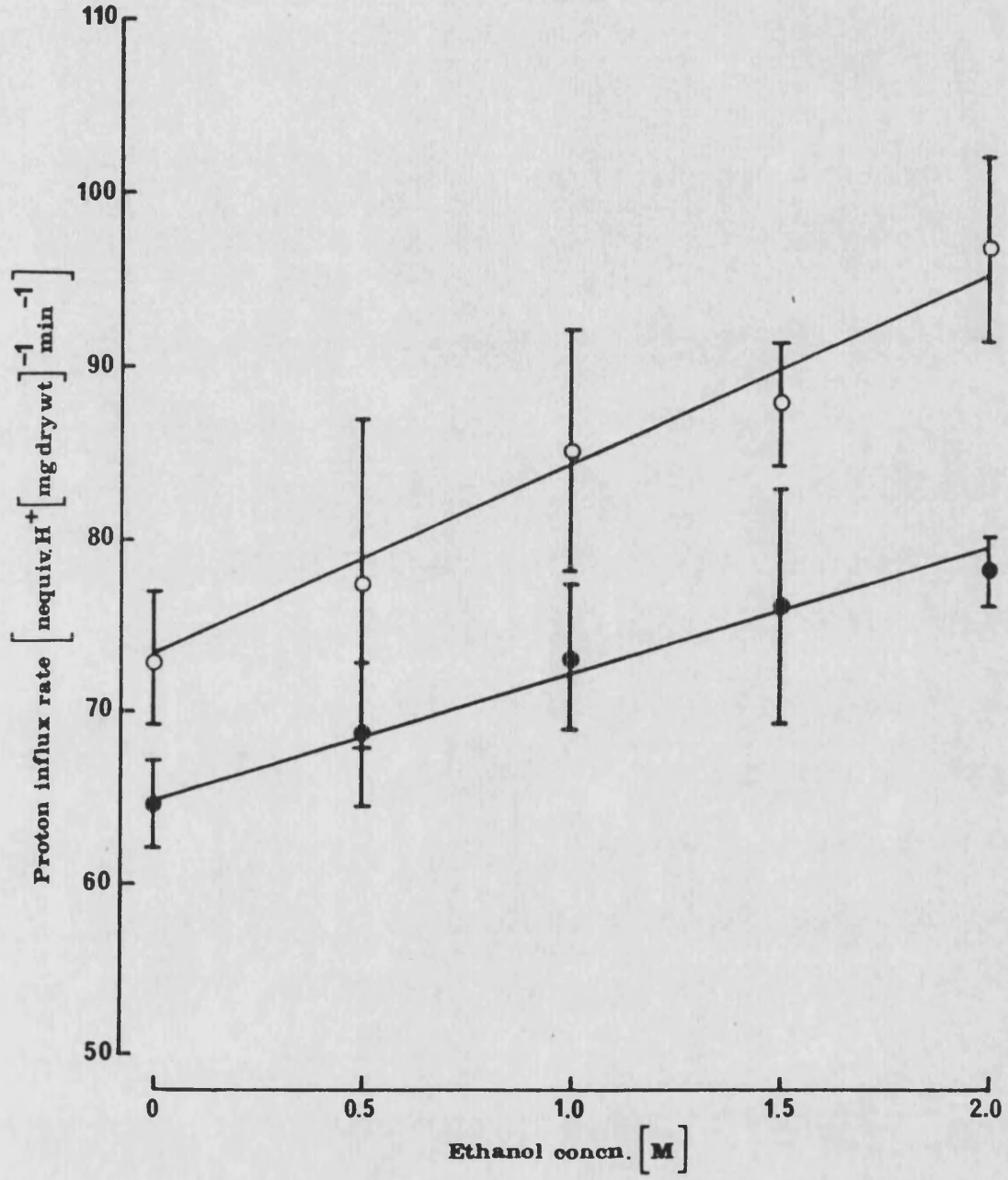


Figure 4. Effect of ethanol on influx of protons into de-energized Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures (○) or 16 h (●) cultures. Vertical bars indicate standard errors.

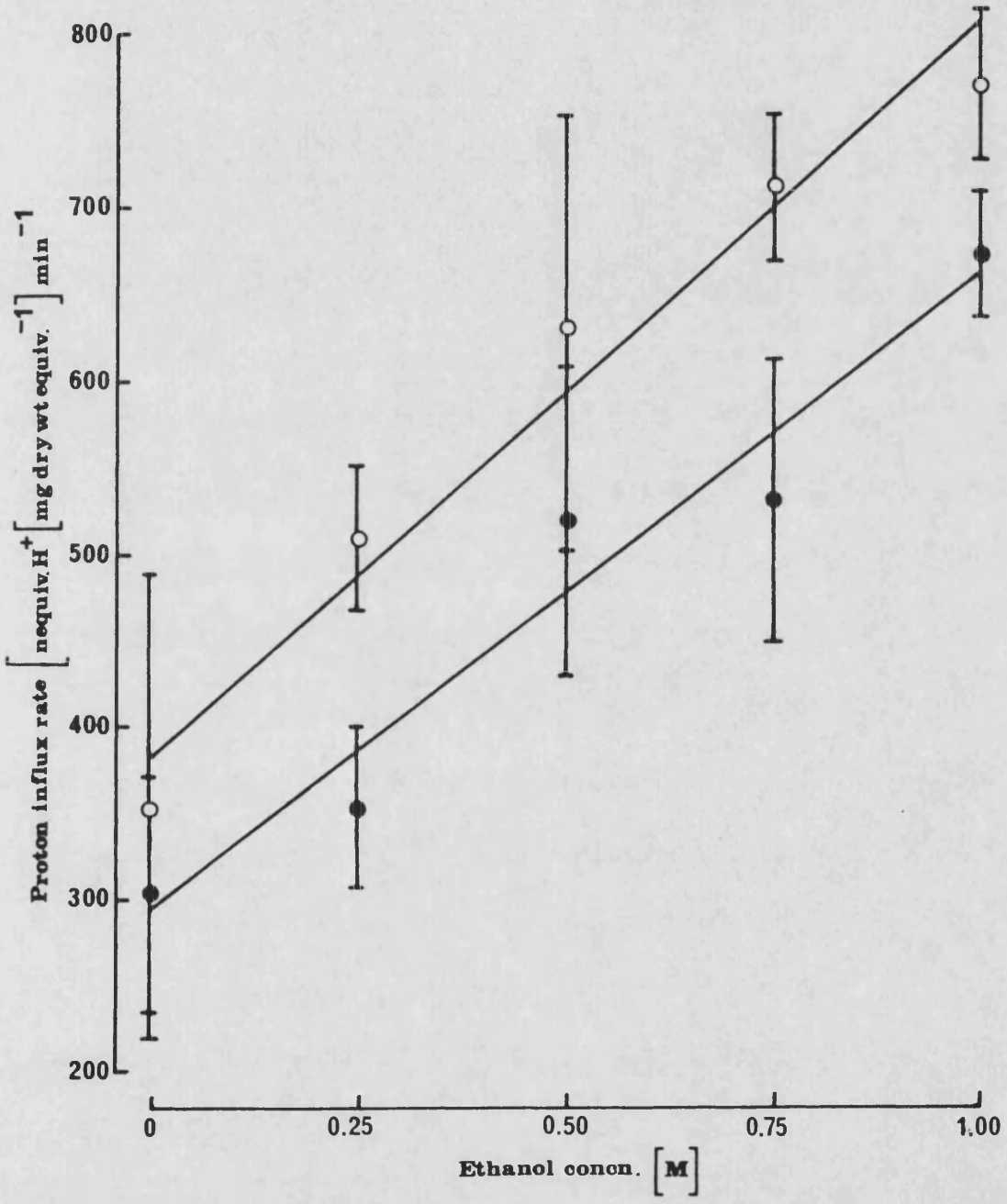


Figure 5. Effect of ethanol on influx of protons into spheroplasts from de-energized Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures (○) or 16 h cultures (●). Vertical bars indicate standard errors.

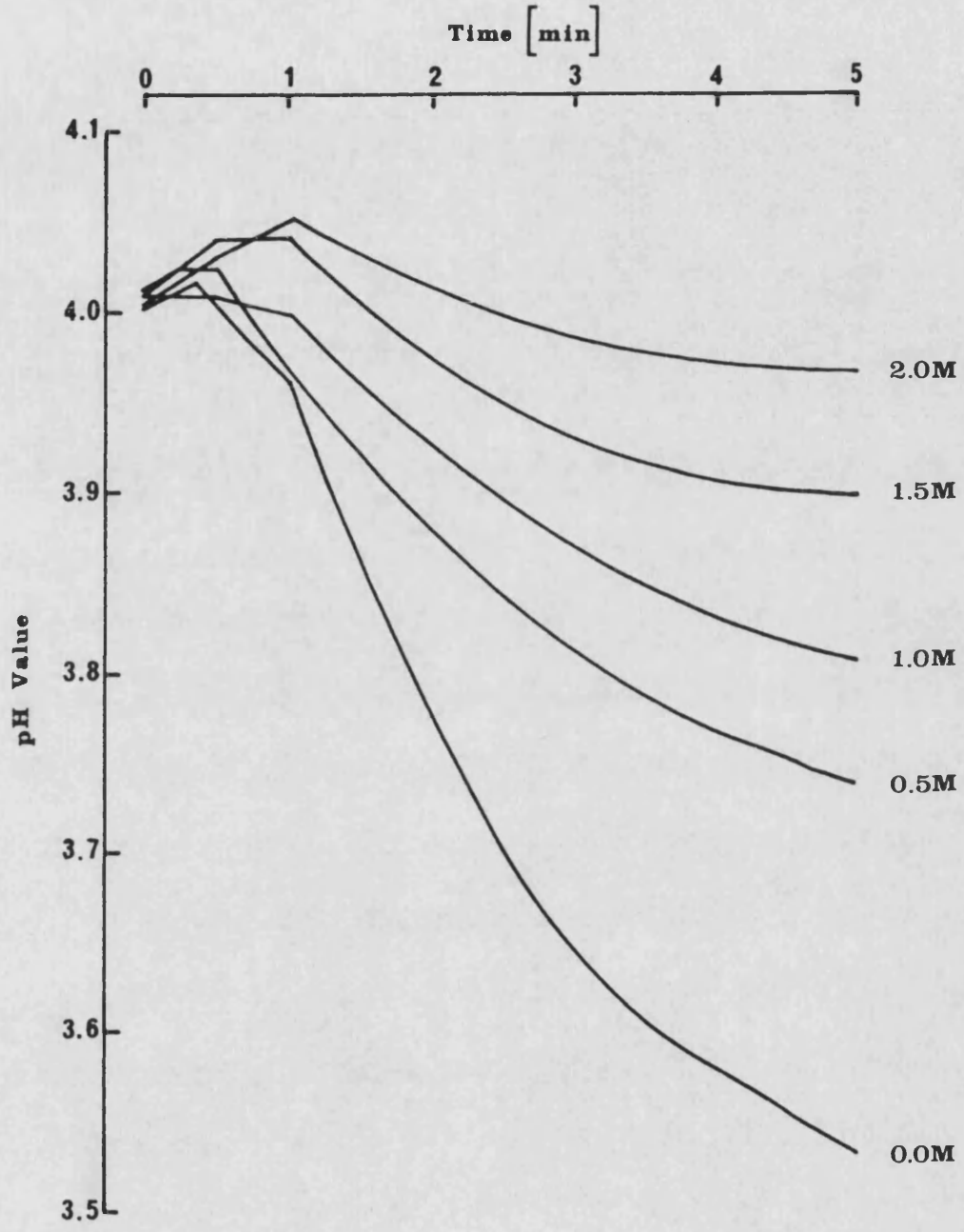


Figure 6. Time-course of acidification of suspensions of energized Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures. The data are representative of those obtained in five separate experiments.

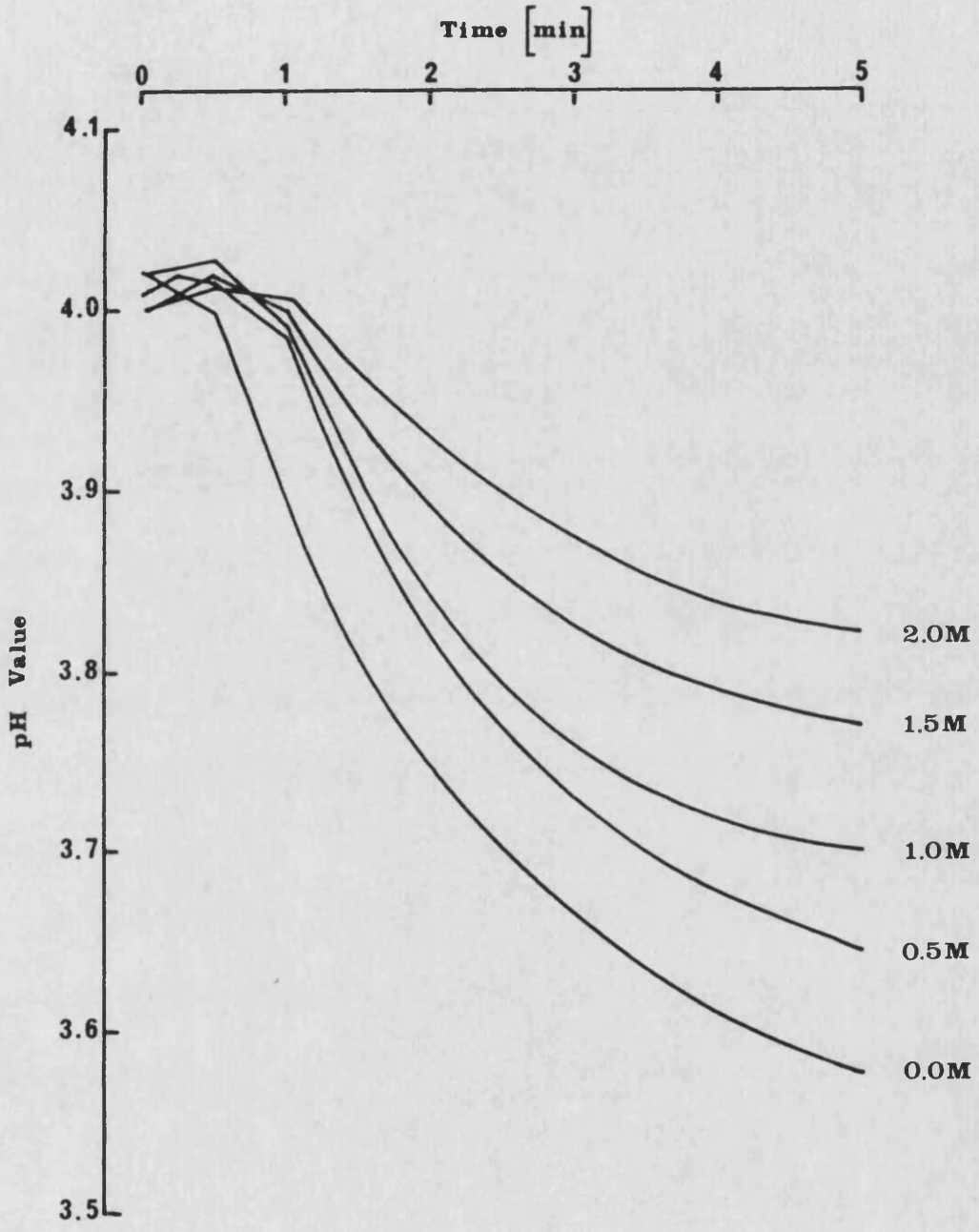


Figure 7. Time-course of acidification of suspensions of energized *Saccharomyces cerevisiae* NCYC 431 harvested from 16 h cultures. The data are representative of those obtained in five separate experiments.

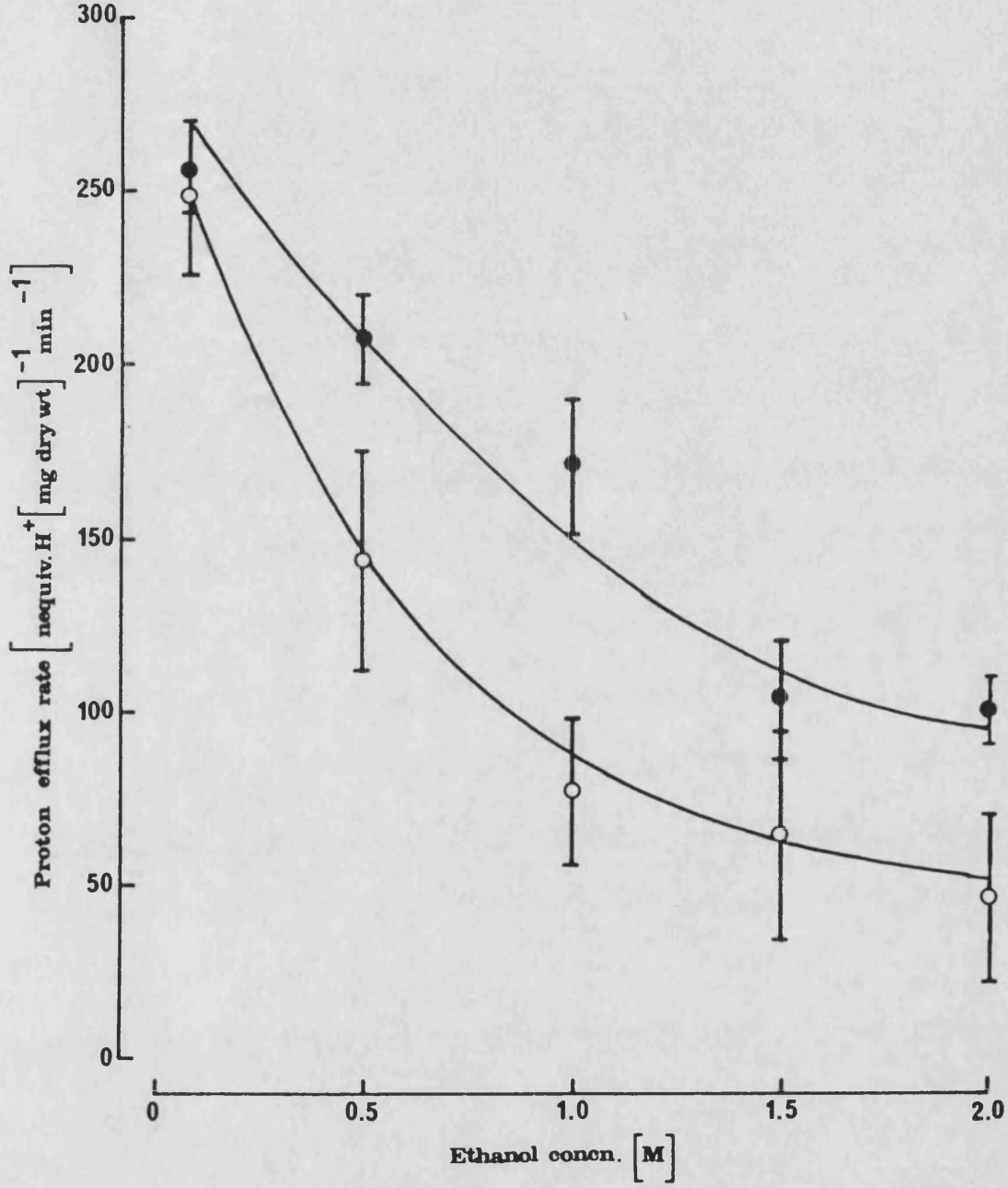


Figure 8. Effect of ethanol on efflux of protons from energized Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures (O) or 16 h cultures (●). Vertical bars indicate standard errors.

Effect of Culture Age and Ethanol on Values for ΔpH, Δψ and Δp

Saccharomyces cerevisiae NCYC 431 accumulated propionic acid under both energized and de-energized conditions (Figs. 9 and 10).

Net accumulation of propionate ceased after 2.5 - 3.0 min. Supplementing suspensions of organisms with ethanol, up to 2.0 M, caused a decrease in the intracellular pH value, as measured by propionate accumulation, which, coupled with the simultaneous increase in extracellular pH value, caused a decrease in ΔpH. With de-energized organisms, the decrease in ΔpH was approximately the same in organisms from 8 h and 16 h cultures (Tables 2 and 3), but, with energized organisms, was proportionately greater with organisms from 8 h compared with 16 h cultures (Tables 4 and 5). TPP^+ was accumulated by de-energized organisms, but not energized organisms, with an equilibrium distribution of this ion being attained after 20 - 30 min incubation (Figs. 11 and 12). Addition of 2,4-DNP to suspensions of de-energized organisms prevented uptake of TPP^+. Ethanol supplementation also decreased the magnitude of Δψ in de-energized organisms, measured by TPP^+ accumulation, 1.0 M-ethanol being sufficient to abolish Δψ in organisms from either 8 h or 16 h cultures (Table 6). However, SCN^- ions were not accumulated to any greater extent by suspensions of de-energized organisms in the presence of 2.0 M ethanol as compared with suspensions of organisms not supplemented with this alkanol (Figs. 13 and 14). The effect of ethanol on values for ΔpH and Δψ lowered the values for Δp (Table 7). The ethanol-induced decrease in Δp was about the same for de-energized organisms from 8 h and 16 h cultures, but for energized organisms was greater for those from 8 h compared with 16 h cultures.

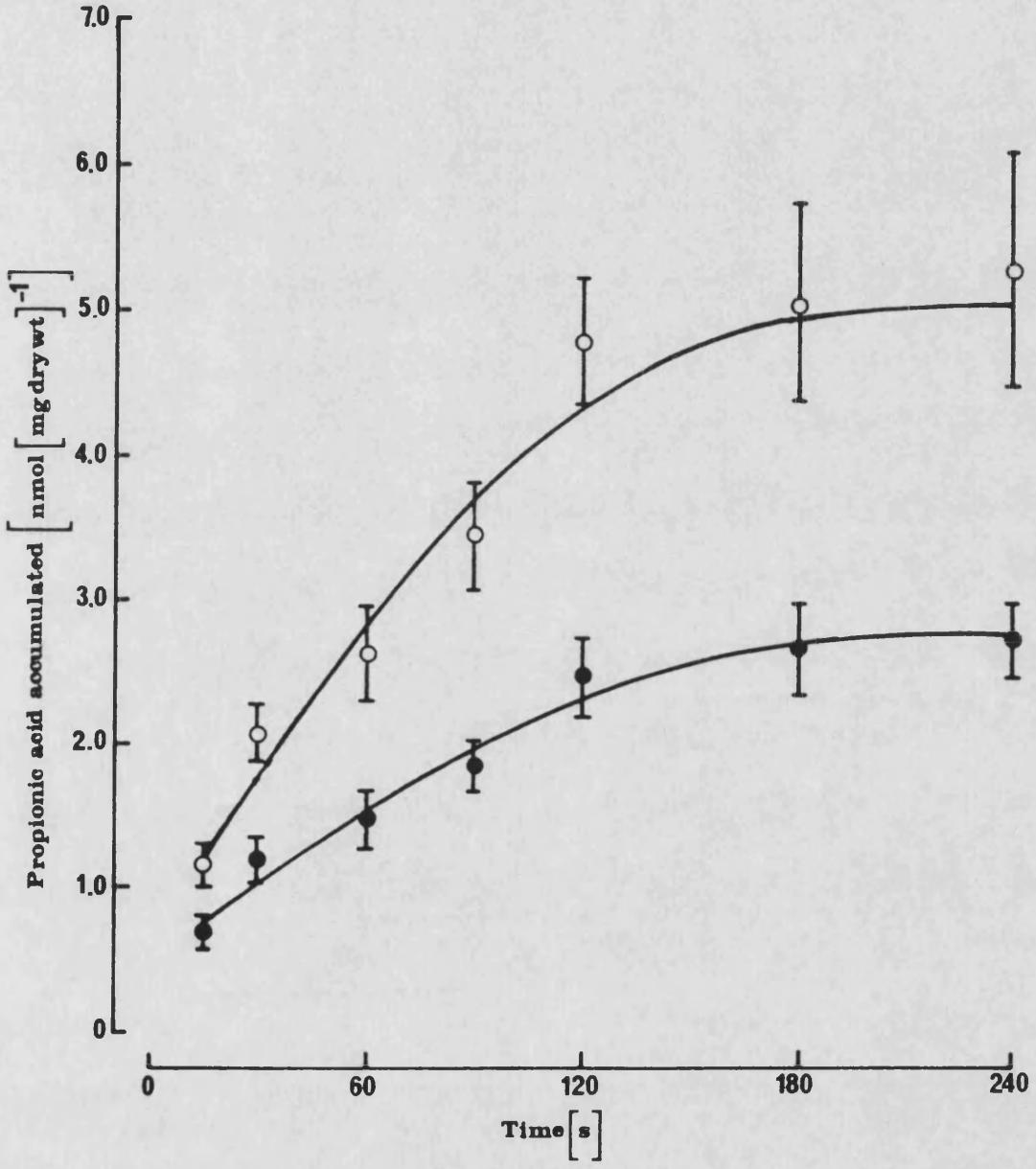


Figure 9. Time-course of propionic acid accumulation by de-energized (●) or energized (○) suspensions of Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures. Vertical bars indicate standard errors.

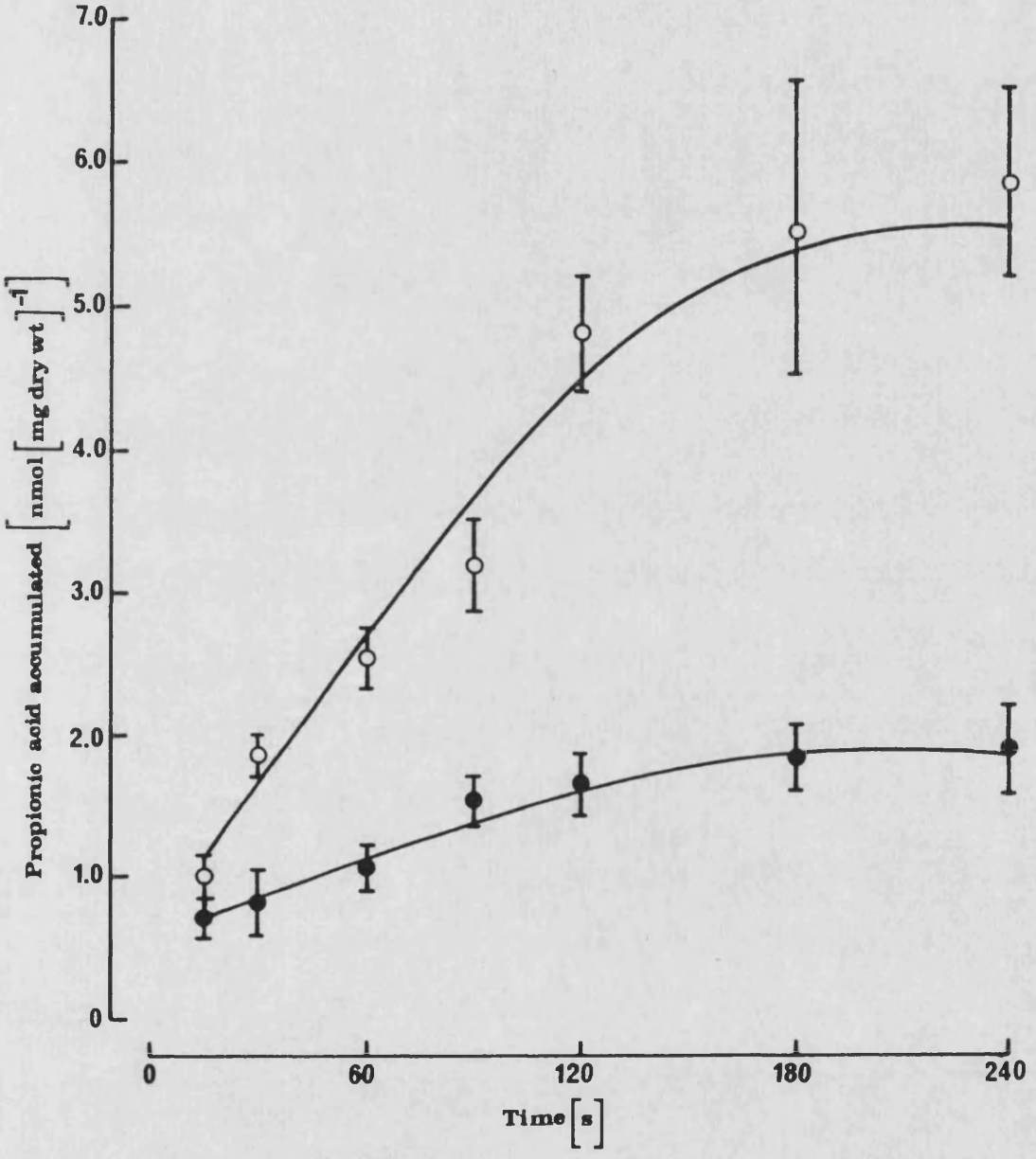


Figure 10. Time-course of propionic acid accumulation by de-energized (●) or energized (○) suspensions of Saccharomyces cerevisiae NCYC 431 harvested from 16 h cultures. Vertical bars indicate standard errors.

Ethanol concn. (M)	Extracellular pH value	Intracellular pH value	ΔpH
0.0	4.68 ± 0.12	5.85 ± 0.11	1.17
0.5	4.73 ± 0.09	5.75 ± 0.06	1.02
1.0	4.81 ± 0.15	5.71 ± 0.08	0.90
1.5	4.82 ± 0.22	5.63 ± 0.08	0.81
2.0	4.85 ± 0.19	5.56 ± 0.12	0.71

Table 2. Effect of ethanol on intracellular and extracellular pH values of suspensions of de-energized *Saccharomyces cerevisiae* NCYC 431 harvested from 8 h cultures. pH values are quoted ± standard error (n=5).

Ethanol concn. (M)	Extracellular pH value	Intracellular pH value	ΔpH
0.0	4.55 ± 0.24	5.90 ± 0.18	1.35
0.5	4.59 ± 0.16	5.79 ± 0.11	1.20
1.0	4.63 ± 0.31	5.69 ± 0.19	1.06
1.5	4.67 ± 0.11	5.65 ± 0.17	0.98
2.0	4.75 ± 0.20	5.60 ± 0.08	0.85

Table 3. Effect of ethanol on intracellular and extracellular pH values of suspensions of de-energized Saccharomyces cerevisiae NCYC 431 harvested from 16 h cultures. pH values quoted are ± standard error (n=5).

Ethanol concn. (M)	Extracellular pH value	Intracellular pH value	ΔpH
0.0	3.55 ± 0.16	6.36 ± 0.14	2.81
0.5	3.66 ± 0.23	6.31 ± 0.14	2.65
1.0	3.93 ± 0.18	6.22 ± 0.44	2.29
1.5	4.14 ± 0.11	6.10 ± 0.08	1.96
2.0	4.20 ± 0.34	5.97 ± 0.12	1.77

Table 4. Effect of ethanol on intracellular and extracellular pH values of suspensions of energized Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures. pH values quoted are ± standard error $\overline{(n=5)}$.

Ethanol concn. (M)	Extracellular pH value	Intracellular pH value	ΔpH
0.0	3.65 ± 0.22	6.52 ± 0.12	2.87
0.5	3.77 ± 0.30	6.50 ± 0.10	2.73
1.0	3.87 ± 0.19	6.44 ± 0.12	2.57
1.5	3.99 ± 0.30	6.37 ± 0.14	2.38
2.0	4.09 ± 0.27	6.37 ± 0.10	2.28

Table 5. Effect of ethanol on intracellular and extracellular pH values of suspensions of energized Saccharomyces cerevisiae NCYC 431 harvested from 16 h cultures. pH values quoted are ± standard error $\overline{(n=5)}$.

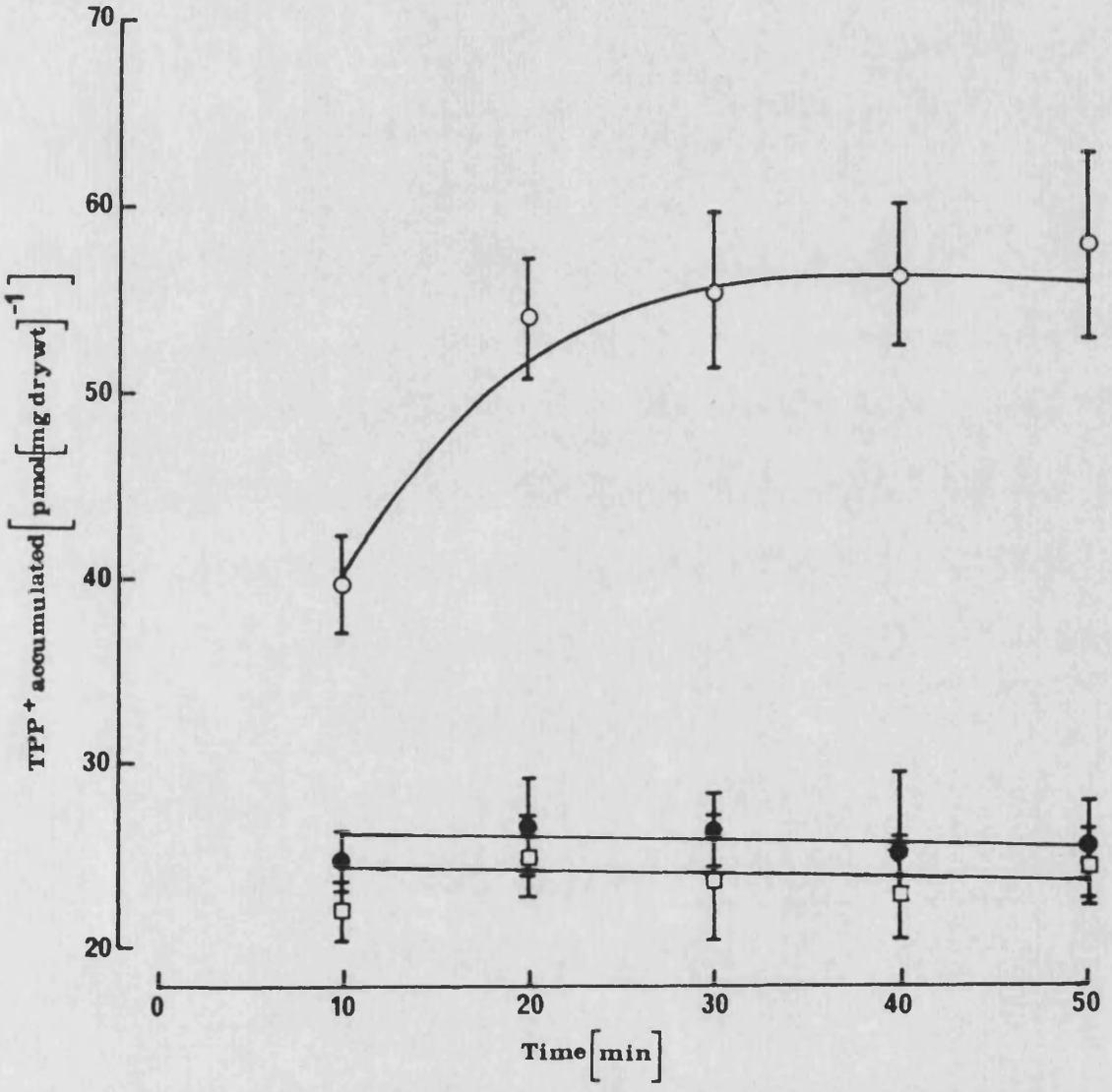


Figure 11. Time-course of TPP^+ accumulation by Saccharomyces cerevisiae NCYC 431 from 8 h cultures. Uptake of TPP^+ was followed in suspensions of de-energized organisms (O), energized organisms (●), or in suspensions of de-energized organisms supplemented with 1 mM-DNP (□). Vertical bars indicate standard errors.

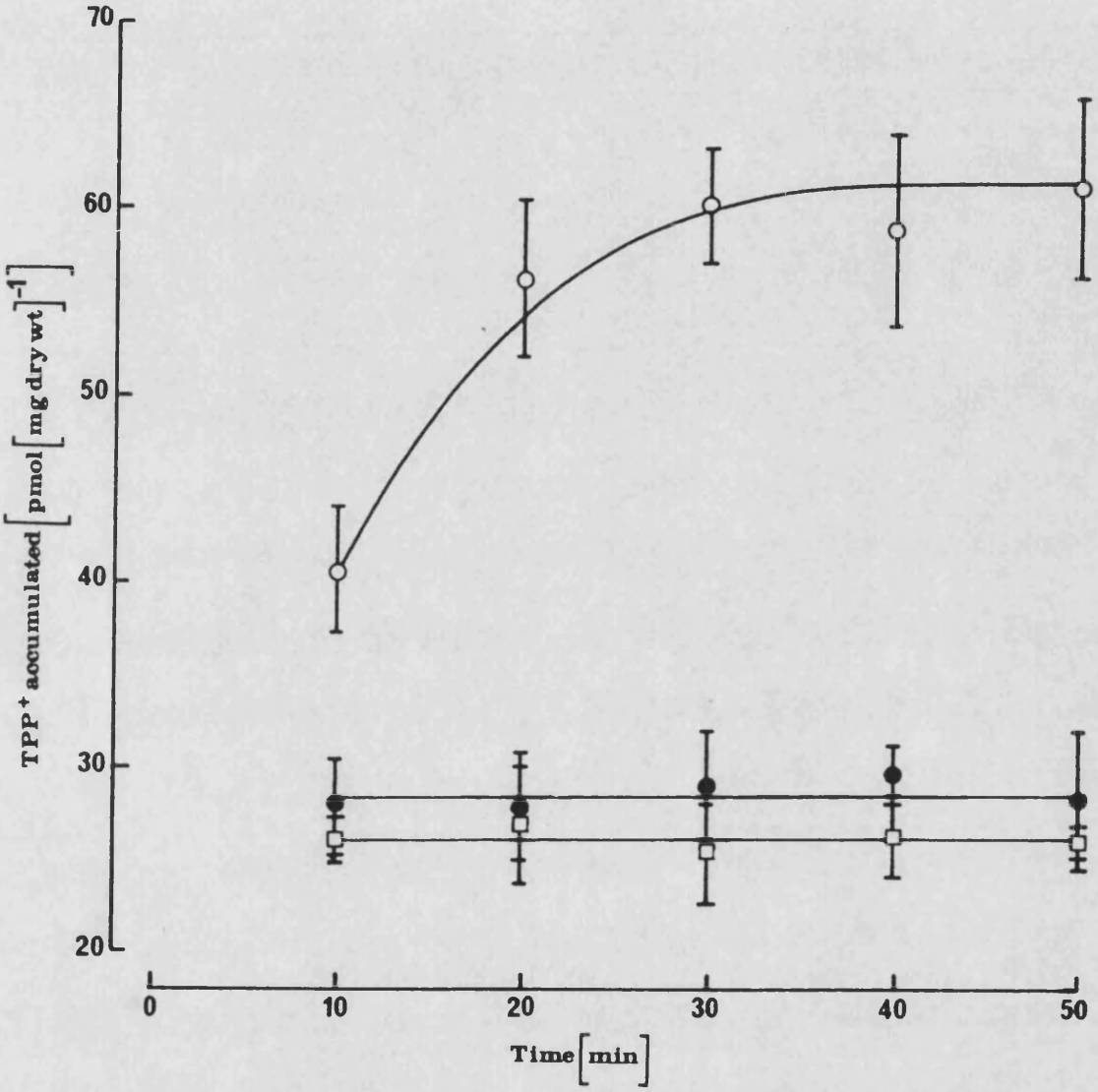


Figure 12. Time-course of TPP^+ accumulation by Saccharomyces cerevisiae NCYC 431 from 16 h cultures. Uptake of TPP^+ was followed in suspensions of de-energized organisms (O), energized organisms (●), or in suspensions of de-energized organisms supplemented with 1 mM-DNP (□). Vertical bars indicate standard errors.

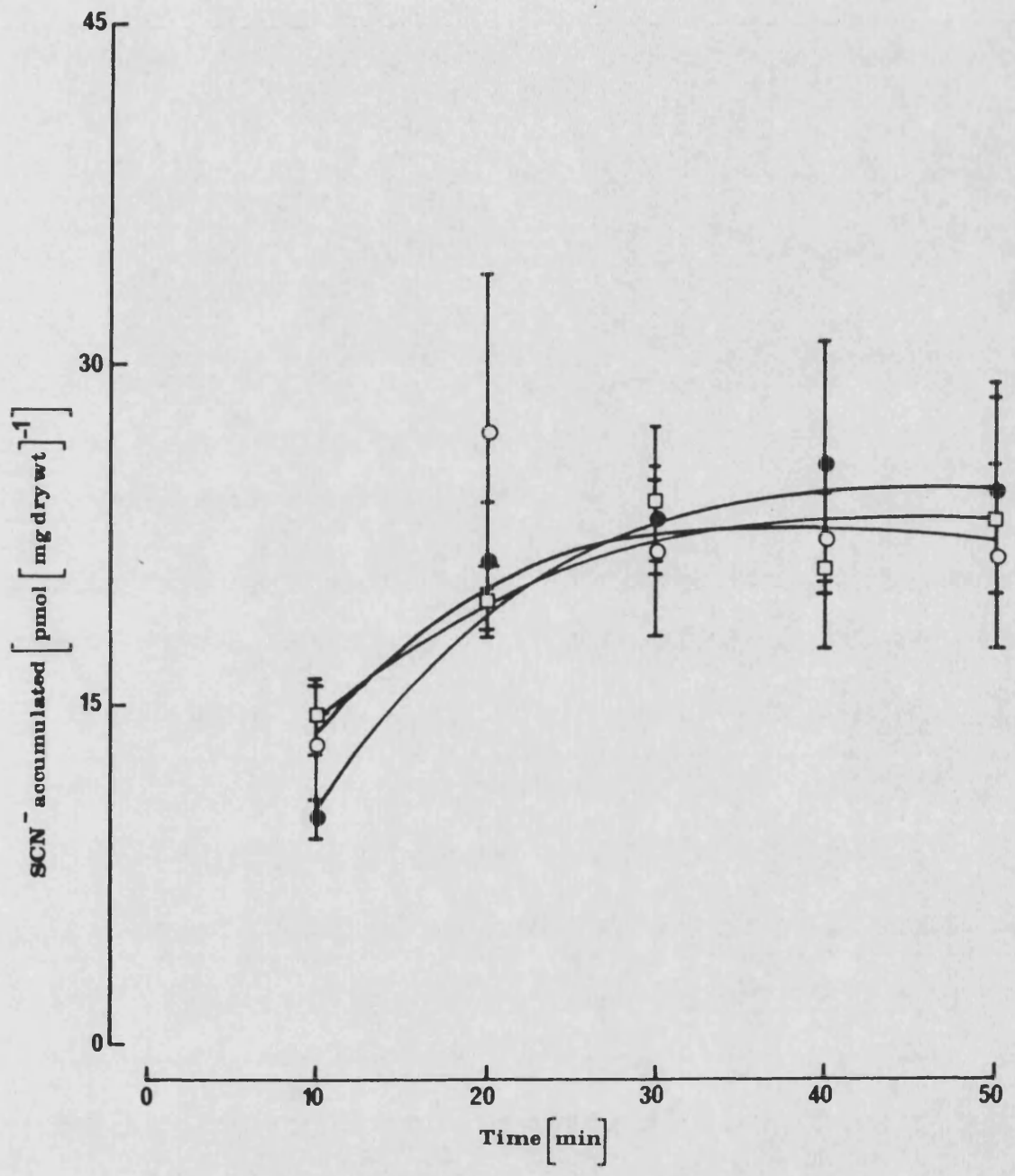


Figure 13. Time-course of SCN^- accumulation by Saccharomyces cerevisiae NCYC 431 from 8 h cultures. Uptake of SCN^- was followed in suspensions of de-energized organisms (O), energized organisms (●), or in suspensions of de-energized organisms supplemented with 2.0 M-ethanol (□). Vertical bars indicate standard errors.

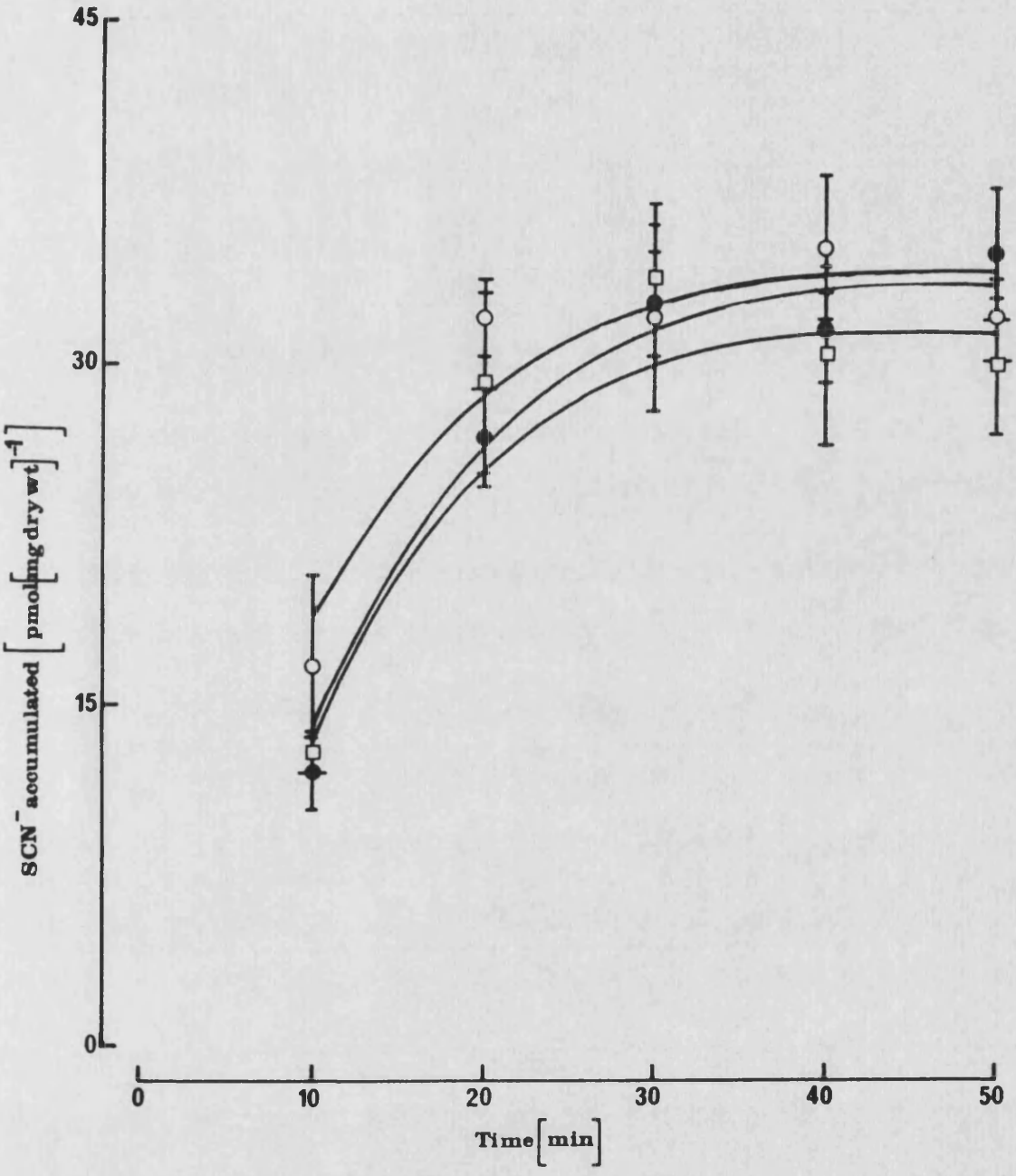


Figure 14. Time-course of SCN^- accumulation by Saccharomyces cerevisiae NCYC 431 from 16 h cultures. Uptake of SCN^- was followed in suspensions of de-energized organisms (O), energized organisms (●), or in suspensions of de-energized organisms supplemented with 2.0 M-ethanol (□). Vertical bars indicate standard errors.

Ethanol concn. (M)	Δψ value (mV)	
	8 h cultures	16 h cultures
0.0	-44 ± 8	-53 ± 8
0.5	-19 ± 12	-41 ± 11
1.0	ND	ND
1.5	ND	ND
2.0	ND	ND

Table 6. Effect of ethanol on Δψ values of suspensions of de-energized Saccharomyces cerevisiae NCYC 431. Values quoted are ± standard error. ND indicates that a Δψ value was not detectable (n=5).

Ethanol concn. (M)	Δp value (mV)			
	8 h cultures		16 h cultures	
	De-energized	Energized	De-energized	Energized
0.0	-113	-165	-132	-169
0.5	-79	-155	-111	-161
1.0	-53	-134	-62	-151
1.5	-48	-115	-58	-140
2.0	-42	-104	-50	-134

Table 7. Effect of culture age and ethanol on the Δp values in de-energized and energized *Saccharomyces cerevisiae* NCYC 431.

KINETIC CHARACTERISTICS OF, AND EFFECT OF ETHANOL ON, THE PLASMA-MEMBRANE ATPASE OF SACCHAROMYCES CEREVISIAE NCYC 431

Comparison of Methods for Plasma-Membrane Isolation

Sucrose density-gradient centrifugation. Electron microscopic examination of samples removed from the 45 - 55% (w/v) sucrose interface of density-gradients showed the presence of membranous material (Plate 1). However, this fraction also contained a number of unlysed spheroplasts as well as other contaminating debris. Isolated membranes could not be observed in samples removed from the 55% - 60% (w/v) sucrose interface of density-gradients. Consequently, only material removed from the 45 - 55% (w/v) sucrose interface was solubilised and assayed for protein content and ATPase activity. Samples from this fraction had a comparatively high protein content and a low level of ATPase activity (Table 8). Indeed, approximately the same level of ATPase activity was obtained by assaying spheroplast lysates (Table 8). In addition, the ATPase activity of fractions from sucrose-density gradients was only moderately sensitive to orthovanadate, a clear indication of the impurity of plasma-membrane preparations obtained by this method from Sacch. cerevisiae NCYC 431 harvested from 16 h cultures.

Attachment of cationically-charged silica microbeads. Plasma-membrane preparations obtained by using cationically-charged silica microbeads had a significantly lower protein content and higher ATPase activity than fractions isolated from sucrose-density gradients (Table 8). Supplementation of spheroplasting buffer with 0.1 M-sodium metabisulphite (Kitamura and Yamamoto,

1981) resulted in a marked improvement in the susceptibility to lysis of spheroplasts derived from organisms from 16 h cultures, such that greater than 99% lysis of spheroplasts was achieved routinely. This improvement in the extent of spheroplast lysis resulted in a further enhancement in the purity of plasma-membrane preparations obtained using silica microbeads, indicated both by the absence of contaminating non-membranous and mitochondrial material when preparations were examined under the electron microscope (Plates 2a and 2b), and the marked increase in orthovanadate-sensitive ATPase activity (Table 8). The attachment of silica microbeads was consequently the method of choice for obtaining plasma membranes for all further experiments on the plasma-membrane ATPase.

Properties of the Plasma-Membrane ATPase

ATPase activity of membranes from organisms from 8 h or 16 h cultures was inhibited by orthovanadate, diethylstilbestrol and DCCD (Figs. 15 and 16). The inhibitor concentrations required to produce half-maximal inhibition of the plasma-membrane ATPase were similar to those previously reported (Blasco et al., 1981; Serrano, 1980; Willsky, 1979). Oligomycin, up to 300 $\mu g\ ml^{-1}$, caused less than 5% inhibition of ATPase activity in membranes from organisms from 8 h and 16 h cultures. The pH optimum of the ATPase activity in membranes from organisms from 8 h cultures was around 6.5, while the optimum for the activity in membranes from organisms from 16 h cultures was near 6.0 (Fig. 17).

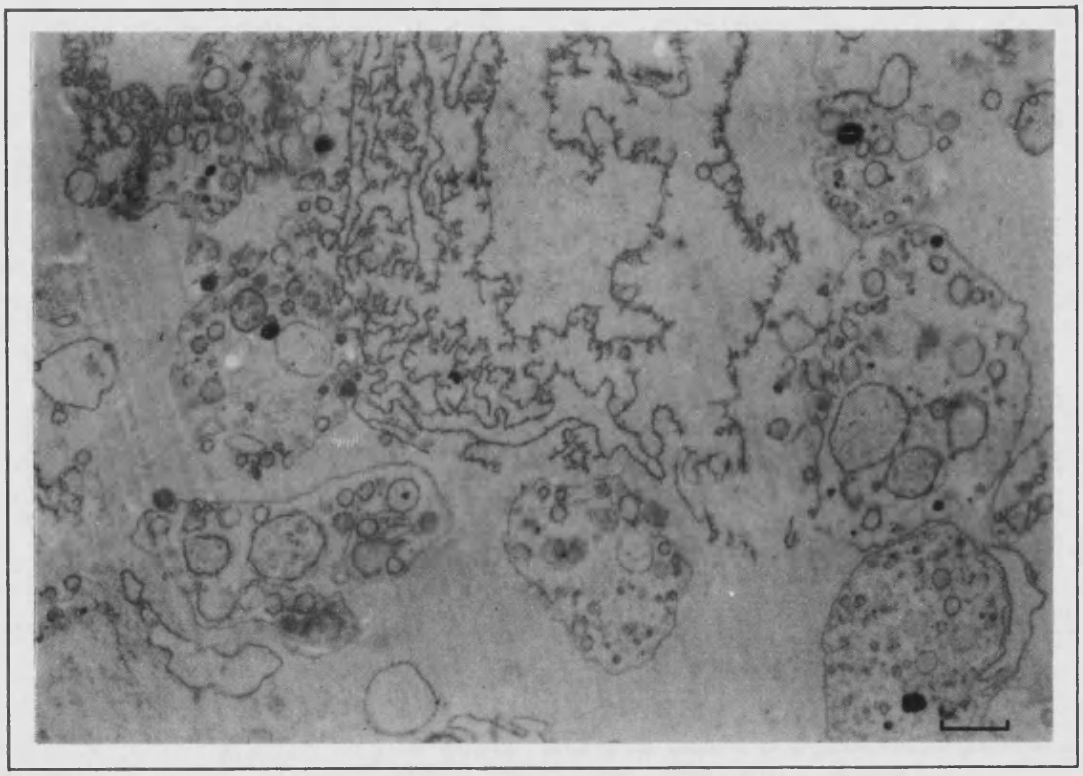
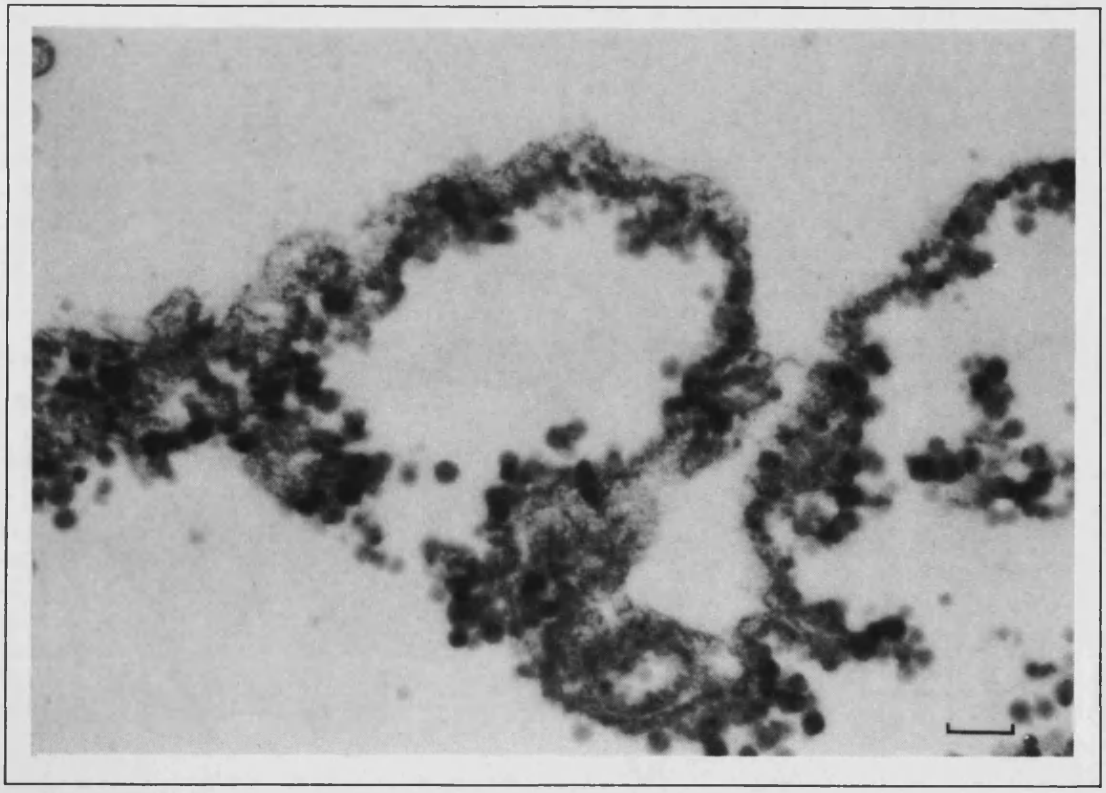


Plate 1. Electron micrograph of a plasma-membrane preparation obtained from Saccharomyces cerevisiae NCYC 431 by sucrose density-gradient centrifugation. Organisms were harvested from 16 h cultures. This micrograph clearly shows the presence of unlysed spheroplasts and other contaminating material in plasma-membrane fractions purified by this method. Bar represents 1.0 µm.

Plate 2a. Electron micrograph of a plasma-membrane preparation obtained from Saccharomyces cerevisiae NCYC 431 by attachment of cationically-charged silica microbeads. Organisms were harvested from 16 h cultures and treated with 0.1 M-sodium metabisulphite prior to spheroplasting. The lack of contamination in this preparation is evident from the absence of granular material in the section. Bar represents 0.5 µm.

Plate 2b. Higher magnification electron micrograph of a plasma-membrane preparation obtained from Saccharomyces cerevisiae NCYC 431 by using silica microbeads, showing the attachment of microbeads to only one face of plasma membranes. Bar represents 0.2 µm.



Source of plasma-membrane preparation	Protein content (mg ml^{-1})	Orthovanadate sensitive ATPase activity (µmol Pi (mg protein)$^{-1}$ min^{-1})	Percent of total ATP-hydrolytic activity sensitive to orthovanadate
Spheroplast lysate	1.80	0.06	29.5
Sucrose-density gradient (fraction removed from 45-55% (w/v) sucrose interface)	1.20	0.11	54.0
Pellet obtained after attachment of cationically-charged microbeads	0.38	0.32	61.2
	0.40*	1.22*	81.1

Table 8. Comparison of methods for isolating plasma membranes from Saccharomyces cerevisiae NCYC 431 harvested from 16 h cultures. All values are the mean of at least two separate determinations. * Indicates sodium metabisulphite (0.1 M) was incorporated in spheroplasting buffer.

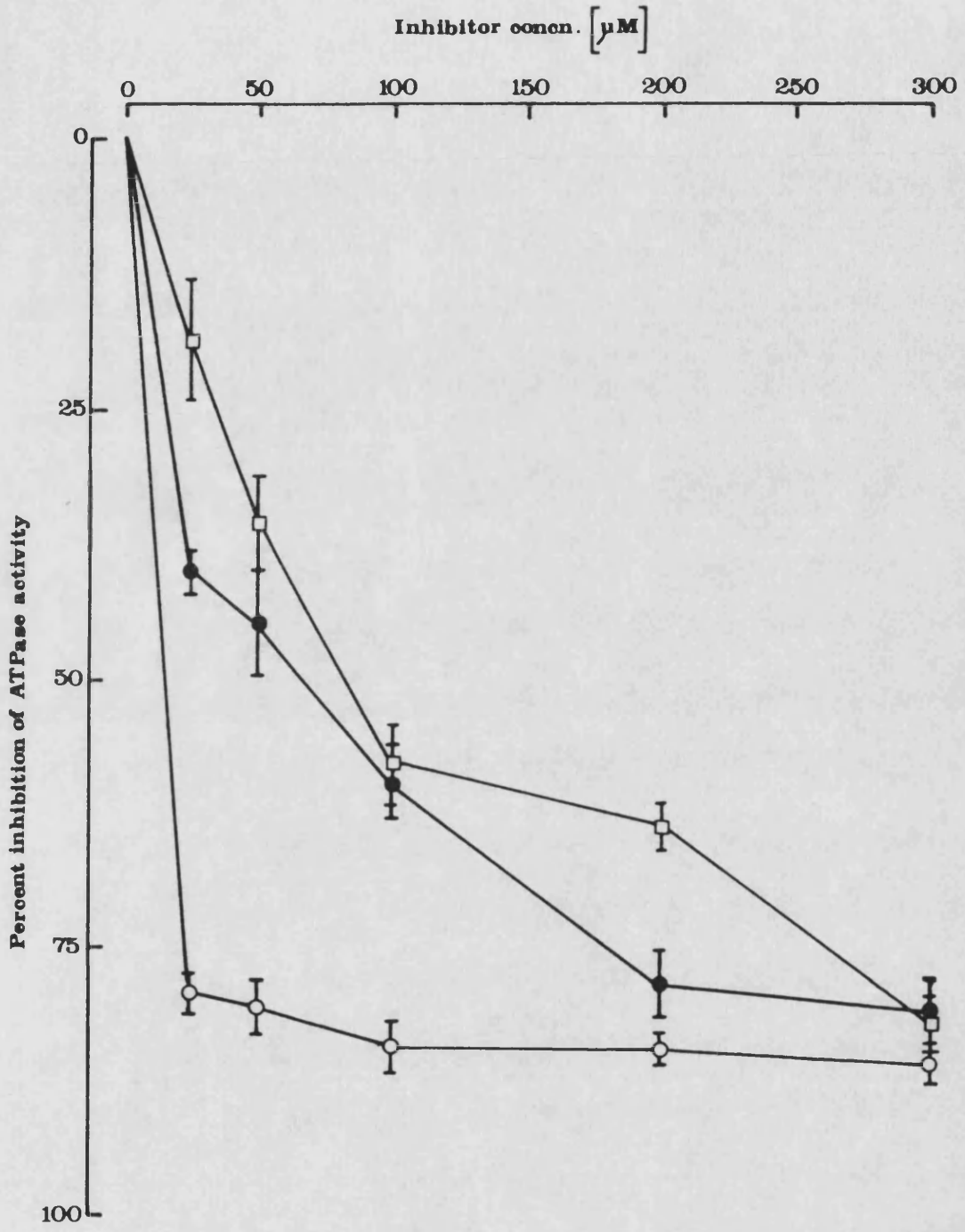


Figure 15. Effect of inhibitors on the ATPase activity of plasma membranes from 8 h cultures of Saccharomyces cerevisiae NCYC 431. (O) indicates response to orthovanadate, (●) diethylstilbestrol and (□) DCCD. Vertical bars indicate standard errors.

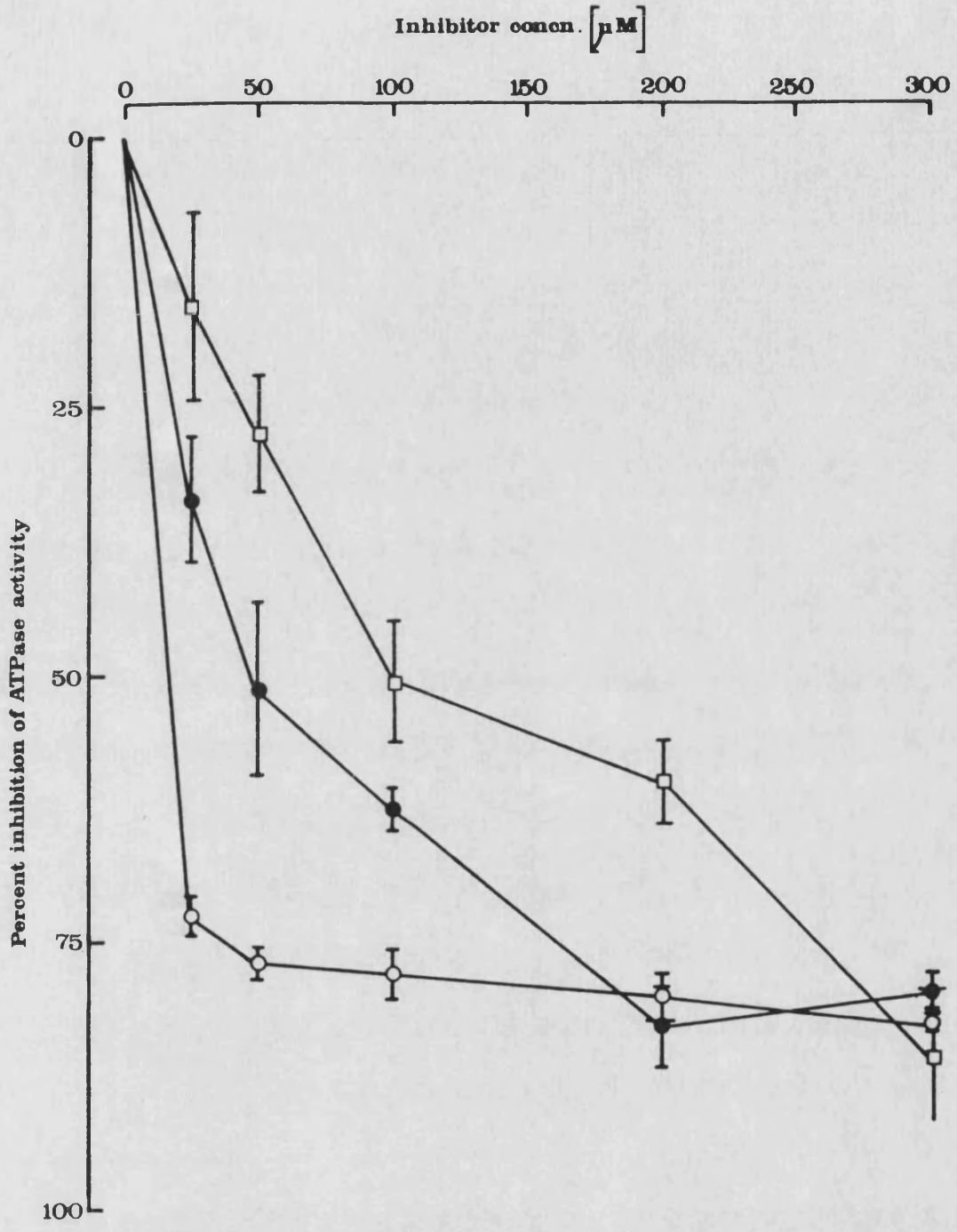


Figure 16. Effect of inhibitors on the ATPase activity of plasma membranes from 16 h cultures of Saccharomyces cerevisiae NCYC 431. (○) indicates response to orthovanadate, (●) diethylstilbestrol and (□) DCCD. Vertical bars indicate standard errors.

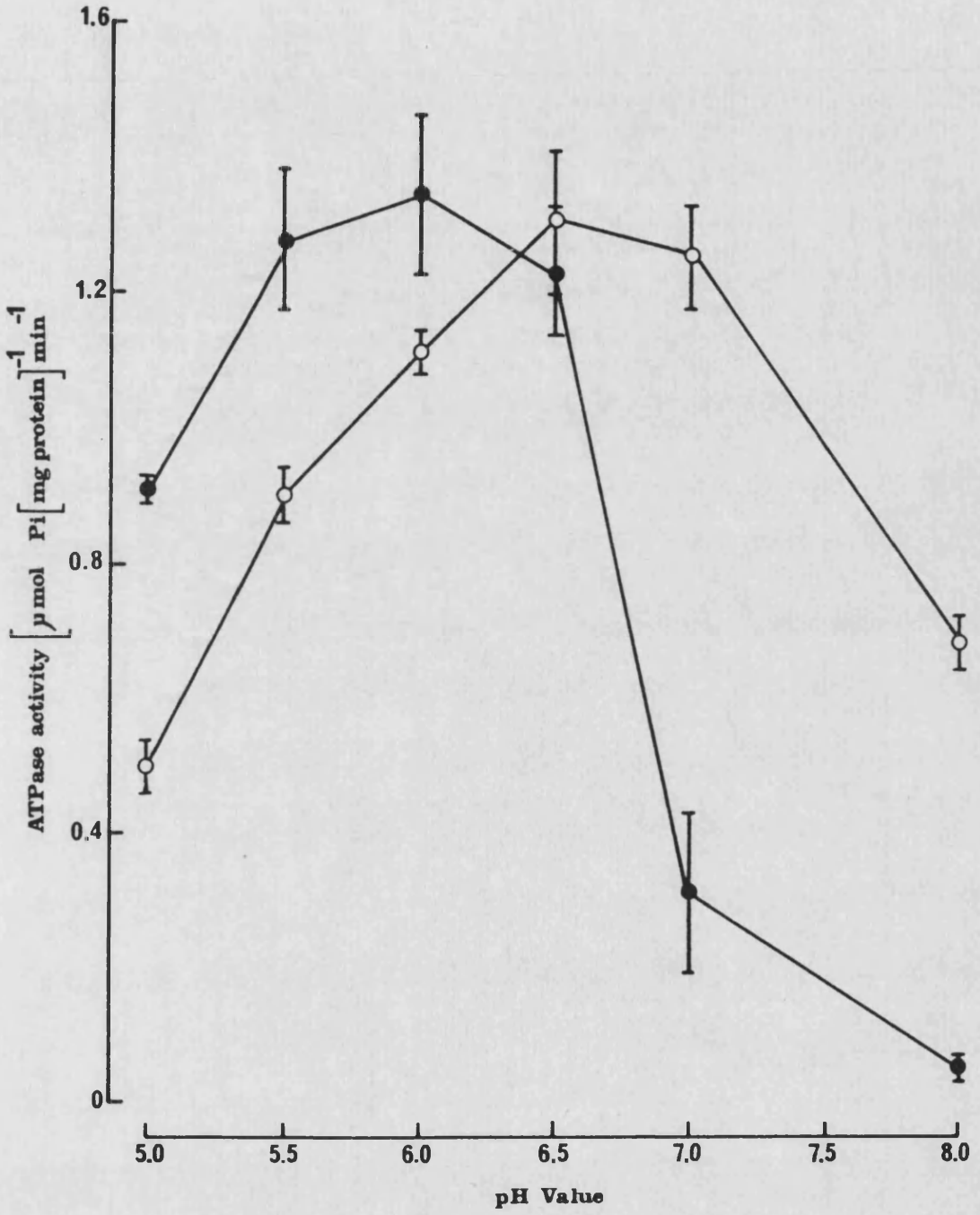


Figure 17. Effect of pH value on the ATPase activity of plasma membranes from 8 h cultures (O) or 16 h cultures (●) of Saccharomyces cerevisiae NCYC 431. Vertical bars indicate standard errors.

Hanes plots (Hanes, 1932) were constructed for ATPase activity of membranes from 8 h and 16 h cultures (Fig. 18). Values for K_m, derived from these plots, were largely unaffected by culture age, whereas V_{max} values differed considerably (Table 9). Dixon plots (Dixon, 1953) showed that ethanol, up to 2.0 M, non-competitively inhibited ATPase activity in membranes from organisms from both 8 h and 16 h cultures (Figs. 19 and 20), although the inhibition constant (K_i) derived from these plots was different for the two preparations.

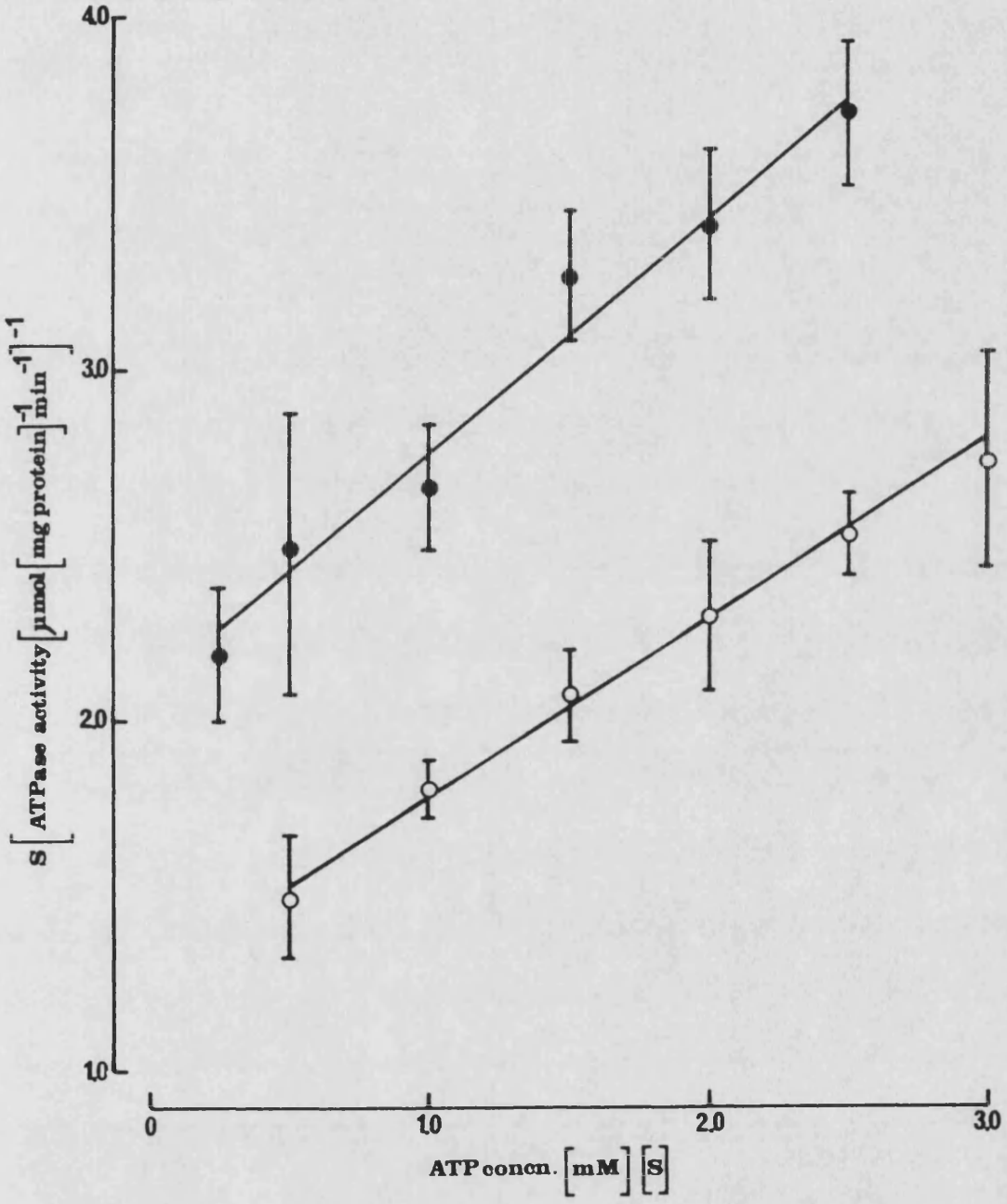


Figure 18. Hanes plots of ATPase activity of plasma membranes from 8 h cultures (O) or 16 h cultures (●) of Saccharomyces cerevisiae NCYC 431. Vertical bars indicate standard errors.

Figure 19. Dixon plot for the effect of ethanol on ATPase activity of plasma membranes from 8 h cultures of Saccharomyces cerevisiae NCYC 431. (O)indicates plasma-membrane preparations were supplemented with 0.5 mM, (●) with 1.0 mM, (□) with 1.5 mM and (■) with 2.0 mM-Na-ATP. Vertical bars indicate standard errors.

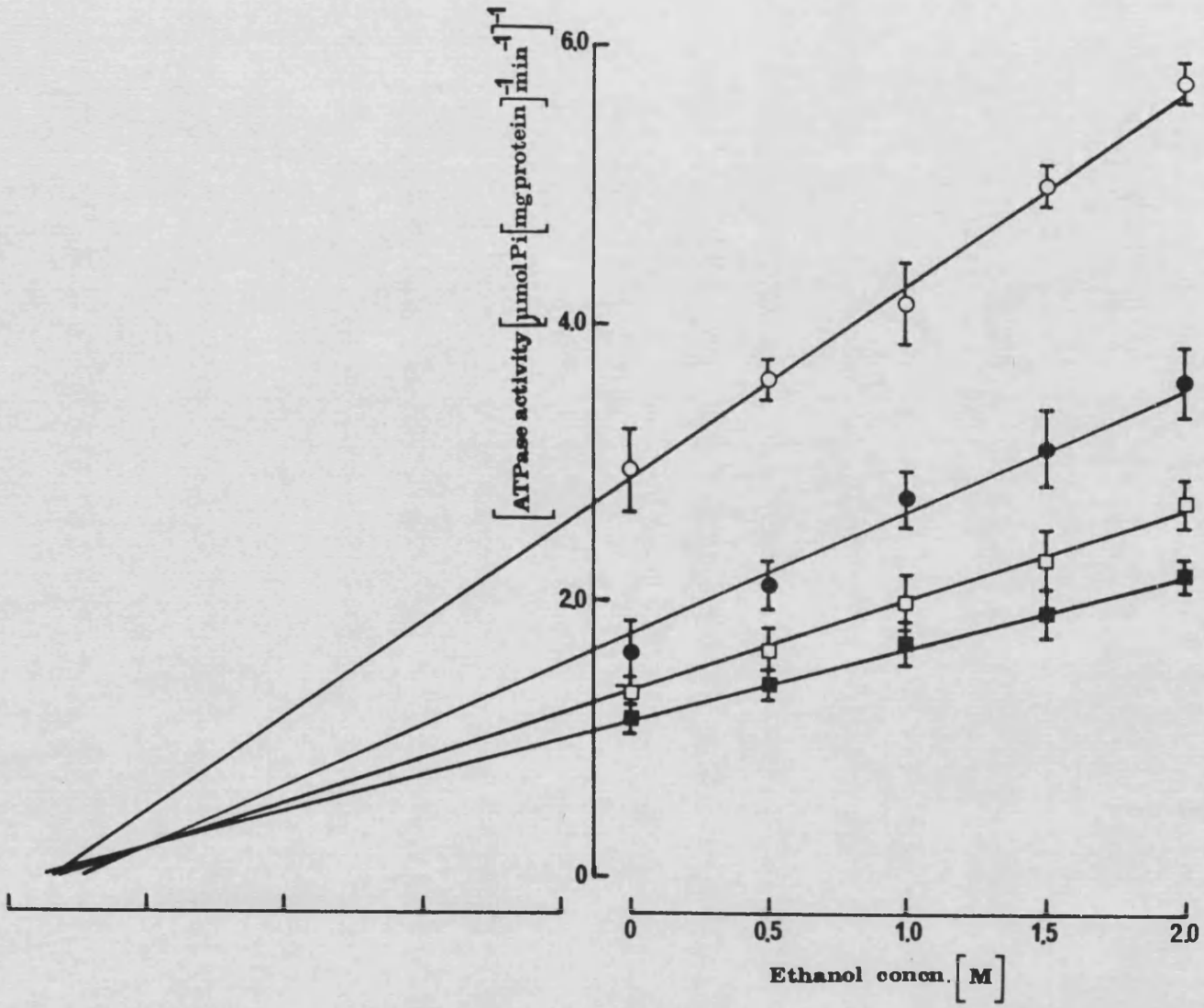
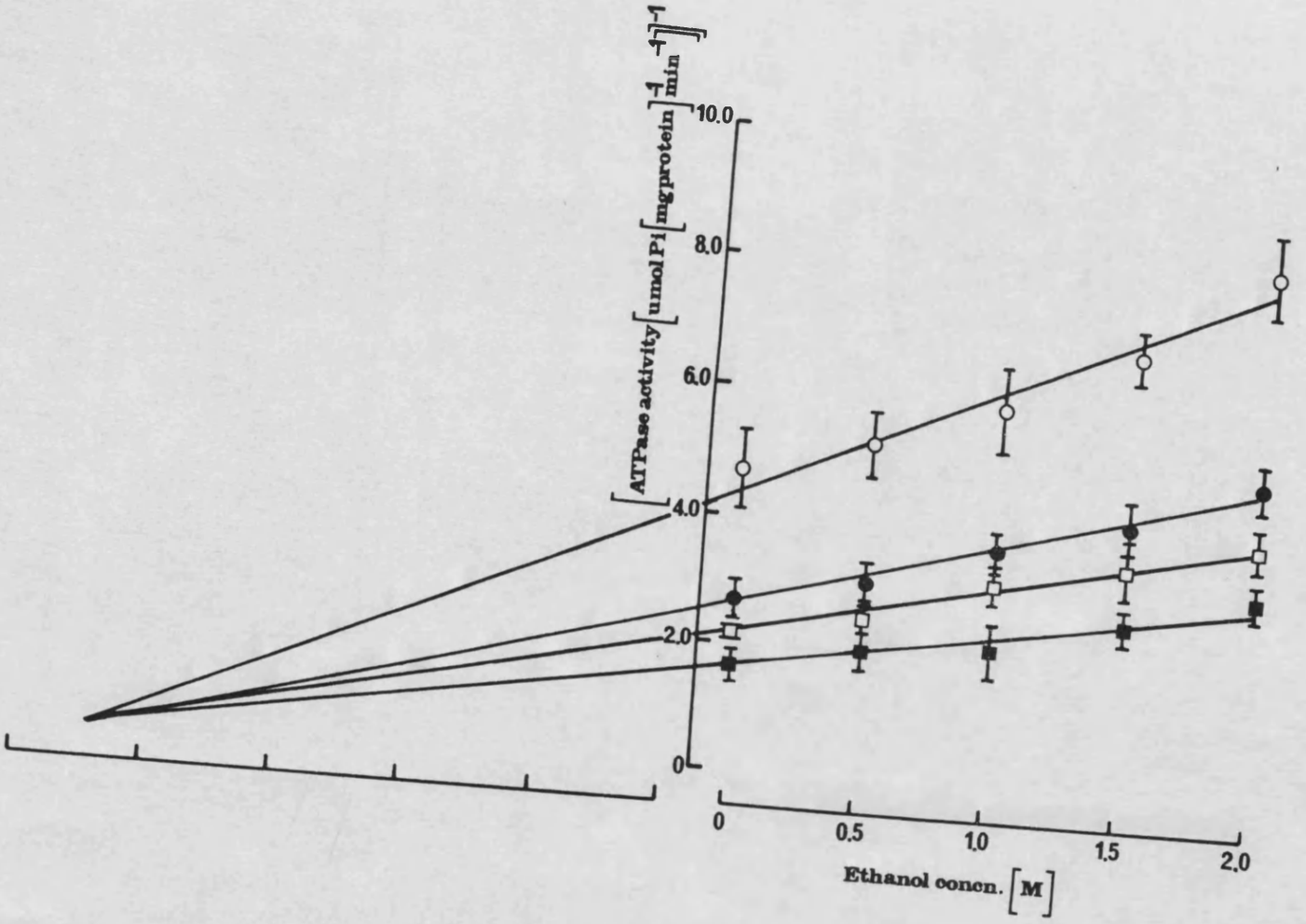

[ATPase activity [µmol Pi [mg protein]$^{-1}$ min^{-1}]$^{-1}$]$^{-1}$
6.0
4.0
2.0
0
0
0.5
1.0
1.5
2.0
Ethanol concn. [M]

Figure 20. Dixon plot for the effect of ethanol on ATPase activity of plasma membranes from 16 h cultures of Saccharomyces cerevisiae NCYC 431. (O) indicates plasma-membrane preparations were supplemented with 0.5 mM, (●) with 1.0 mM, (□) with 1.5 mM and (■) with 2.0 mM-Na-ATP. Vertical bars indicate standard errors.


ATPase activity [μmol Pi [mgprotein]-1 min-1]-1
10.0
8.0
6.0
4.0
2.0
0
0
0.5
1.0
1.5
2.0
Ethanol concn. [M]

Constant	Value for ATPase from organisms grown for:	
	8 hours	16 hours
K_m [ATP] (mM)	2.47 ± 0.08	2.62 ± 0.32
V_{max} (μmol Pi (mg protein)$^{-1}$min^{-1})	1.97 ± 0.09	1.33 ± 0.02
K_i [EtOH] (M)	1.87 ± 0.12	2.19 ± 0.08

Table 9. Kinetic constants for the plasma-membrane ATPase of Saccharomyces cerevisiae NCYC 431. K_m and V_{max} values were derived from Hanes (1932) plots and K_i values from Dixon (1953) plots. All values are quoted ± standard error.

PHOSPHOLIPID AND FATTY-ACYL COMPOSITIONS OF PLASMA MEMBRANES ISOLATED FROM SACCHAROMYCES CEREVISIAE NCYC 431

Lipid extracts from plasma-membrane preparations, obtained using cationically-charged silica microbeads, were comprised mainly of phospholipid and free sterol; some triglyceride and sterol ester was also present (Fig. 21). Extracts did not, however, contain any free fatty acid. The phospholipid fraction of plasma-membrane lipids contained predominantly phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine (Fig. 22). Phosphatidylethanolamine and phosphatidylcholine were the principal phospholipids, the former being present in greater proportion in membranes from organisms from 8 h compared with 16 h cultures. Phosphatidylinositol was present in about the same proportion in membranes from organisms from 8 h and 16 h cultures, while the proportion of phosphatidylserine increased with increasing culture age. The major fatty-acyl residues in all plasma-membrane phospholipids were $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$. However, the fatty-acyl composition of individual phospholipids differed in organisms from 8 h as compared with 16 h cultures, the degree of unsaturation of phospholipids decreasing with culture age (Tables 11 and 12). Of the four major phospholipids, phosphatidylethanolamine contained the highest proportion of unsaturated fatty-acyl residues, phosphatidylserine constituted the most saturated phospholipid class, while phosphatidylinositol showed greatest conservation of fatty-acyl unsaturation with culture age.

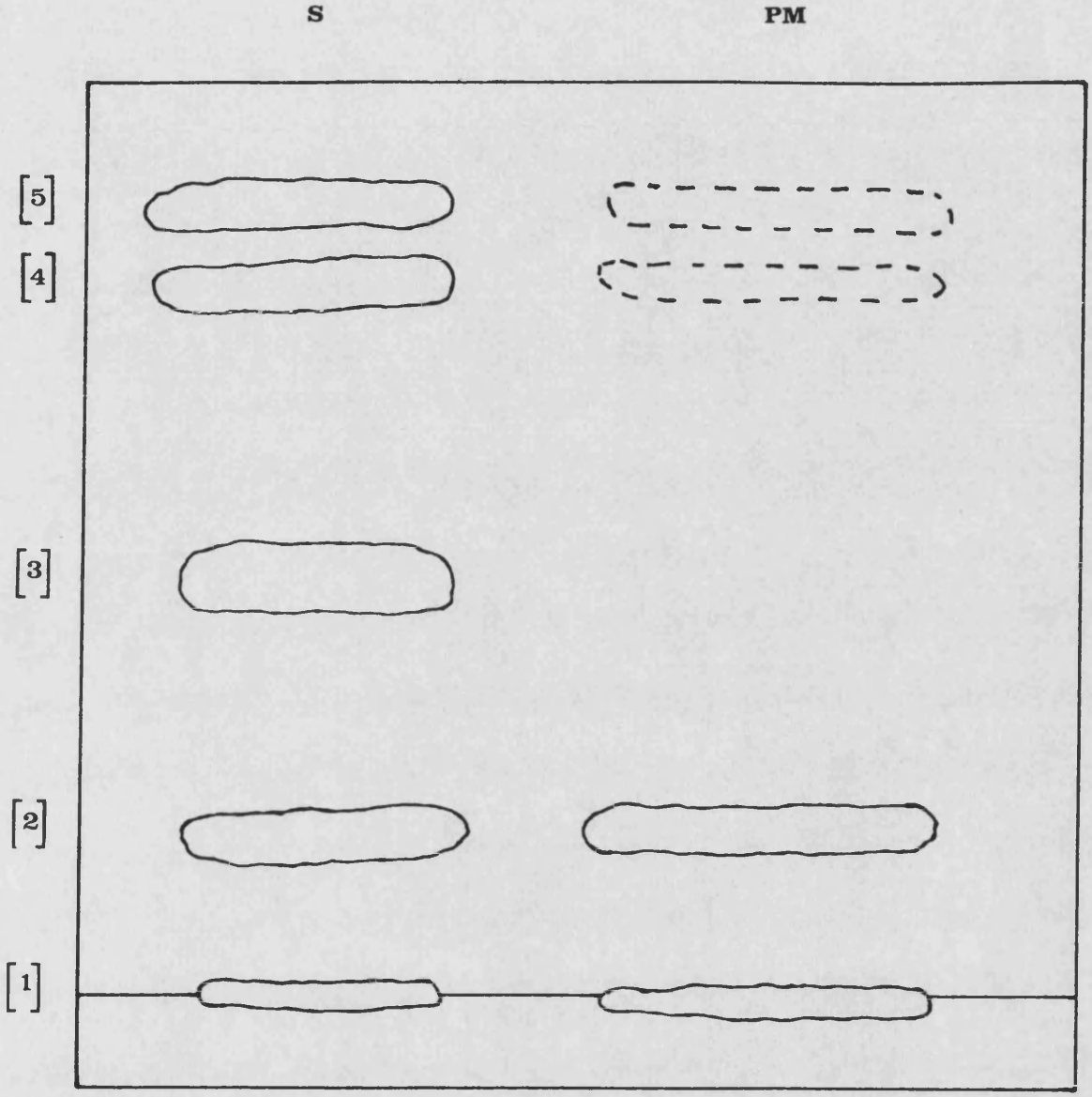
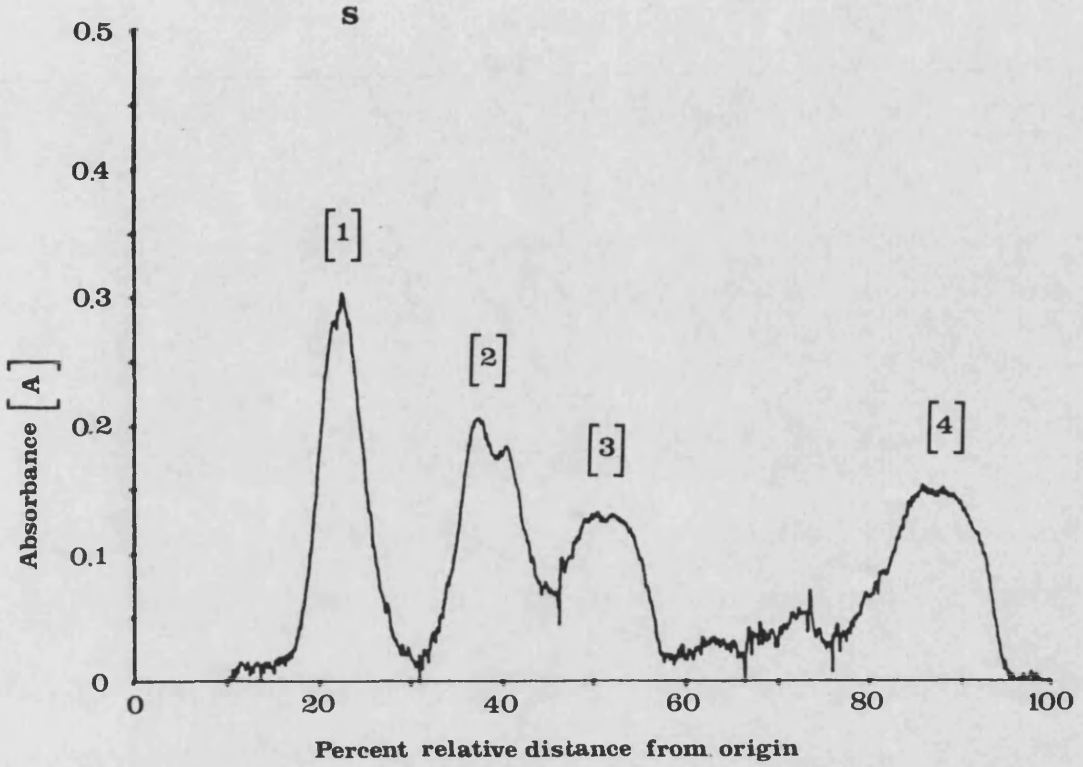
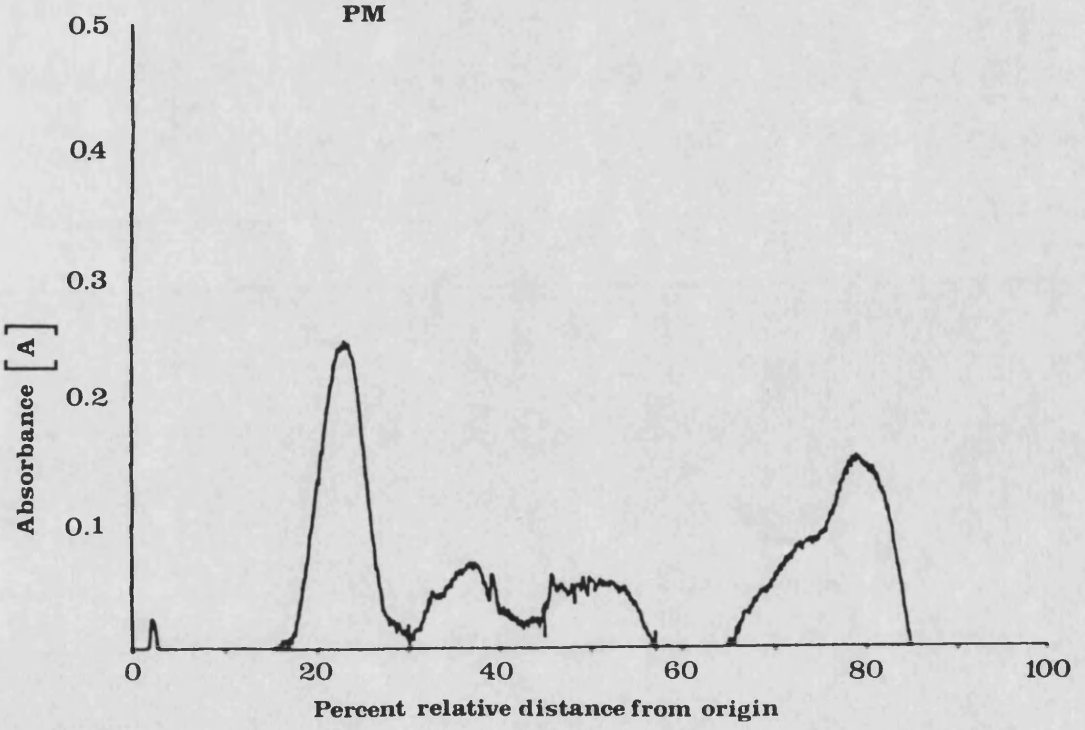


Figure 21. Lipids of plasma membranes(PM) isolated from organisms from 16 h cultures of Saccharomyces cerevisiae NCYC 431 separated by thin-layer chromatography in a solvent system of petroleum ether (60°C - 80°C)/diethyl ether/ acetic acid (70:30:1, by vol.). Standard lipid mix (S) contained phospholipid (1), free sterol (2), free fatty acid (3), triglyceride (4) and sterol ester (5). Solid lines indicate clearly defined bands, broken lines indicate diffuse bands.

Figure 22. Scanning-densitometer trace of phospholipids of plasma membranes (PM) isolated from Saccharomyces cerevisiae NCYC 431 from 16 h cultures. Phospholipids were separated by thin-layer chromatography in a solvent system of chloroform/methanol/acetic acid (65:25:8, by vol.). Standard phospholipid mix (S) contained phosphatidylcholine (1), phosphatidylethanolamine (4), phosphatidylinositol (3) and phosphatidylserine (2). Traces are representative of three separate experiments.


S
0.5
0.4
0.3
0.2
0.1
0
Absorbance [A]
[1]
[2]
[3]
[4]
0
20
40
60
80
100
Percent relative distance from origin


PM
0.5
0.4
0.3
0.2
0.1
Absorbance [A]
0
20
40
60
80
100
Percent relative distance from origin

Phospholipid	Percentage of total phospholipid Organisms grown for:	
	8 h	16 h
Phosphatidylethanolamine	46.32 ± 5.44	31.14 ± 2.24
Phosphatidylcholine	32.01 ± 3.60	35.28 ± 4.23
Phosphatidylinositol	18.49 ± 3.11	20.37 ± 5.13
Phosphatidylserine	3.06 ± 0.33	12.94 ± 2.33

Table 10. Phospholipid composition of plasma membranes from *Saccharomyces cerevisiae* NCYC 431 grown for 8 h or 16 h. Values quoted are ± standard error.

Fatty-acyl residue	Percentage of the total phospholipid class			
	PC	PE	PI	PS
12:0	1.98 ± 0.46	0.23 ± 0.08	0.41 ± 0.29	1.83 ± 0.16
14:0	2.14 ± 0.14	1.92 ± 0.33	1.10 ± 0.11	1.73 ± 0.21
14:1	0.71 ± 0.15	0.22 ± 0.05	0.81 ± 0.09	1.37 ± 0.22
16:0	22.70 ± 2.27	28.98 ± 1.21	32.49 ± 1.63	38.82 ± 1.75
16:1	46.22 ± 2.17	42.06 ± 1.78	28.67 ± 0.87	20.32 ± 3.80
18:0	4.41 ± 0.90	3.92 ± 0.24	9.30 ± 1.58	24.37 ± 2.95
18:1	21.69 ± 1.53	30.40 ± 2.04	26.72 ± 2.52	11.42 ± 1.41
Δ mol^{-1}	0.69	0.73	0.56	0.33

Table 11. Fatty-acyl composition of phospholipids in plasma membranes of Saccharomyces cerevisiae NCYC 431 harvested after 8 h incubation. Values are quoted ± standard error.

Fatty-acyl residue	Percentage of the total phospholipid class			
	PC	PE	PI	PS
12:0	1.34 ± 0.61	0.82 ± 0.18	1.71 ± 0.42	3.83 ± 0.67
14:0	2.79 ± 0.27	1.85 ± 0.21	2.48 ± 0.53	3.26 ± 0.43
14:1	1.38 ± 0.15	0.68 ± 0.10	1.69 ± 0.37	1.00 ± 0.36
16:0	33.50 ± 1.44	26.92 ± 2.64	27.40 ± 3.14	40.67 ± 5.04
16:1	34.25 ± 0.93	42.19 ± 4.47	31.19 ± 1.34	15.23 ± 3.25
18:0	9.73 ± 1.67	7.67 ± 0.56	16.91 ± 0.79	23.39 ± 3.10
18:1	16.95 ± 2.01	20.69 ± 1.99	18.01 ± 2.24	11.09 ± 1.50
Δmol^{-1}	0.53	0.64	0.51	0.27

Table 12. Fatty-acyl composition of phospholipids in plasma membranes of Saccharomyces cerevisiae NCYC 431 harvested after 16 h incubation. Values are quoted ± standard error.

EFFECT OF ETHANOL ON GLYCINE ACCUMULATION BY SACCHAROMYCES CEREVISIAE NCYC 431

Woolf-Hofstee (Hofstee, 1959) plots for accumulation of glycine by organisms from 8 h and 16 h cultures were concave, indicating the presence of two separable glycine accumulation systems (Figs. 23 and 24), which differ in affinity for glycine and velocity of solute accumulation (Table 13). The high-affinity system was not detectable in the GAP-less mutant (Fig. 25), although the K_T and V_{max} values of the low-affinity system were similar in the mutant and wild-type organism (Table 13). Dixon (1953) plots showed that ethanol caused non-competitive inhibition of glycine accumulation by the high-affinity system in energized and de-energized organisms (Figs. 26 - 29). Values for K_i derived from these plots were virtually the same in organisms from 8 h and 16 h cultures, but with energized organisms, the value was greater for organisms from 16 h cutures compared with 8 h cultures (Table 14). The inhibitory effect of glycine accumulation closely paralleled the decrease in Δp caused by ethanol (Table 7). This correspondence was observed with organisms from 8 h and 16 h cultures and in suspensions containing 50 - 100 μM glycine (Figs. 30 - 35).

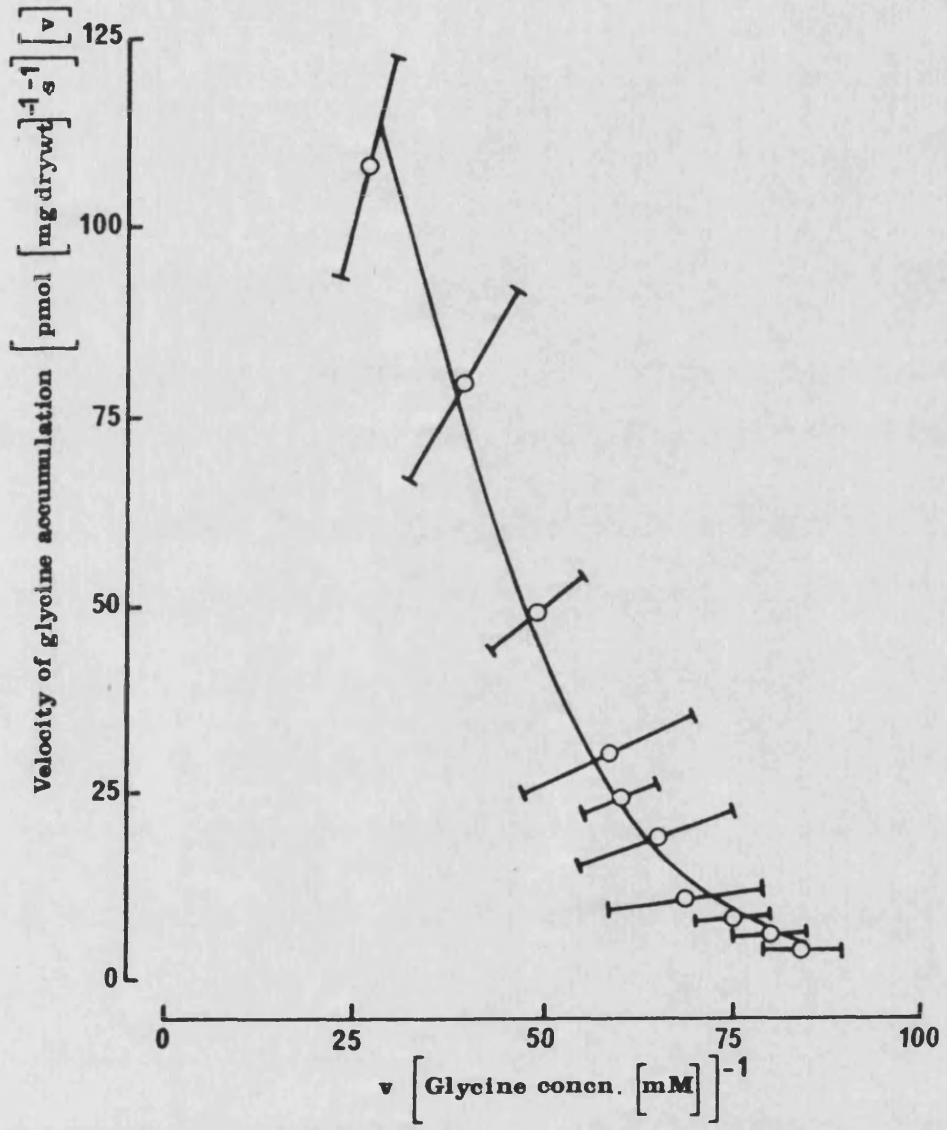


Figure 23. Woolf-Hofstee plot for accumulation of glycine by Saccharomyces cerevisiae NCYC 431 from 8 h cultures. Bars indicate standard errors.

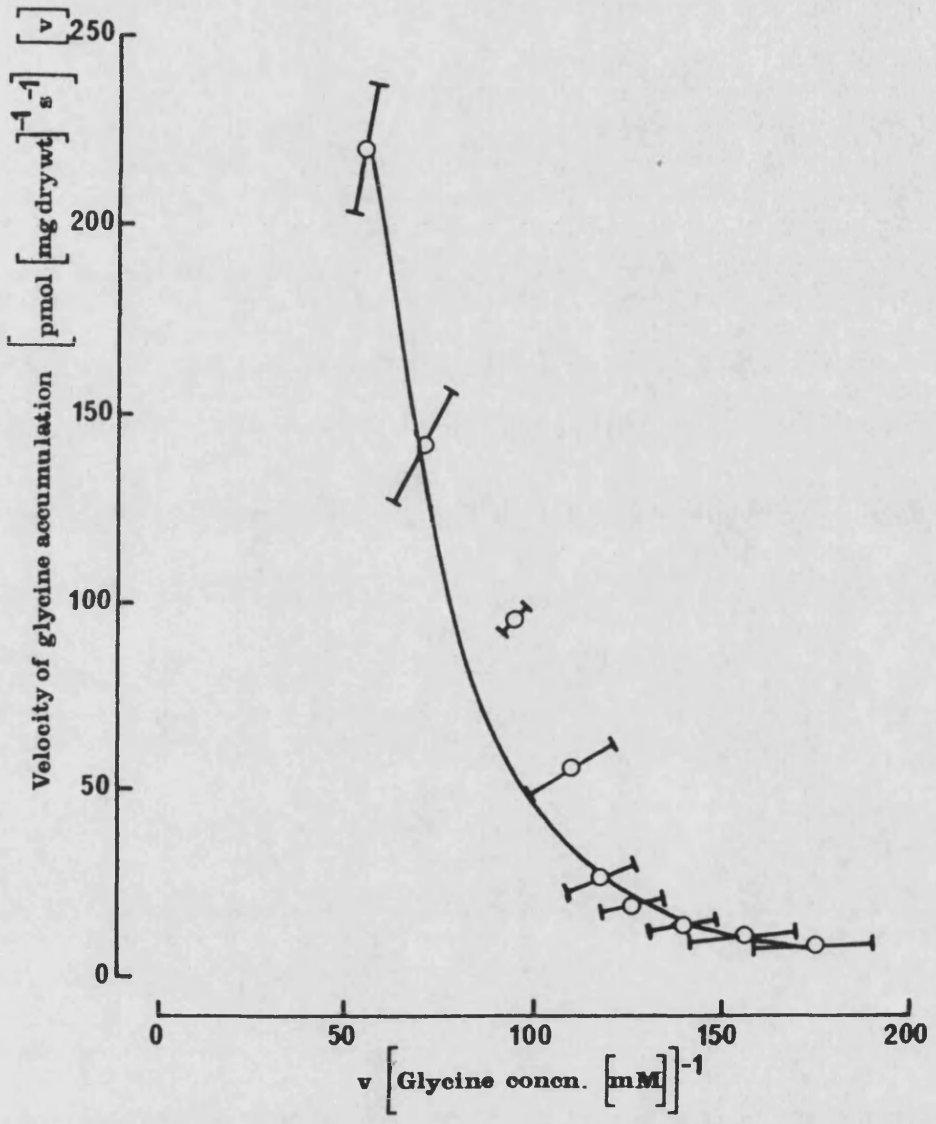


Figure 24. Woolf-Hofstee plot for accumulation of glycine by Saccharomyces cerevisiae NCYC 431 from 16 h cultures. Bars indicate standard errors.

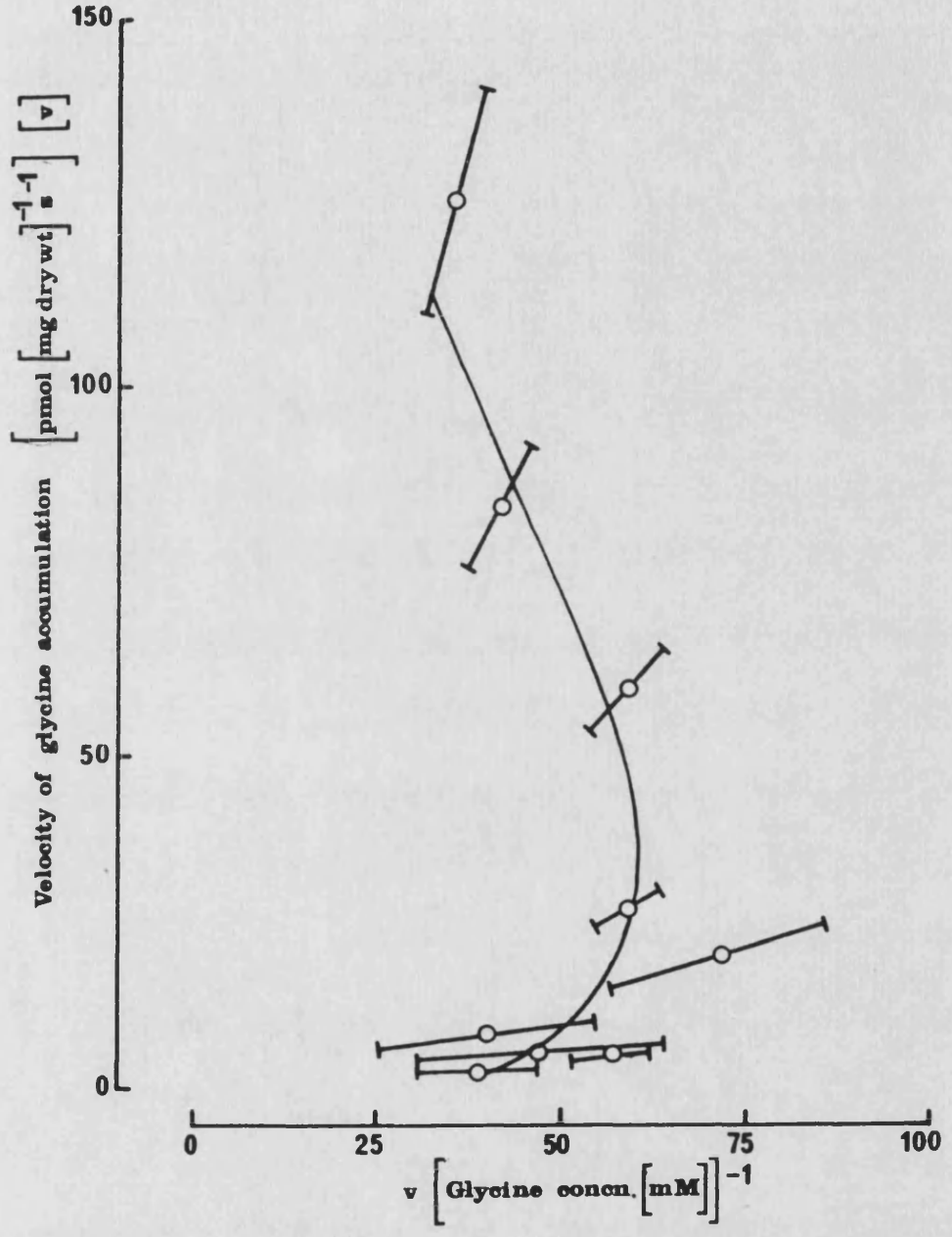


Figure 25. Woolf-Hofstee plot for accumulation of glycine by CC1 mutant of Saccharomyces cerevisiae NCYC 431. Organisms were harvested from 16 h cultures. Bars indicate standard errors.

Constant	Affinity of system	Values* for organisms from: cultures grown for: 8 h	16 h
K_T	High	65 ± 21	93 ± 42
	Low	2250 ± 48	2860 ± 80
	Low+	ND	2380 ± 270
V_{max}	High	7.80 ± 0.96	8.21 ± 1.52
	Low	275.90 ± 18.60	352.80 ± 26.90
	Low+	ND	181.80 ± 30.20

Table 13. Kinetic constants for accumulation of glycine by Saccharomyces cerevisiae NCYC 431. The units for K_T and V_{max} are μM and pmol (mg dry wt)$^{-1}s^{-1}$, respectively.

* Where necessary values were corrected as described by Neal(1972).

\+ Indicates kinetic constants determined for GAP-less mutant, CC1. ND indicates that kinetic constants were not determined under these conditions. All values are quoted ± standard error.

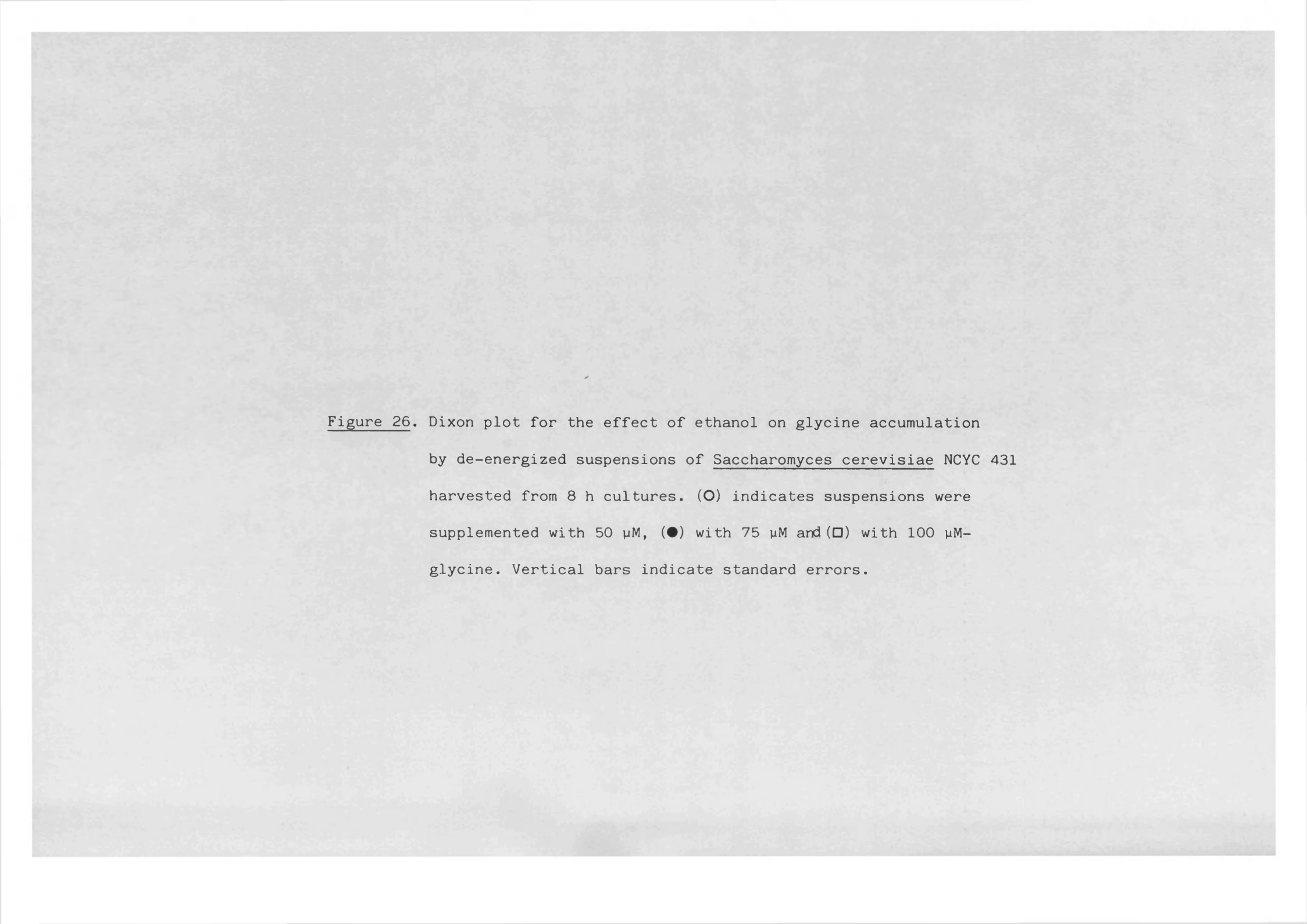
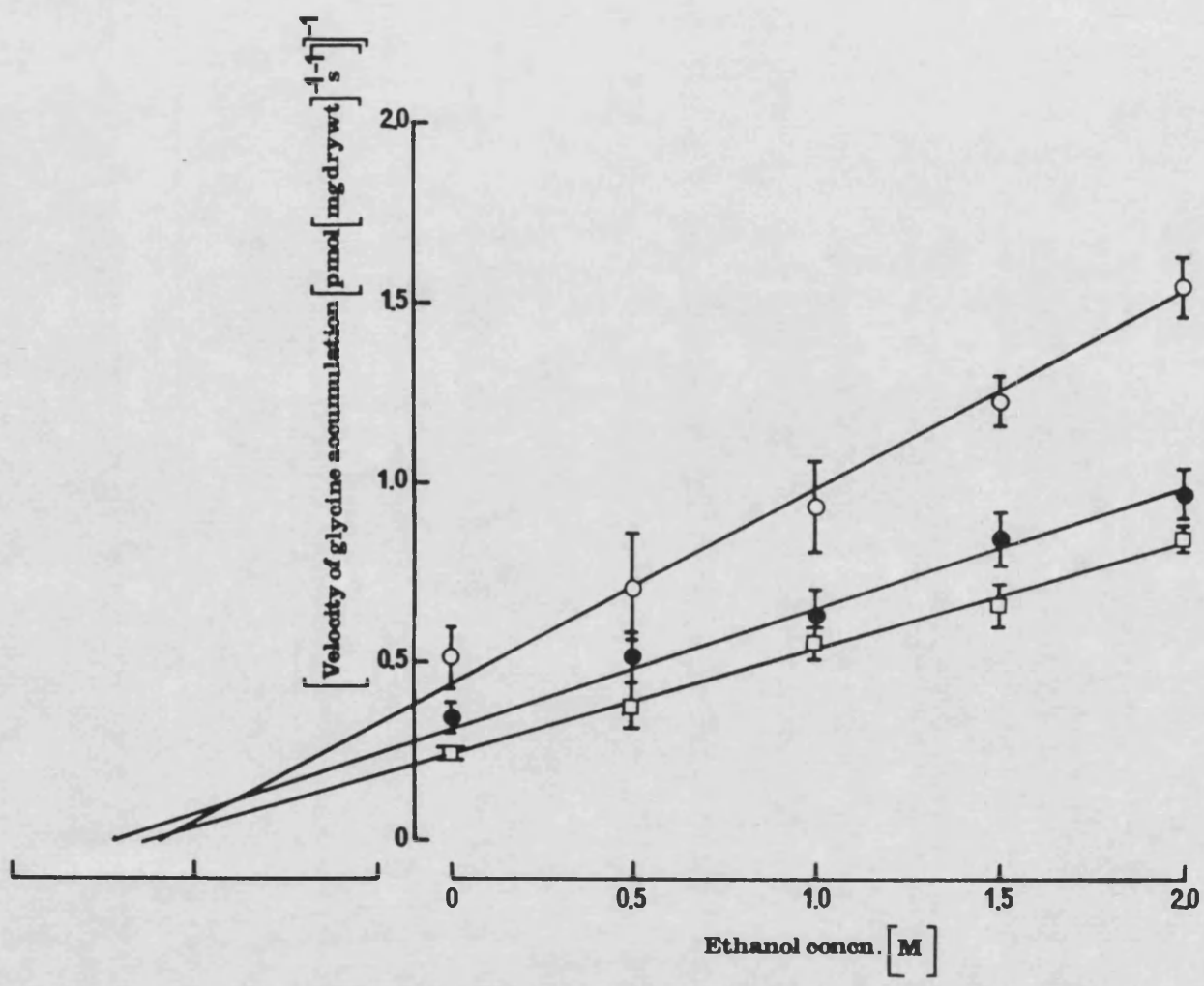


Figure 26. Dixon plot for the effect of ethanol on glycine accumulation by de-energized suspensions of Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures. (O) indicates suspensions were supplemented with 50 μM, (●) with 75 μM and (□) with 100 μM-glycine. Vertical bars indicate standard errors.


Velocity of glycine accumulation [pmol [mg dry wt]^-1 s^-1]^-1
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
Ethanol concn. [M]

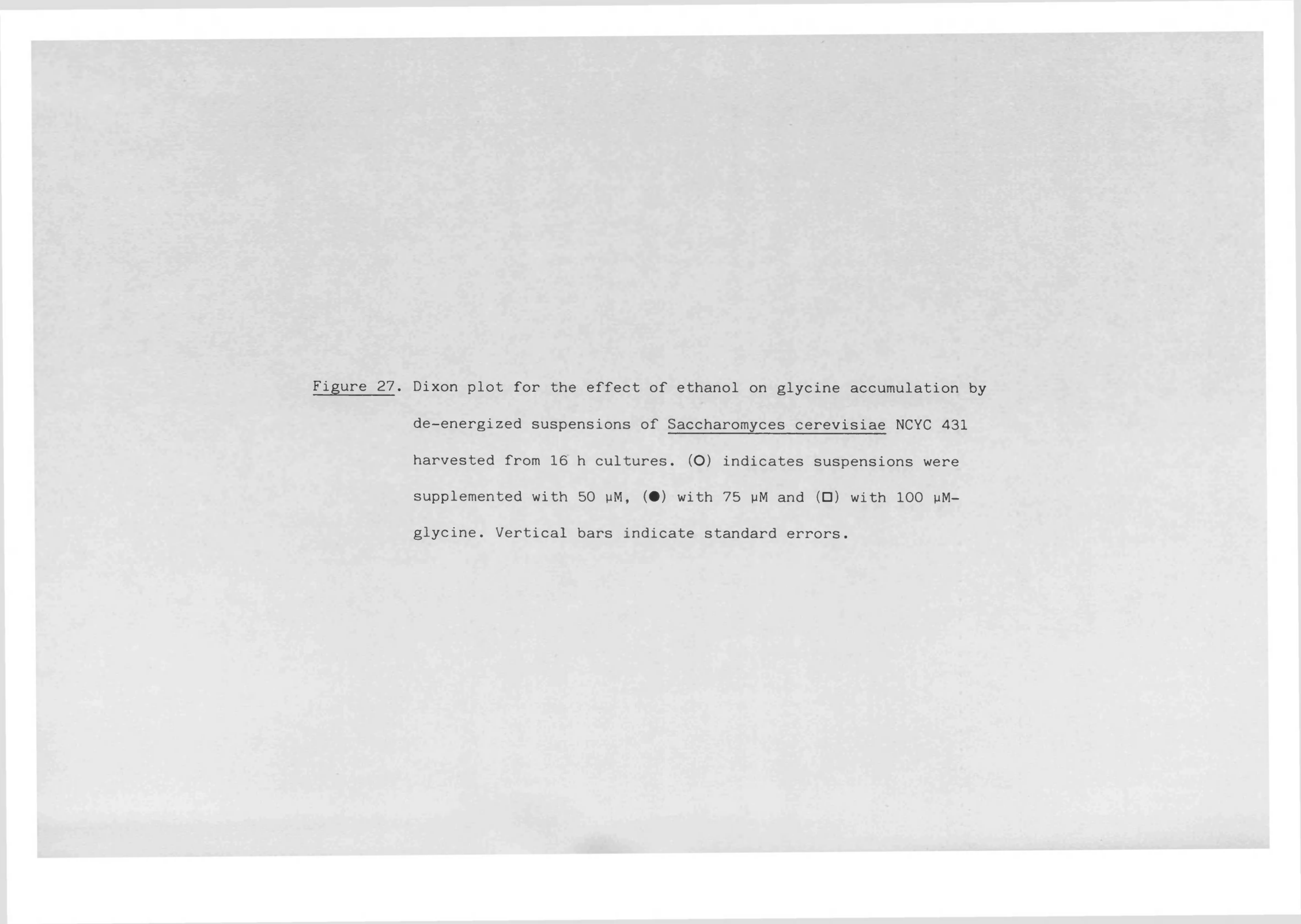


Figure 27. Dixon plot for the effect of ethanol on glycine accumulation by de-energized suspensions of Saccharomyces cerevisiae NCYC 431 harvested from 16 h cultures. (O) indicates suspensions were supplemented with 50 μM, (●) with 75 μM and (□) with 100 μM-glycine. Vertical bars indicate standard errors.

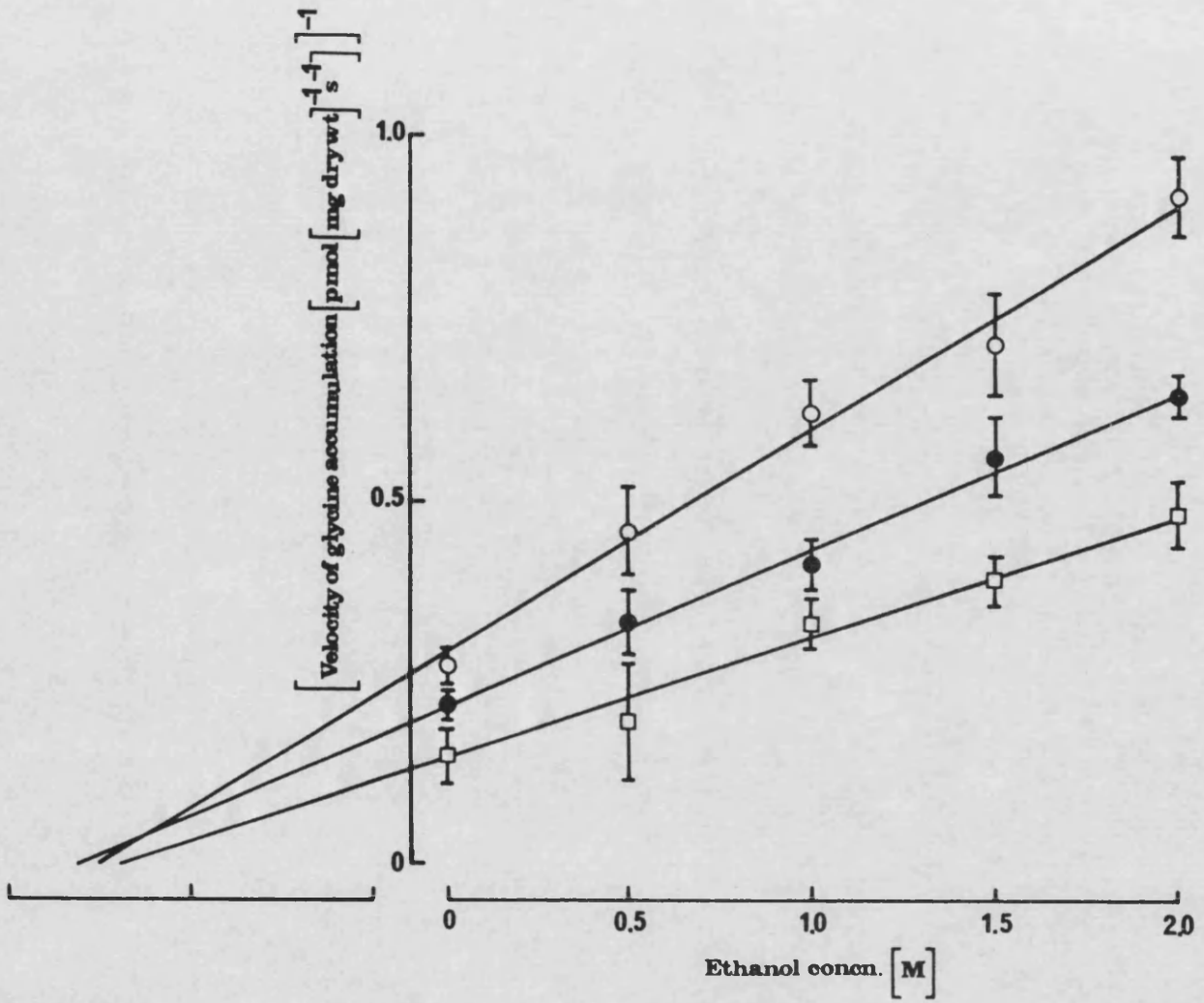

[Velocity of glycine accumulation [p mol [mg dry wt]$^{-1}$ s^{-1}]$^{-1}$]$^{-1}$
1.0
0.5
0
0
0.5
1.0
1.5
2.0
Ethanol concn. [M]

Figure 28. Dixon plot for the effect of ethanol on glycine accumulation by energized suspensions of Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures. (O) indicates suspensions were supplemented with 50 μM, (●) 75 μM and (□) 100 μM-glycine. Vertical bars indicate standard errors.

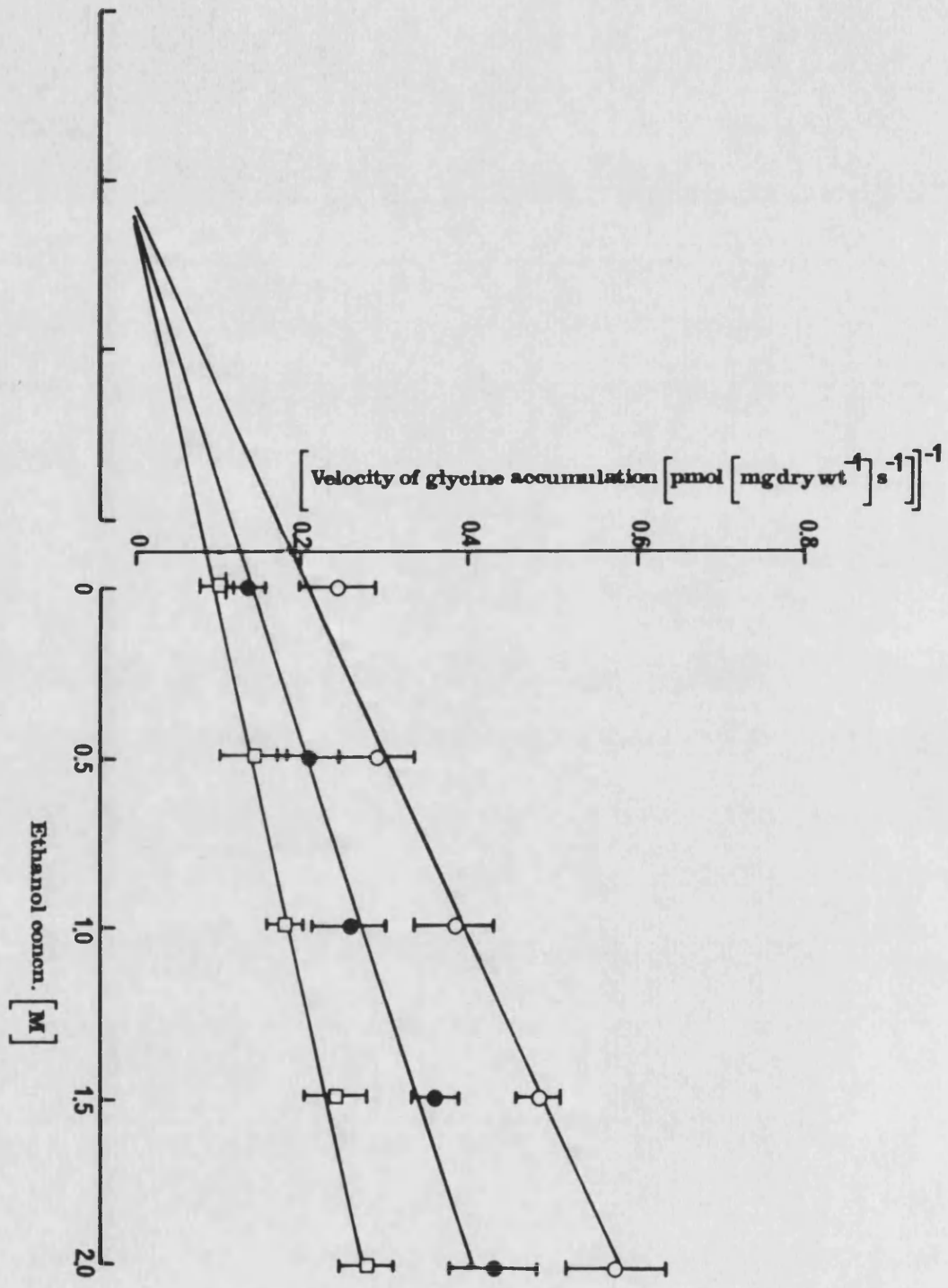
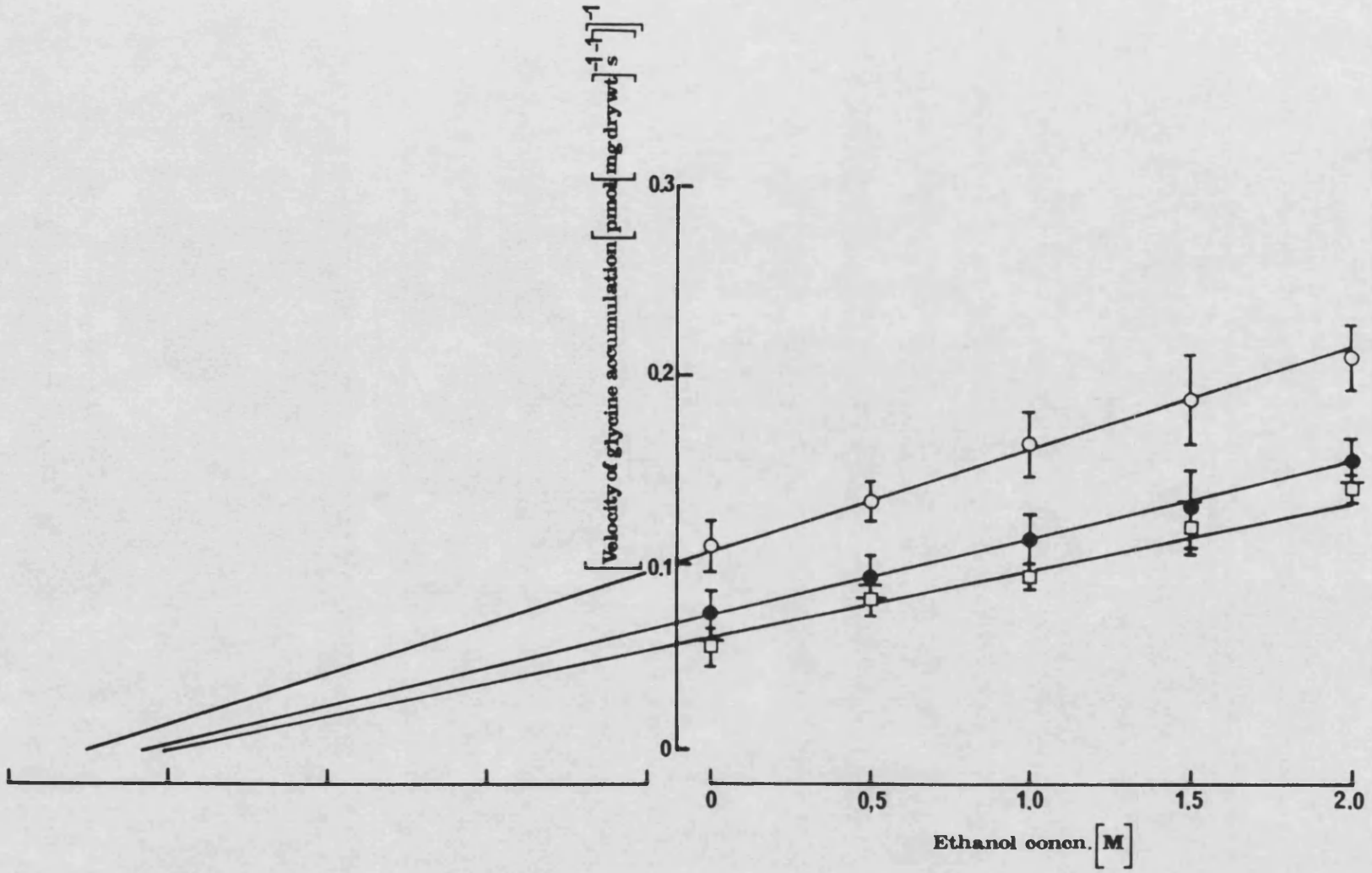

Velocity of glycine accumulation [pmol [mg dry wt^{-1}] s^{-1}]$^{-1}$
0
0.2
0.4
0.6
0.8
Ethanol conen. [M]
0
0.5
1.0
1.5
2.0

Figure 29. Dixon plot for the effect of ethanol on glycine accumulation by energized suspensions of Saccharomyces cerevisiae NCYC 431 harvested from 16 h cultures. (○) indicates suspensions were supplemented with 50 μM, (●) with 75 μM and (□) with 100 μM-glycine. Vertical bars indicate standard errors.

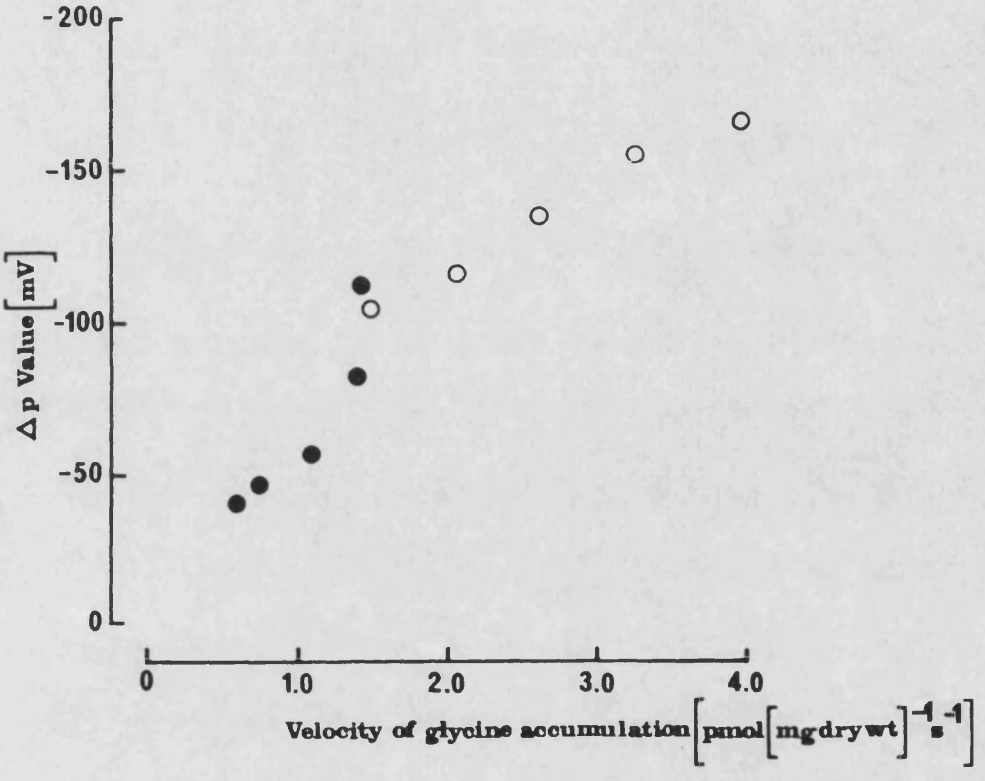

Velocity of glycine accumulation [pmol [mg dry wt]^-1 s^-1]^-1
0.3
0.2
0.1
0
0
0,5
1.0
1.5
2.0
Ethanol conen. [M]

State of energization of organisms	K_i [Ethanol] values (M) for organisms harvested from:	
	8 h cultures	16 h cultures
Energized	0.97 ± 0.10	1.55 ± 0.11
De-energized	0.71 ± 0.09	0.74 ± 0.08

Table 14. Inhibition constant (K_i) values for the effect of ethanol on glycine accumulation by Saccharomyces cerevisiae NCYC 431 by the high-affinity glycine-uptake system. Values are quoted ± standard error.

Figure 30. Relationship between the effect of ethanol on the velocity of glycine accumulation and on the Δp generated by Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures.(●) indicates the response of de-energized organisms and (O) of energized organisms. Data are for glycine accumulation by suspensions containing 50 μM-glycine.

Figure 31. Relationship between the effect of ethanol on the velocity of glycine accumulation and on the Δp generated by Saccharomyces cerevisiae NCYC 431 harvested from 16 h cultures. (●) indicates the response of de-energized organisms and (O) of energized organisms. Data are for glycine accumulation by suspensions containing 50 μM-glycine.


-200
-150
-100
-50
0
Δp Value [mV]
0
1.0
2.0
3.0
4.0
Velocity of glycine accumulation [pmol [mg dry wt]$^{-1}$ s^{-1}]

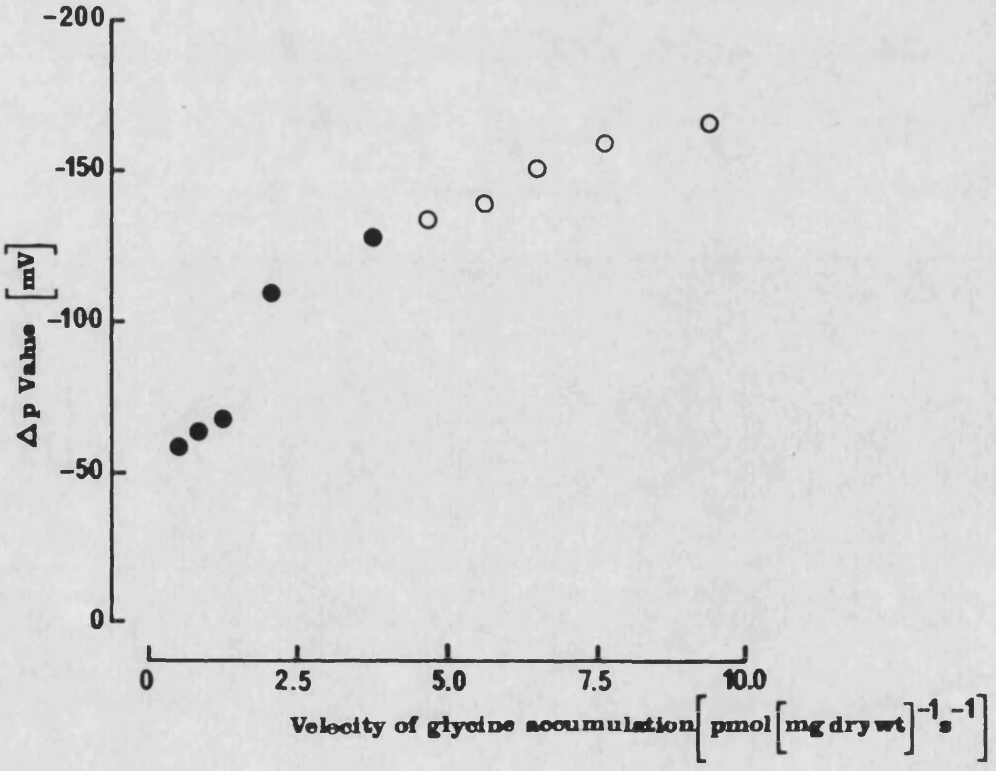
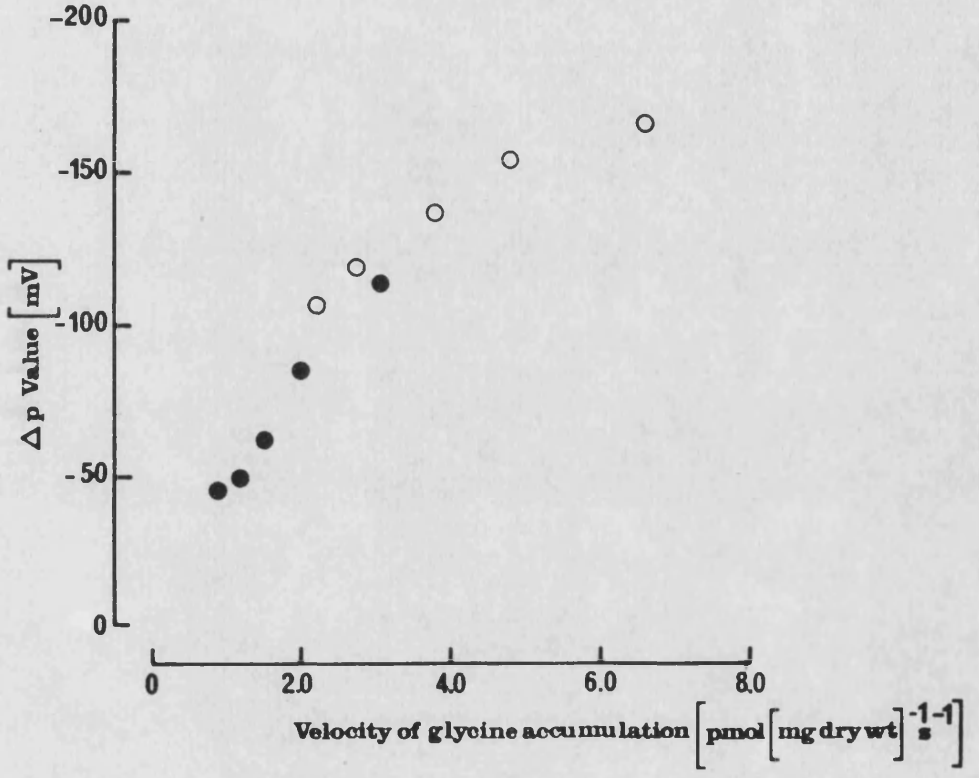

-200
-150
-100
-50
0
Δp Value [mV]
0
2.5
5.0
7.5
10.0
Velocity of glycine accumulation [pmol [mg dry wt]$^{-1}$ s^{-1}]

Figure 32. Relationship between the effect of ethanol on the velocity of glycine accumulation and on the Δp generated by Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures. (●) indicates the response of de-energized organisms and (O) of energized organisms. Data are for glycine accumulation by suspensions containing 75 µM-glycine.

Figure 33. Relationship between the effect of ethanol on the velocity of glycine accumulation and on the Δp generated by Saccharomyces cerevisiae NCYC 431 harvested from 16 h cultures. (●) indicates the response of de-energized organisms and (O) of energized organisms. Data are for glycine accumulation by suspensions containing 75 µM-glycine.


-200
-150
-100
-50
0
Δp Value [mV]
0
2.0
4.0
6.0
8.0
Velocity of glycine accumulation [pmol [mg dry wt]^-1 s^-1]

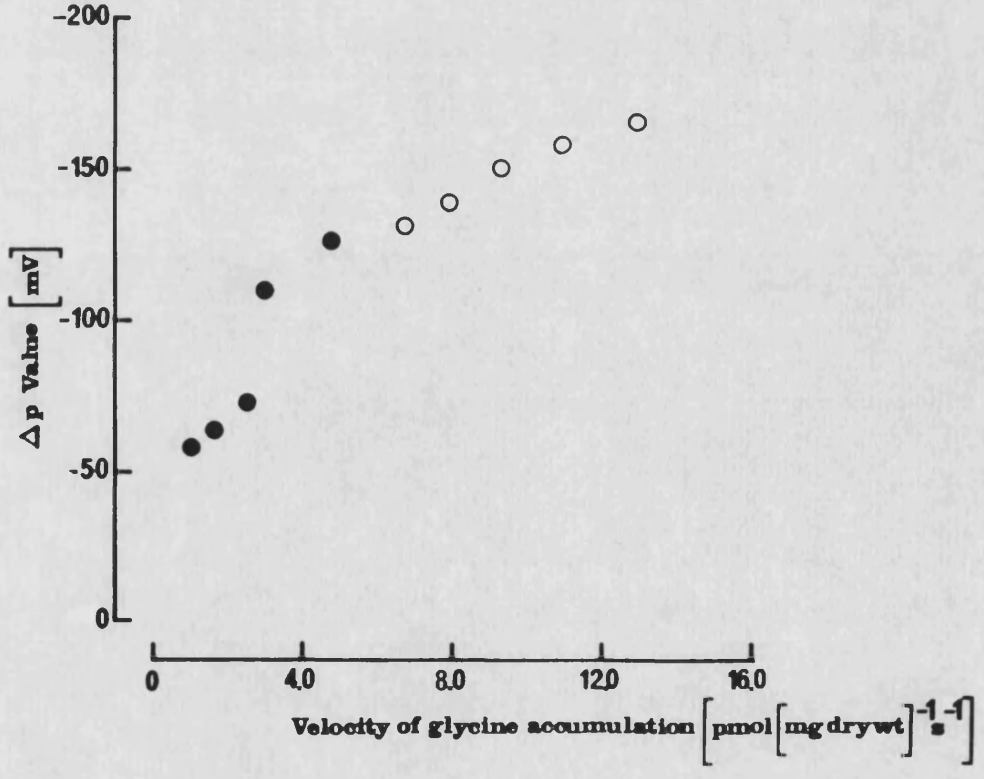
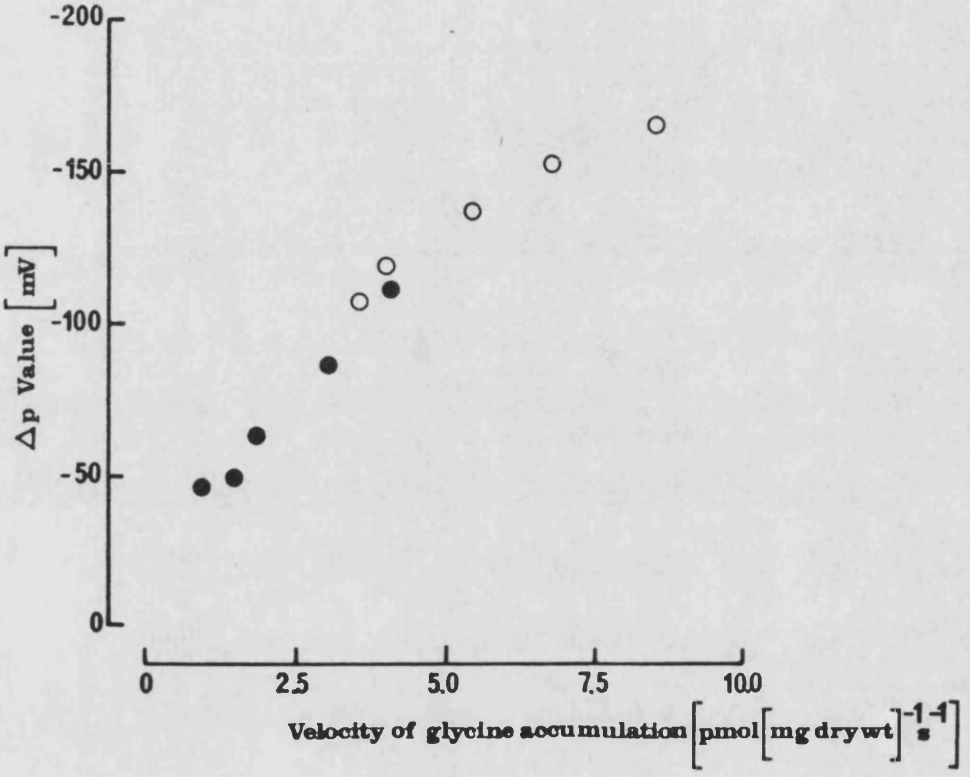
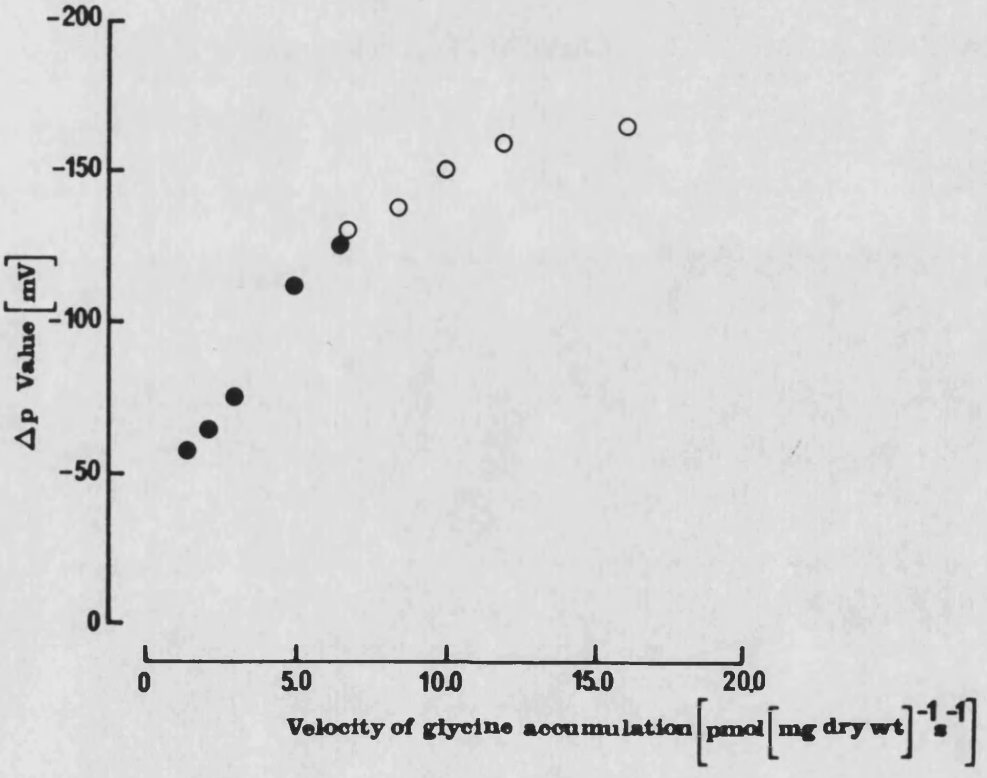

-200
-150
-100
-50
0
Δp Value [mV]
0
4.0
8.0
12.0
16.0
Velocity of glycine accumulation [pmol [mg dry wt]^-1 s^-1]

Figure 34. Relationship between the effect of ethanol on the velocity of glycine accumulation and on the Δp generated by Saccharomyces cerevisiae NCYC 431 harvested from 8 h cultures. (●) indicates the response of de-energized organisms and (O) of energized organisms. Data are for glycine accumulation by suspensions containing 100 μM-glycine.

Figure 35. Relationship between the effect of ethanol on the velocity of glycine accumulation and on the Δp generated by Saccharomyces cerevisiae NCYC 431 harvested from 16 h cultures. (●) indicates the response of de-energized organisms and (O) of energized organisms. Data are for glycine accumulation by suspensions containing 100 μM-glycine.


-200
-150
-100
-50
0
Δp Value [mV]
0
2.5
5.0
7.5
10.0
Velocity of glycine accumulation [pmol [mg dry wt]^-1 s^-1]


-200
-150
-100
-50
0
Δp Value [mV]
0
5.0
10.0
15.0
20.0
Velocity of glycine accumulation [pmol [mg dry wt]^-1 s^-1]

DISCUSSION

To examine the inhibitory effects of ethanol on micro-organisms, in this study Sacch. cerevisiae, research must obviously be undertaken which seeks to determine the response of metabolic functions to the presence of this alcohol. To be of greater value, however, such experiments should be conducted on populations of organisms which vary in their ability to resist the deleterious effects of ethanol. In this way, primary and secondary effects of this inhibitor can hopefully be distinguished. A previous study in this laboratory (Beavan, 1982) demonstrated that culture age influences the ability of Sacch. cerevisiae NCYC 431, when grown under self-induced anaerobic conditions, to ferment glucose in the presence of ethanol. Beavan reported that 1.0 M-ethanol completely inhibited fermentation of glucose by organisms from mid-exponential phase (8 h) cultures of Sacch. cerevisiae NCYC 431. The same concentration of ethanol, however, caused only a 12% decrease in the fermentative activity of organisms from early stationary phase (16 h) cultures. Data presented in this thesis show that populations of organisms from 16 h cultures of Sacch. cerevisiae NCYC 431 are also able to retain viability, in the presence of ethanol, to a greater extent than populations of organisms from 8 h cultures. These results suggest that, during the transition from the exponential to stationary phase of growth, almost certainly as a consequence of the accumulation of ethanol in fermenting cultures, alterations in cellular physiology occur which result in a general enhancement of the ability of organisms to resist the inhibitory effects of ethanol. It is reasonable to assume,

therefore, that metabolic functions which constitute primary targets for the inhibitory action of ethanol on growth, fermentation or viability will be less sensitive to this alkanol in organisms from 16 h cultures as compared with organisms from 8 h cultures.

EFFECT OF ETHANOL ON THE PROTON-MOTIVE FORCE ACROSS THE PLASMA MEMBRANE OF *SACCHAROMYCES CEREVISIAE* NCYC 431

An increase in the rate of passive influx of protons following addition of ethanol to de-energized organisms confirms the report of Leão and van Uden (1984b) that this alkanol dissipates proton gradients. Rates of passive influx of protons into de-energized organisms appear to be strongly dependent on the pH value of experimental suspensions. The rates recorded in this study, at a pH value of 4.0, are approximately four times greater than those measured by Leão and van Uden (1984b) at a pH value of 5.25. Furthermore, Ballarin-Denti *et al.* (1984) found that, at extracellular pH values near to neutrality, rates of passive influx of protons into *Sacch. cerevisiae* were even lower, some one order of magnitude less than the rates reported by Leao and van Uden (1984b) under comparable conditions. The considerable increase in the rate of proton influx when spheroplasts rather than intact organisms were examined demonstrates the large proton-buffering capacity of the yeast cell wall.This ability of the cell wall to buffer protons may have some importance in intra-cellular pH regulation. The possible physiological role of proton buffering by the yeast wall is further indicated by the finding that rates of proton influx were similar with spheroplasts from organisms from 8 h or 16 h cultures, suggesting that the different rates observed with intact organisms can be attributed to the greater capacity of the wall of organisms from 16 h cultures to buffer protons. This increase in proton-buffering capacity could simply be due to the presence of additional cell-wall material in organisms from 16 h cultures as compared with

organisms from 8 h cultures. Alternatively, it might represent an adaptive response of organisms designed to minimise the disadvantageous effects on intracellular pH value possibly produced as a consequence of the culture age-dependent decrease in the pH value of cultures of Sacch. cerevisiae NCYC 431 (Beavan, 1982).

The rapid extracellular acidification produced by energized Sacch. cerevisiae NCYC 431 results in a net efflux of protons, presumably facilitated by the activity of the Mg^{2+}-dependent, proton-translocating ATPase located in the plasma membrane (Willsky, 1979; Malpartida and Serrano, 1981b). The acidification curves obtained were similar in shape to those obtained with other strains of Sacch. cerevisiae in unbuffered suspensions (Sigler et al., 1981; Pascual et al., 1983; Leão and van Uden, 1984b). The noticeable lag between glucose addition and the onset of acidification was also observed by Sigler et al. (1981). These workers demonstrated that the length of this lag period depends on the extent of cell starvation, disappearing in organisms starved for more than 3 h. Both the increase in final extracellular pH value and the decrease in initial net proton efflux rate observed following addition of ethanol to suspensions were greater with organisms from 8 h as compared with 16 h cultures. Leão and van Uden (1984b) suggested that the increase in final extracellular pH value can be attributed to the ethanol-induced enhancement of the rate of passive influx of protons. It is possible, however, that other factors are involved in both the ethanol-mediated increase in the final extracellular pH value and the ability of the alcohol to decrease the initial

net proton efflux rate. One obvious possibility, which merited further investigation, is that ethanol either directly or indirectly inhibits the activity of the plasma-membrane ATPase. This effect, augmented by the ethanol-induced increase in passive influx of protons, could contribute to the observed decrease in the rate and extent of proton efflux. The potential complexity of ethanol effects on proton exchange across the plasma membrane of energized Sacch. cerevisiae is suggested by the non-linear relationship between increasing ethanol concentration and decreasing net proton efflux rate. This deviation from linearity might result from a summation of the effects of ethanol on active efflux and passive influx of protons, or may perhaps be an artefact produced as a consequence of the ability of the cell wall to buffer protons and hence distort the true extent of proton extrusion. It was unfortunate that, because of the instability of spheroplasts in glucose-containing buffer, the importance of the cell wall under energized conditions could not be assessed.

With de-energized organisms, ethanol caused a progressive decrease in both components which contribute to the magnitude of the Δp, namely, $\Delta\psi$ and ΔpH. This strongly suggests that the proton-uncoupling action of ethanol is alone sufficient to dissipate Δp, particularly since the enhancement of proton influx induced by ethanol appeared to be sufficient to both increase the extracellular pH value and decrease the intracellular pH value of suspensions. Similar effects on ΔpH and $\Delta\psi$ can be induced by addition of yeast killer toxin to suspensions

of toxin-sensitive Sacch. cerevisiae (de la Peña et al., 1981). de la Peña and his colleagues concluded that yeast killer toxin acts as a proton uncoupler and, furthermore, these workers indicated that even partial dissipation of ΔpH or $\Delta\psi$, produced by enhanced proton leakage across the plasma membrane, could have a profound effect on the biochemical and physiological state of the organism. However, when examining the effect of ethanol on Δp, no significant difference could be detected between de-energized organisms from 8 h and 16 h cultures. This finding supports the previously expounded idea that the extent of proton uncoupling in organisms from 8 h and 16 h cultures is very similar. It therefore seems that, if maintenance of a Δp is important for Sacch. cerevisiae NCYC 431 to tolerate ethanol, then the uncoupling effect of ethanol on the plasma-membrane proton gradient is not the determining parameter.

Assuming that the extent of proton uncoupling induced by ethanol is the same in energized and de-energized organisms (Leão and van Uden, 1984b), then the notion that factors other than simple uncoupling are involved in the effect of ethanol on Δp is supported by the data obtained with energized organisms. The inability of energized organisms to accumulate TPP^+ can be attributed directly to the extracellular acidification produced if suspensions of Sacch. cerevisiae are supplied with glucose. A pH-dependent, reciprocal relationship exists between ΔpH and $\Delta\psi$ in both Sacch. cerevisiae (Boxman et al., 1984) and Rh. glutinis (Hauer et al., 1981), in order to maintain Δp at a reasonably constant value. Consequently, at extracellular pH

values of less than 4.0, Sacch. cerevisiae does not establish a negative $\Delta\psi$. Höfer and Künemund (1984) showed that, at low extracellular pH values, a positive $\Delta\psi$ could be established in Rh. glutinis. Saccharomyces cerevisiae NCYC 431, however, did not accumulate SCN^- ions to an appreciably greater extent under conditions where no accumulation of TPP^+ occurred as compared with conditions under which negative $\Delta\psi$ values were determinable. It must be concluded, therefore, that the accumulation of SCN^- ions by Sacch. cerevisiae occurs in a manner not directly related to the polarity of the plasma membrane. Two main possibilities could account for my inability to measure a positive $\Delta\psi$ value in Sacch. cerevisiae NCYC 431. Firstly, the plasma membrane of this organism could be impermeable to SCN^- ions, the apparent accumulation of thiocyanate simply representing non-specific binding of ions to sites in the cell wall. Secondly, intracellular non-specific binding could be responsible for the observed SCN^- accumulation, in which case a positive $\Delta\psi$ cannot presumably be established by Sacch. cerevisiae under the conditions examined.

The state of energization of Sacch. cerevisiae NCYC 431 exerts a considerable influence over the intracellular pH value of organisms. The intracellular pH value of energized organisms was considerably higher than the corresponding value for de-energized organisms. A similar difference between internal pH values of energized and de-energized Sacch. cerevisiae, of the order of 0.5 - 0.7 pH units, has been reported previously (Conway and Downey, 1950; Riemersma and Alsbach, 1974). The major difference between energized and de-energized organisms, as far as the

energetics of the plasma membrane is concerned, is that under de-energized conditions the proton-translocating plasma-membrane ATPase is inactive. Thus the differences in internal pH values of energized and de-energized organisms implicates, if only indirectly, the plasma-membrane ATPase in intracellular pH regulation in Sacch. cerevisiae, a possibility already considered by Sanders et al. (1981). In addition, since the dissipative effect of ethanol on Δp differed in energized organisms from 8 h and 16 h cultures, it seems even more likely that ethanol has an inhibitory effect on the plasma-membrane ATPase of Sacch. cerevisiae. This effect could perhaps be more pronounced on the ATPase of organisms from 8 h as compared with 16 h cultures. Consequently, an inhibitory effect of ethanol on the plasma-membrane ATPase might be a crucial factor in the ability of this alkanol to affect the functioning of the plasma membrane of Sacch. cerevisiae.

Indirect evidence also suggests a possible physiological significance of the dissipative effect of ethanol on the Δp of energized organisms. The crucial role of Δp in the energization of solute uptake by Sacch. cerevisiae has become well established (Eddy, 1978; 1982). Addition of 1.5 M-ethanol to cultures of Sacch. cerevisiae NCYC 431, after 8 h incubation under self-induced anaerobic conditions, causes an immediate cessation of growth (Beavan et al., 1982). Since this concentration of ethanol is sufficient to cause an appreciable decrease in the Δp of energized organisms, it is possible that dissipation of the Δp could, via the obviously deleterious effects this would have on

the ability of organisms to accumulate metabolically important solutes, be responsible for the inhibitory effect of ethanol on growth.

In an attempt to elucidate further the mechanisms of ethanol-mediated dissipation of Δp and to obtain more direct evidence for the physiological significance of Δp dissipation, both the effect of ethanol on the plasma-membrane ATPase of Sacch. cerevisiae NCYC 431 and the effect of this alkanol on glycine accumulation by the GAP of this organism were examined.

THE PLASMA-MEMBRANE ATPASE OF SACCHAROMYCES CEREVISIAE

Isolation of Plasma Membranes from Saccharomyces cerevisiae NCYC 431

The attachment of cationically-charged silica microbeads to spheroplasts proved to be the more successful and efficient method of isolating plasma membranes from Sacch. cerevisiae NCYC 431 harvested from 16 h cultures. Plasma-membrane preparations obtained from sucrose-density gradients were comparatively impure, containing considerable numbers of unlysed spheroplasts. This contamination of plasma-membrane fractions, in conjunction with difficulties in assessing the effectiveness of lysophosphatidyl-choline in solubilising the enzyme prior to assay, probably accounts for the low ATPase activity associated with plasma-membrane preparations obtained by sucrose density-gradient centrifugation. The inadequacy of this method, under the conditions examined, is illustrated by the fact that the ATPase activity of plasma membranes purified by sucrose density-gradient centrifugation was only slightly higher than the activity of this enzyme in unpurified spheroplast lysates.

The use of cationically-charged silica microbeads resulted in several important improvements in the efficiency of plasma-membrane isolation. Firstly, the overall time of plasma-membrane purification was halved from the 6 h necessary to obtain a plasma-membrane preparation by sucrose density-gradient centri-fugation. Secondly, the protein content of plasma-membrane preparations was significantly lower than that of solubilised sucrose-density gradient fractions and, almost certainly as a direct consequence of this, the orthovanadate-sensitive ATPase

activity of plasma-membrane preparations was increased considerably. Nonetheless, levels of ATPase activity in the initial plasma-membrane preparations obtained using silica microbeads were still markedly lower than the 1.6 - 1.8 μmol Pi (mg protein)$^{-1}$min^{-1} reported by Schmidt et al. (1983) for plasma membranes isolated by the same method. The main problem appeared to be that, although Sacch. cerevisiae NCYC 431 harvested from 16 h cultures can be converted into spheroplasts, under normal conditions the cell walls of only about 90% of organisms are susceptible to treatment with Zymolyase. The majority of previous investigations on the plasma-membrane ATPase, including the study of Schmidt et al. (1983), have used mid-exponential phase Sacch. cerevisiae as the source of plasma-membrane material. Consequently, these workers experienced no difficulty in converting intact organisms into spheroplasts prior to the isolation of plasma membranes.

The protein content of yeast cell walls increases as cultures enter stationary phase (Nurminen, 1976). This additional protein prevents Zymolyase from gaining access to wall polysaccharide and,consequently, the effectiveness of this enzyme in converting intact cells into spheroplasts is decreased. Kitamura and Yamamoto (1981) found that incorporation of sodium sulphite into the incubation medium considerably improved the susceptibility of stationary-phase organisms to Zymolyase treatment, presumably by breaking disulphide bonds in wall proteins and thereby increasing the extent of Zymolyase penetration into the interior of cell walls. By incorporating sodium

metabisulphite into spheroplasting buffer, I was able to obtain greater than 99% conversion of organisms, from 16 h cultures of Sacch. cerevisiae NCYC 431, into spheroplasts. Plasma-membrane preparations subsequently obtained from sulphite-treated organisms possessed considerably more ATPase activity than membranes isolated from untreated organisms. The absence of contaminating material, when plasma-membrane preparations were viewed in the electron microscope, also indicates the effectiveness of this method for purifying plasma membranes. The clearly visible attachment of silica microbeads to only the external face of plasma membranes attests to the controlled nature of spheroplast lysis. Premature lysis of spheroplasts, before adhesion of cationally-charged microbeads, would result in both the interior and exterior faces of membranes becoming coated with silica. More importantly, under such circumstances, membranes of intracellular organelles might also become coated with silica and, consequently, could contaminate plasma-membrane preparations.

Analysis of the lipid composition of plasma membranes also indicated the purity of preparations obtained using silica microbeads. Thin-layer chromatographic analysis of total lipid extracts from membranes isolated using cationically-charged microbeads showed that, of the lipid classes present, phospholipids and free sterols predominate. Plasma-membrane preparations appeared to contain only comparatively minor amounts of triglycerides and sterol esters, neutral lipids mainly associated with intracellular organelles and membranes (Kramer et al., 1978).

Characteristics of, and Effect of Ethanol on, the Plasma-Membrane ATPase of Saccharomyces cerevisiae NCYC 431

The ATPase activity present in plasma membranes isolated using silica microbeads showed many of the characteristic features of the plasma-membrane ATPase of Sacch. cerevisiae. Firstly, ATPase activity was inhibited by specific inhibitors of the plasma-membrane ATPase of Sacch. cerevisiae but not by inhibitors of the mitochondrial ATPase of this organism (Serrano, 1978, 1980). Secondly, ATPase activity was optimal at pH values of 6.0 - 6.5, a distinguishing property of the plasma membrane-bound enzyme (Willsky, 1979; McDonough et al., 1980). Interestingly, the optimum pH value for plasma-membrane ATPase activity decreased as the age of culture from which organisms were harvested increased. This is possibly due to culture age-dependent variations in the in vivo environment of the enzyme. Since the extracellular pH value of self-induced anaerobic cultures is known to decrease appreciably during the transition from the exponential to the stationary phase of growth (Beavan , 1982), it is conceivable that similar, if smaller, shifts in intracellular pH value also occur. This possibility is enhanced by the fact that the external pH value of suspensions of Sacch. cerevisiae is known to influence the internal pH value of organisms (de la Peña et al., 1982). An alteration in the intracellular pH value of organisms might lead to a modification in the conformational properties of the plasma-membrane ATPase to ensure retention of activity.

The kinetic characteristics of the plasma-membrane ATPase of Sacch. cerevisiae NCYC 431 are similar to those previously reported for the plasma-membrane ATPase of this organism. My measured K_m values of 2.5 - 2.6 mM are in broad agreement with the 0.7 mM value reported by Willsky (1979) and the K_m value of 1.7 mM published by Borst-Pauwels and Peters (1981). Values for V_{max} of 1.3 - 1.9 μmol Pi (mg protein)$^{-1}$min^{-1} are also similar to previously reported values for activity of the plasma membrane ATPase of Sacch. cerevisiae (Ahlers et al., 1978; Serrano, 1978; Schmidt et al., 1983). That the V_{max} value for the ATPase in plasma membranes from organisms from 16 h cultures is lower than the corresponding value for plasma membranes from organisms from 8 h cultures confirms the report of Tuduri et al. (1985) of a decline in the activity of this enzyme during the late-exponential phase of growth. Tuduri and his coworkers found a 50 - 60% decrease in the plasma-membrane ATPase activity of Sacch. cerevisiae during the last cell division of exponential growth.

Ethanol non-competitively inhibits plasma-membrane ATPase activity. This effect was more pronounced on the plasma-membrane ATPase of organisms cultured for 8 h rather than 16 h. When considered in conjunction with the finding that ethanol inhibits ATPase activity, as judged by K_i values, in the same range of concentrations at which it dissipates Δp, it is tempting to suggest that the differential effect of ethanol on the plasma-membrane ATPase is responsible for culture age-dependent differences in the extent of ethanol-induced dissipation of Δp.

It should be noted, however, that to demonstrate such a relationship more conclusively presents considerable practical difficulties.

At the present time comparatively little is known about regulation of the ATP-hydrolytic and proton-pumping functions of the plasma-membrane ATPase, although the stoichiometry of proton ejection to ATP hydrolysis has been elucidated in model proteoliposomes (1:1, Malpartida and Serrano, 1981b). It is not known, however, how these two functions are linked and controlled *in vivo* where there are likely to be numerous regulatory influences on ATPase activity. The extent of ATPase regulation in *Sacch. cerevisiae* is perhaps suggested by my finding that, although the V_{max} value of the plasma-membrane ATPase of organisms from 16 h cultures is significantly lower than the corresponding value for organisms from 8 h cultures, the magnitude of Δp, in the absence of ethanol, is more or less identical in organisms from both culture ages. As further evidence of the complexity of the relationship between Δp generation and ATPase activity, de la Peña *et al.* (1981) showed that loss of the ability to pump protons across the plasma membrane cannot always be related to an inhibition of the ATP-hydrolytic activity of the plasma-membrane ATPase. Both de la Peña *et al.* (1981) and Eilam *et al.* (1984) showed that DCCD inhibits both net proton efflux by energized *Sacch. cerevisiae* and the ATPase activity of plasma membranes purified from this organism. de la Peña and his colleagues (1981), however, reported that a related carbodiimide, although capable of producing a similar effect to DCCD on the ability of organisms to pump protons, was comparatively ineffective at preventing ATP hydrolysis by purified plasma

membranes. These data illustrate the difficulty of interpreting the results obtained from my studies on the effect of ethanol on plasma-membrane energetics. All inhibitors of the plasma-membrane ATPase so far examined, in addition to inhibiting ATPase activity, cause deleterious, non-specific effects on the permeability properties of the plasma membrane perhaps of a similar nature to those produced by ethanol. Hence, the DCCD-induced decrease in the rate of proton ejection observed by de la Peña et al. (1981) is probably a composite effect of, firstly, an inhibition of the plasma-membrane ATPase and, secondly, an increase in the proton permeability of the plasma membrane. The other carbodiimide studied, 1-ethyl-3-(3-diethylaminopropyl) carbodiimide, is presumably relatively ineffective as an inhibitor of the plasma-membrane ATPase but can still uncouple proton gradients and hence decrease net proton efflux rates. it is therefore important to determine more precisely the relative contributions of the proton-uncoupling and ATPase-inhibiting effects of ethanol to the overall effect of this alkanol on Δp. The only rational way to investigate this problem is to study how inhibition of ATPase activity affects Δp generation.

One possible way to investigate this problem would be to use a specific inhibitor of the plasma-membrane ATPase which effectively, in other words at low concentrations, inhibits this enzyme in intact organisms. This would overcome the problems associated with the inhibitors examined so far since, at micromolar concentrations, undesirable non-specific effects of the inhibitor should be negligible. The use of such an inhibitor could enable the effect of ATPase inhibition on proton translocation across the plasma

membrane to be examined directly; however, no such inhibitor has yet been found for the fungal plasma-membrane ATPase. In the absence of this ideal solution, one available possibility is to purify the plasma-membrane ATPase and then reconstitute it in liposomes. Such a manipulation has already been carried out with Sacch. cerevisiae by Malpartida and Serrano (1981a). In this environment, albeit somewhat artificial, it might be feasible to examine the effect of plasma-membrane ATPase inhibitors on the ability of the purified enzyme to establish a $\Delta\psi$ across the liposomal membrane. Hopefully, under these conditions, only low concentrations of ATPase inhibitors would be required to cause an appreciable loss of ATPase activity, hence overcoming the problem of non-specific membrane permeability effects. Unfortunately, addition of ethanol to such a system would reveal little since, even at low concentrations, this alkanol can presumably act as a proton uncoupler as well as an inhibitor of ATPase activity.

Can Alterations in Plasma-Membrane Phospholipid Composition Explain Culture Age-Dependent Variations in the Properties of the Plasma-Membrane ATPase of Saccharomyces cerevisiae NCYC 431?

Lipid compositions of plasma-membrane preparations obtained using silica microbeads were similar to those reported for plasma membranes of Sacch. cerevisiae isolated using alternative techniques. The proportions of phospholipid classes in plasma membranes were very similar to those discovered by Longley et al. (1968) and Suomalainen and Nurminen (1970). The absence of free fatty acids from lipid extracts suggests that incorporation of p-chloromercuribenzoate successfully prevented degradation of

phospholipids during extraction of lipids from plasma membranes. The changes that occurred in the overall fatty-acyl composition of plasma-membrane phospholipids as cultures progressed from the exponential to the stationary phase of growth were similar to those reported by Beavan et al. (1982) for whole-cell phospholipids, suggesting that culture age has a similar effect on the degree of unsaturation of phospholipids in most types of yeast membrane. In addition, the present study revealed that changes in the relative proportions of phospholipids and in the fatty-acyl composition of individual phospholipid classes accompany the transition from the exponential to the stationary phase of growth. The relative proportions of phosphatidylethanolamine and phosphatidylserine, but not of phosphatidylcholine or phosphatidylinositol, in plasma membranes varied with culture age. Interestingly, only phosphatidylinositol retained the same degree of fatty-acyl unsaturation in plasma membranes from organisms from 8 h and 16 h cultures. This might suggest that this phospholipid supports some plasma-membrane function which, associated with a critical degree of fatty-acyl unsaturation, is of considerable physiological importance to the yeast. When the changes in fatty-acyl composition of individual phospholipid classes are taken into consideration, the overall degree of fatty-acyl unsaturation of plasma-membrane phospholipid, as indicated by the Δmol^{-1} value, decreases from 0.67 in organisms from 8 h cultures to 0.53 in organisms from 16 h cultures.

Since the proton-translocating ATPase of Sacch. cerevisiae is a plasma membrane-bound enzyme, it is likely that changes in plasma-membrane lipid composition will affect some, if not all, the

properties of this enzyme. By adding lysophosphatidylcholine to the purified plasma-membrane ATPase of Schizosacch. pombe, Dufour and Goffeau (1980) effected an increase in the activity of the enzyme from 1 μmol Pi (mg protein)$^{-1}$min^{-1} to 26 umol Pi (mg protein)$^{-1}$ min^{-1}. Consequently, the lipid environment of the plasma-membrane ATPase appears to have a profound influence on its activity. It is conceivable, therefore, that the alterations in phospholipid and fatty-acyl composition which accompany the transition from the exponential to the stationary growth phase of self-induced anaerobic cultures of Sacch. cerevisiae NCYC 431 are responsible, at least in part, for the alterations observed in the properties of the plasma-membrane ATPase.

This possibility is strengthened by the fact that variations in the lipid composition of plasma membranes of Sacch. cerevisiae have already been shown to influence the kinetic parameters of the proton-translocating ATPase (Ahlers et al., 1980; Stadtlander et al., 1982). Stadtlander and his colleagues demonstrated that changes in phospholipid head-group orientation, as well as in fatty-acyl chain length and degree of unsaturation, modify the kinetic characteristics of the yeast plasma-membrane ATPase. Although both the K_m and V_{max} value of the plasma-membrane ATPase were affected, the influence of Mg^{2+} on the activity of the enzyme was most strongly influenced by alterations in plasma-membrane lipid composition. By culturing organisms at temperatures greater than 30°C, an increase in the affinity of Mg^{2+} for its 'activator' site on the plasma-membrane ATPase was induced by Stadtlander and his coworkers. This resulted in a broadening of the range of Mg^{2+}

concentrations at which ATPase activity was maximal, an effect, according to Stadtlander et al. (1982), which was due to the decreased level of unsaturated fatty-acyl residues in phospholipids of plasma membranes from organisms grown at higher temperatures. This evidence, however, seems to contradict my findings. Plasma membranes from organisms from 16 h cultures contain a lower proportion of unsaturated fatty-acyl residues than plasma membranes from organisms from 8 h cultures. Consequently, the plasma-membrane ATPase of organisms cultured for 16 h should be stimulated to a greater extent by Mg^{2+} ions. On the contrary, however, ATPase activity was greater in plasma membranes from organisms from 8 h cultures, suggesting that something more physiologically fundamental than an alteration in affinity for Mg^{2+} is responsible for the culture age-dependent differences in plasma-membrane ATPase activity. One alternative possibility is that, rather than the fatty-acyl composition of phospholipids, phospholipid head-group configuration might affect ATPase activity. Indeed, Stadtlander et al. (1982) found that increasing the proportion of phosphatidylethanolamine in plasma membranes increased the affinity of the plasma-membrane ATPase for its substrate. This might account for the marginally lower K_m value of the plasma-membrane ATPase of organisms from 8 h rather than 16 h cultures. The simplest explanation for the culture age-dependent differences in V_{max} value of the plasma-membrane ATPase is that organisms from 8 h cultures contain more functional ATPase molecules in their plasma membranes than organisms from 16 h cultures. Although the protein content of plasma membranes from organisms from both culture ages was similar, this does not necessarily indicate that the relative proportions of the numerous membrane proteins

do not vary with culture age. A reduction in the number of ATPase molecules present in the plasma membrane would decrease the overall ATPase activity of membranes without affecting the affinity of the enzyme for its substrate.

Since plasma-membrane lipids are known to be targets for the inhibitory action of ethanol on Sacch. cerevisiae (Ingram and Buttke, 1984), it is possible that changes in the lipid composition of plasma membranes could be responsible for the differences seen in the response of the plasma-membrane ATPase, of organisms from 8 h or 16 h cultures, to ethanol. Moreover, it is quite likely that perturbations in lipid structure are directly responsible for the inhibitory effect of ethanol on the plasma-membrane ATPase of Sacch. cerevisiae. Grisham and Barnett (1973) found that alkanols non-competitively inhibited the $[Na^+-K^+]$-ATPase of sheep kidney medulla and, furthermore, the effectiveness of this inhibition was directly related to the hydrophobicity of the alkanol. These workers concluded that inhibition of $[Na^+-K^+]$-ATPase activity by ethanol occurs as a consequence of the ability of this alcohol to alter the lipid structure of the plasma membrane, not by a direct interaction between the alkanol and a hydrophobic protein component of the enzyme. From a consideration of my data, this explanation seems equally likely to apply to the effect of ethanol on the plasma-membrane ATPase of Sacch. cerevisiae. The K_i [ethanol] values for the plasma-membrane ATPase obtained in this study, around 2.0 M, are considerably lower than those reported by other workers for the effect of this alkanol on enzymes found in the cytoplasm of Sacch. cerevisiae. The K_i[ethanol] value for hexokinase, the

most ethanol sensitive of the glycolytic enzymes, is 4.1 M (Millar et al., 1982). Assuming that ethanol inhibits hexokinase activity via a direct effect on the enzyme molecule, it appears that this alkanol is relatively ineffective as a protein-denaturing agent. Consequently, it is reasonable to suggest that ethanol, rather than denaturing the ATPase protein, inhibits ATPase activity by altering the conformation of the lipid environment of the enzyme.

It is possible, therefore, that the alterations in lipid composition, particularly the decrease in phospholipid fatty-acyl unsaturation, which occur as culture age increases could confer a protective effect on the plasma-membrane ATPase. A similar situation has already been shown to occur in higher animals. Waring et al. (1981) found that ethanol-fed rats had increased proportions of saturated fatty-acyl residues in their mitochondrial-membrane phospholipids. Interestingly, this increase in fatty-acyl saturation was most pronounced in the cardiolipin component of membrane phospholipid. Cardiolipin is closely associated with the electron-transport chain and $F_1 - F_0$ ATPase of the inner mitochondrial membrane. In a previous study, Rottenberg et al. (1980) had reported that levels of $F_1 - F_0$ ATPase activity in mitochondria from ethanol-treated rats were lower than in untreated animals but, significantly, the $F_1 - F_0$ ATPase of ethanol-fed animals was less sensitive to inhibition by ethanol. Is it possible, therefore, that the decrease in the degree of fatty-acyl unsaturation of plasma-membrane phospholipids of Sacch. cerevisiae NCYC 431 from 16 h as compared with 8 h cultures is responsible for both the culture age-dependent decrease in plasma-membrane ATPase activity and the corresponding

increase in the ability of this enzyme to resist inhibition by ethanol? An overall decrease in phospholipid fatty-acyl unsaturation might result in a less fluid plasma membrane and this could limit the mobility of intrinsic proteins, such as the plasma-membrane ATPase, perhaps adversely affecting their activity (McMurchie and Raison, 1979). Conversely, however, a decrease in membrane fluidity could restrict the partioning of ethanol into the plasma membrane hence decreasing the extent of alkanol-mediated membrane disruption (Michaelis and Michaelis, 1983).

The gross lipid analyses carried out in this thesis cannot reveal the precise nature of the lipid environment of the plasma-membrane ATPase. Highly localised alterations in phospholipid, or perhaps even sterol, composition could be important in determining the activity and ethanol sensitivity of this enzyme. Intrinsic proteins are known to act as focal points for lipid domains of greater or lesser fluidity than the bulk lipid of the membrane (Rank et al., 1978; Sandermann, 1978) and it is quite conceivable that the immediate lipid environment of the plasma-membrane ATPase differs from the overall lipid composition of the plasma membrane. Only detailed bio-physical studies on the plasma membrane of Sacch. cerevisiae, utilising spin-labelled fatty acid and phospholipid probes, could reveal the existence and location of lipid domains of varying fluidity. However, the influence of phospholipid head-group orientation or phospholipid fatty-acyl unsaturation and chain length on the response of the plasma-membrane ATPase to ethanol could be examined by varying the lipid environment of the purified enzyme and then measuring rates of ATP hydrolysis in the presence and absence of ethanol.

Attempts to relate culture age-associated variations in plasma-membrane ATPase activity to differences in the ability of organisms to establish and maintain a Δp across the plasma membrane can, at this stage, only be speculative. Strong circumstantial evidence does,nonetheless, indicate that an inhibitory effect of ethanol on the plasma-membrane ATPase of Sacch. cerevisiae is, if only in part, responsible for the ability of this alkanol to de-energize the plasma membrane of this organism.

TRANSPORT OF GLYCINE BY SACCHAROMYCES CEREVISIAE

In order to determine the extent to which ethanol-mediated inhibition of solute uptake into Sacch. cerevisiae is due to the dissipative effect of this alkanol on Δp, an examination of the characteristics of, and effect of ethanol on, glycine transport by this organism was undertaken. Glycine was chosen as the solute to be studied for three main reasons. Firstly, glycine is one of a number of amino acids for which, at the time, there was little evidence to suggest that Sacch. cerevisiae synthesises a specific transport system or systems. Consequently, the GAP constitutes the principal means of glycine uptake into this organism (Cooper, 1982). This absence of a glycine-specific carrier should have, at least in theory, precluded the need to differentiate between glycine uptake by two or more separate systems. Secondly, from a purely chemical point of view, glycine is the simplest of the amino acids. Since glycine does not possess an asymmetric carbon atom, it can exist in only one stereochemical form and, therefore, there is no need to take into account the presence of both D- and L-enantiomers in experimental suspensions. As the affinity of stereoisomers of amino acids for transport systems differs considerably, this could affect the measurement of kinetic parameters. Thirdly, and most importantly, Ballarin-Denti et al. (1984) reported that glycine accumulation by Sacch. cerevisiae has no appreciable effect on the intracellular pH value of organisms, even under de-energized conditions. This finding is of significance because, under de-energized conditions, the proton-translocating ATPase is inactive and, consequently, co-transport of amino acids and protons might be expected to lower intracellular pH values. In the absence of such a change in internal pH value, it is reasonable

to assume that the Δp values previously measured in Sacch. cerevisiae NCYC 431 will not be markedly altered during the course of glycine transport experiments.

Characteristics of Glycine Transport in Saccharomyces cerevisiae NCYC 431

Glycine uptake into Sacch. cerevisiae NCYC 431 is mediated by two distinct transport systems. The kinetic parameters of glycine accumulation by the high-affinity system reported in this thesis are similar to previously published values for high-affinity glycine uptake by Sacch. cerevisiae. Eddy et al. (1970b) reported a K_T value of 64 μM for glycine uptake by the high-affinity carrier of Sacch. cerevisiae (Sacch. carlsbergensis), while Ballarin-Denti et al. (1984) recorded a K_T value of 380 μM for glycine transport by the same system. Ballarin-Denti and his coworkers attributed the discrepancy between their estimate of K_T and that of Eddy et al. (1970b) to the fact that the simultaneous recording of ^{31}P-NMR spectra necessitated the employment of very high cell density suspensions, approximately 20 mg dry wt ml^{-1}, in transport experiments. Both Eddy et al. (1970b) and Ballarin-Denti et al. (1984) reported the presence, in Sacch. cerevisiae, of a second glycine-uptake system. These investigators determined K_T values for glycine transport by this system of 2.8 mM and 13.7 mM, respectively. Assuming the value quoted by Ballarin-Denti and his colleagues is again an overestimate, then these data are in agreement with my own on glycine uptake by the low-affinity system.

Importantly, neither of the two previous reports on the kinetics of glycine accumulation by Sacch. cerevisiae contained any information on which amino-acid transport systems are responsible for high- and low-affinity glycine uptake. Ballarin-Denti et al. (1984) assumed that high-affinity uptake of glycine occurs via the GAP, although no evidence was presented to substantiate this claim. Indeed, under the experimental conditions employed in the present study, and in the investigation of Ballarin-Denti and his coworkers, the GAP should have been fully repressed since organisms grown in the presence of NH_4^+ ions were used to examine glycine uptake and no obvious attempts were made to derepress the GAP prior to the commencement of experimentation. However, both Grenson et al. (1970) and Calderbank et al. (1985) found that nitrogen-repressed Sacch. cerevisiae could carry out high-affinity transport of L-alanine, another amino acid believed to depend predominantly on the GAP for its uptake, albeit at significantly lower rates than in organisms derepressed for the GAP. It seems, therefore, that some residual GAP activity is present in Sacch. cerevisiae even under fully repressed conditions. This could explain the high-affinity, low-velocity glycine uptake observed in Sacch. cerevisiae NCYC 431.

By obtaining a mutant of Sacch. cerevisiae NCYC 431 lacking a functional GAP, I was able to demonstrate conclusively that high-affinity glycine uptake in this organism occurs via the GAP. The selection of GAP-less mutants, by the method of Rytka (1975), depends for its success on the fact that, although specific amino-acid permeases exhibit a pronounced preference for L-stereoisomers of amino acids,

the GAP shows an equal affinity for both D- and L-enantiomers. Since D-amino acids are toxic to wild-type Sacch. cerevisiae, mutants lacking the GAP can be easily distinguished by their ability to grow on nitrogen-poor media containing D-amino acids. The mutant CC1, isolated by this method, grew normally under self-induced anaerobic conditions but only possessed the low-affinity glycine carrier. The K_T and V_{max} values for glycine uptake by this system were similar in both the GAP-less mutant and the wild-type organism. Consequently, it seems clear that high-affinity glycine uptake into Sacch. cerevisiae is mediated by the GAP and this system was subsequently studied with respect to the effect of ethanol on glycine transport.

The nature of the low-affinity glycine-uptake system remains unclear. Since the K_T value of this transport system was considerably higher, and the V_{max} value lower, than previously reported values for the uptake of principal substrates by specific amino-acid permeases (Gregory et al., 1982; Calderbank, 1984), it seems unlikely that this carrier functions primarily to transport glycine. Competition studies, using a number of different amino acids, might be beneficial in determining the specificity, and hence defining the physiological role, of this amino-acid transport system.

It is interesting to note that culture age appears to affect the kinetics of low-affinity glycine uptake by Sacch. cerevisiae, the K_T and V_{max} values for the low-affinity carrier being lower in organisms from 8 h cultures as compared with organisms from 16 h cultures. GAP-mediated glycine uptake was apparently unaffected by culture age, indicating that the GAP is not expressed to an appreciably

greater extent in organisms cultured for 16 h rather than 8 h. It is possible that the changes in plasma-membrane lipid composition already discussed in terms of their influence on plasma-membrane ATPase activity are also responsible for culture age-dependent changes in the activity of solute-transport systems. Enrichment of plasma membranes of Sacch. cerevisiae with phosphatidylcholine or phosphatidylethanolamine causes a significant decrease in the rate of amino-acid uptake by organisms (Trivedi et al., 1982, 1983). Alterations in the degree of fatty-acyl unsaturation of plasma-membrane phospholipids also appear to influence the kinetics of certain specific amino-acid permeases (Calderbank et al., 1984). It seems possible, therefore, that the changes in plasma-membrane lipid composition which occur as culture age increases may influence the kinetic characteristics of low-affinity glycine transport.

Effect of Ethanol on Glycine Accumulation by Saccharomyces cerevisiae NCYC 431

My data on the effect of ethanol on glycine accumulation agree with the report of Leão and van Uden (1984a) that inhibition of GAP activity by this alkanol is non-competitive. Leão and van Uden (1984a) successfully correlated the extent of alkanol-mediated inhibition of the GAP with the degree of alkanol hydrophobicity. Consequently, these workers suggested that inhibition of GAP activity by ethanol could occur by one or more related effects, each involving a hydrophobic target site. The potential mechanisms invoked by Leão and van Uden (1984a) to explain the inhibitory action of alkanols on glycine transport were, principally, depolarization of the plasma membrane and damaging configurational changes

of the GAP produced either through direct interaction of alkanols with the transport proteins or via alkanol-induced changes in their lipid environment or by a combination of both of these effects.

In this study I have been able to resolve, at least to some extent, this apparent ambiguity about how ethanol affects the functioning of the GAP of Sacch. cerevisiae. My data strongly suggest that, at least at concentrations of 2.0 M or below, the inhibitory effect of ethanol on glycine uptake by the GAP occurs as a direct consequence of the dissipative effect of this alkanol on the Δp across the plasma membrane. This conclusion is supported in part by the fact that the K_i[ethanol]values, under both energized and de-energized conditions, show that the magnitude of ethanol-mediated inhibition of glycine uptake is strongly dependent on the extent of Δp dissipation produced by ethanol. Under de-energized conditions, no difference was observed in the extent to which ethanol inhibited GAP activity in organisms from 8 h or 16 h cultures. However, under energized conditions, organisms from 8 h cultures were markedly more sensitive to ethanol-induced inhibition of glycine uptake than organisms from 16 h cultures. This finding directly parallels my previous discovery on ethanol-mediated dissipation of the Δp. In addition to this evidence, the linear relationship demonstrated between the rate of glycine uptake by the GAP and the magnitude of Δp, both in the presence and absence of ethanol, further illustrates the direct effect that Δp dissipation has on glycine transport. If a direct, or indirect, inactivation of the GAP occurred at ethanol concentrations of greater than 1.0 M, for example, the relationship between the magnitude of Δp and the rate of glycine uptake would be expected

to deviate from linearity. On the contrary, the strong correlation between plasma-membrane energization and uptake of this amino acid, under all experimental conditions examined, bears a striking similarity to the effect addition of proton-uncoupling agents has on rates of solute accumulation by proton-symport operated permeases (Misra and Höfer, 1975; Keenan and Rose, 1979).

Consequently, at least as far as the GAP is concerned, a primary reason for the inhibitory effect of ethanol on amino-acid uptake appears to be the ability of this alkanol to dissipate the Δp across the plasma membrane. Obviously, it remains to be seen if this strong relationship between Δp magnitude and solute-uptake rate also holds for other proton-symport mediated transport systems present in the plasma membrane of Sacch. cerevisiae. Previous studies have shown that ethanol non-competitively inhibits uptake of ammonium ions (Leão and van Uden, 1983) and maltose (Loureiro-Dias and Peinado, 1982), and it would be of considerable interest to discover whether the inhibitory effect of ethanol on the accumulation of these solutes is due to the dissipation of Δp produced by this alkanol.

Perhaps of greater immediacy, however, is the need to examine the nature of ethanol-induced inhibition of glucose transport in Sacch. cerevisiae. Since glycolytic enzymes are relatively insensitive to ethanol, an inhibitory effect of ethanol on glucose uptake could be a determining factor in the ability of this alkanol to decrease the fermentative activity of organisms. Although Leão and van Uden (1982b) showed that ethanol non-competitively inhibits glucose uptake, these workers used xylose, an analogue of glucose, to

investigate the effect of alkanols on solute uptake by the constitutive monosaccharide carrier of Sacch. cerevisiae. Glucose transport is now known to occur by two separate systems, a low-affinity system mediated by facilitated diffusion and a high-affinity system dependent on glycolytic kinases for its operation (Bisson and Fraenkel, 1983). Since xylose uptake can be effected by both carriers (Busturia and Lagunas, 1986), the data of Leão and van Uden (1982b) probably only show a composite effect of ethanol on the two glucose permeases. Consequently, the inhibition constants quoted by these workers cannot be compared to similar values determined for single solute-transport systems. Glucose transport by either carrier does not occur via a proton symport and it would therefore be of great interest to compare the effect of ethanol on glucose uptake with the inhibition of proton-symport operated solute transport produced by this alkanol. This comparison could, hopefully, enable two useful advances to be made in the area of ethanol tolerance. Firstly, the development of an overall hypothesis to explain how ethanol-mediated inhibition of solute transport is effected and, secondly, the proposal of a cohesive model to define how ethanol effects on the plasma membrane might account for the inhibitory action of this alkanol on growth, fermentation and the retention of viability by Sacch. cerevisiae.

REFERENCES

AGUILERA, A. and BENITEZ, T. (1985). Role of mitochondria in ethanol tolerance of Saccharomyces cerevisiae. Archives of Microbiology **142**, 389-392.

AHLERS, J. (1984). Effects of different salts on the plasma membrane ATPase and on proton transport in yeast. Canadian Journal of Biochemistry and Cell Biology **62**, 998-1005.

AHLERS, J., AHR, E. and SEYFARTH, A. (1978). Kinetic characteristics of plasma-membrane ATPase from Saccharomyces cerevisiae. Molecular and Cellular Biochemistry **22**, 39-49.

AHLERS, J., RADE, S. and STADTLANDER, K. (1980). Influence of growth conditions on the composition of the plasma membrane from yeast and on kinetic properties of the membrane-bound ATPase and the purine transport system. Hoppe-Seylers Zeitschrift für Physiologische Chemie **361**, 1261-1262.

ALDERMANN, B. and HÖFER, M. (1984). Fractionation of membranes from Metschnikowia reukaufii protoplasts. Evidence for a plasma-membrane-bound ATPase. Journal of General Microbiology **130**, 711-723.

ARMSTRONG, W.McD. and ROTHSTEIN, A. (1964). Discrimination between alkali metal cations by yeast. I. Effect of pH on uptake. Journal of General Physiology **48**, 61-71.

BALLARIN-DENTI, A., DEN HOLLANDER, J.A., SANDERS, D., SLAYMAN, C.W. and SLAYMAN, C.L. (1984). Kinetics and pH-dependence of glycine-proton symport in Saccharomyces cerevisiae. Biochimica et biophysica acta **778**, 1-16.

BARTS, P.W.J.A. and BORST-PAUWELS, G.W.F.H. (1983). Effects of membrane potential and surface potential on the kinetics of solute transport. Biochimica et biophysica acta **813**, 51-60.

BARTS, P.W.J.A., HOEBRICHTS, J.A., KLAASEN, A. and BORST-PAUWELS, G.W.F.H. (1980). Uptake of the lipophilic cation dibenzyldimethylammonium into Saccharomyces cerevisiae. Interaction with the thiamine transport system. Biochimica et biophysica acta **597**, 125-136.

BEAVAN, M.J. (1982). Production and tolerance of ethanol in Saccharomyces cerevisiae. Ph.D. Thesis, University of Bath, U.K.

BEAVAN, M.J., CHARPENTIER, C. and ROSE, A.H. (1982). Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in Saccharomyces cerevisiae NCYC 431. Journal of General Microbiology **128**, 1447-1455.

BERNHEIM, F. (1978). The effect of aliphatic alcohols on efflux of H^+ and K^+ from Pseudomonas aeruginosa. Microbios Letters **9**, 91-95.

BISSON, L.F. and FRAENKEL, D.G. (1983). Involvement of kinases in glucose and fructose uptake by Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America **80**, 1730-1734.

BLASCO, F., CHAPUIS, J.-P and GIORDANI, R. (1981). Characterization of the plasma membrane ATPase of Candida tropicalis. Biochimie **63**, 507-514.

BOGGS, J.M. (1980). Intermolecular hydrogen bonding between lipids: influence on organization and function of lipids in membranes. Canadian Journal of Biochemistry **58**, 755-770.

BOGONEZ, E., MACHADO, A. and SATRUSTEGUI, J. (1983). Ammonia accumulation in acetate-growing yeast. Biochimica et biophysica acta **733**, 234-241.

BORST-PAUWELS, G.W.F.H. (1981). Ion transport in yeast. Biochimica et biophysica acta **650**, 88-127.

BORST-PAUWELS, G.W.F.H. and DOBBELMANN, J. (1972). Determination of the yeast cell pH. Acta botanica neerlandica **21**,149-154.

BORST-PAUWELS, G.W.F.H. and JAGER, S. (1969). Inhibition of phosphate and arsenate uptake in yeast by monoiodoacetate, fluoride, 2,4-dinitrophenol and acetate. Biochimica et biophysica acta **172**, 399-406.

BORST-PAUWELS, G.W.F.H. and PETERS, P.H.J. (1981). Factors affecting the inhibition of yeast plasma membrane ATPase by vanadate. Biochimica et biophysica acta **642**, 173-181.

BORST-PAUWELS, G.W.F.H., THEUVENET, A.P.R. and STOLS, A.L.H. (1983). All or none interactions of inhibitors of the plasma membrane ATPase with Saccharomyces cerevisiae. Biochimica et biophysica acta **732**, 186-192.

BORST-PAUWELS, G.W.F.H., WOLTERS, G.H.J. and HENRICKS, J.J.G. (1971). The interaction of 2,4-dinitrophenol with anaerobic Rb^+ transport across the yeast cell membrane. Biochimica et biophysica acta **225**, 269-276.

BOULTON, A.A. (1965). Some observations on the chemistry and morphology of the membranes released from yeast protoplasts by osmotic shock. Experimental Cell Research **37**, 343-351.

BOWMAN, B.J. and SLAYMAN, C.W. (1977). Characterization of plasma membrane adenosine triphosphatase of Neurospora crassa. Journal of Biological Chemistry **252**, 3357-3363.

BOWMAN, B.J. and SLAYMAN, C.W. (1979). The effects of vanadate on the plasma membrane ATPase of Neurospora crassa. Journal of Biological Chemistry **254**, 2928-2934.

BOWMAN, B.J., BERENSKI, C.J. and YUNG, C.Y. (1985). Size of the plasma membrane H^+-ATPase from Neurospora crassa determined by radiation inactivation and comparison with the sarcoplasmic reticulum Ca^{2+}-ATPase from skeletal muscle. Journal of Biological Chemistry **260**, 8726-8730.

BOWMAN, B.J., BLASCO, F. and SLAYMAN, C.W. (1981). Purification and characterization of the plasma membrane ATPase of Neurospora crassa. Journal of Biological Chemistry **256**, 12343-12349.

BOXMAN, A.W., DOBBELMANN, J. and BORST-PAUWELS, G.W.F.H. (1984). Possible energization of K^+ accumulation into metabolizing yeast by the proton motive force. Binding correction to be applied in the calculation of the yeast plasma membrane potential from tetraphenylphosphonium distribution. Biochimica et biophysica acta **772**, 51-57.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72**, 248-254.

BRECKENRIDGE, W.C., GOMBOS, G. and MORGAN, I.G. (1972). The lipid composition of adult rat brain synaptosomal plasma membranes. Biochimica et biophysica acta **266**, 695-707.

BROCKLEHURST, R., GARDNER, D. and EDDY, A.A. (1977). The absorption of protons with α-methylglucoside and α-thioethylglucoside by the yeast NCYC 240. Biochemical Journal **162**, 591-599.

BROWN, S.W., OLIVER, S.G., HARRISON, D.E.F. and RIGHALETO, R.C. (1981). Ethanol inhibition of yeast growth and fermentation: differences in the magnitude and complexity of the effect. European Journal of Applied Microbiology and Biotechnology **11**, 151-155.

BUSTURIA, A. and LAGUNAS, R. (1986). Catabolite inactivation of the glucose transport system in Saccharomyces cerevisiae. Journal of General Microbiology **132**, 379-385.

CALDERBANK, J. (1984). Plasma-membrane fatty-acyl unsaturation in Saccharomyces cerevisiae and accumulation of amino acids and peptides. Ph.D. Thesis, University of Bath, U.K.

CALDERBANK, J., KEENAN, M.H.J. and ROSE, A.H. (1985). Plasma-membrane phospholipid unsaturation affects expression of the general amino-acid permease in Saccharomyces cerevisiae Y185. Journal of General Microbiology **131**, 57-65.

CALDERBANK, J., KEENAN, M.H.J., ROSE, A.H. and HOLMAN, G.D. (1984). Accumulation of amino acids by Saccharomyces cerevisiae Y185 with phospholipids enriched in different fatty-acyl residues: a statistical analysis of data. Journal of General Microbiology **130**, 2817-2824.

CAREY, V.C. and INGRAM, L.O. (1983). Lipid composition of Zymomonas mobilis: Effects of ethanol and glucose. Journal of Bacteriology **154**, 1291-1300.

CHANEY, L.K. and JACOBSON, B.S. (1983). Coating cells with colloidal silica for high yield isolation of plasma membrane sheets and identification of transmembrane proteins. Journal of Biological Chemistry **258**, 10062-10072.

CHIN, J.H. and GOLDSTEIN, D.B. (1977a). Effects of low concentrations of ethanol on the fluidity of spin-labelled erythrocyte and brain membranes. Molecular Pharmacology 13, 435-441.

CHIN, J.H. and GOLDSTEIN, D.B. (1977b). Drug tolerance in biomembranes: a spin label study of the effects of ethanol. Science **196**, 684-685.

CHIN, J.H. and GOLDSTEIN, D.B. (1984). Cholesterol blocks the disordering effects of ethanol in biomembranes. Lipids **19**, 929-935.

COCKBURN, M., EARNSHAW, P. and EDDY, A.A. (1975). The stoichiometry of the absorption of protons with phosphate and L-glutamate by yeasts of the genus Saccharomyces. Biochemical Journal **146**, 705-712.

CONWAY, E.J. and BRADY, T. (1947). Mechanism of high acid production by yeast and its bearing on hydrochloric acid formation in the stomach. Nature **159**, 137-138.

CONWAY, E.J. and BRADY, T.G. (1950). Biological production of acid and alkali. I. Quantitative relations of succinic and carbonic acids to the potassium and hydrogen exchange in fermenting yeast. Biochemical Journal **47**, 360-369.

CONWAY, E.J. and DOWNEY, M. (1950). pH values of the yeast cell. Biochemical Journal **47**, 355-360.

CONWAY, E.J. and DUGGAN, F. (1958). A cation carrier in the yeast cell wall. Biochemical Journal **69**, 269-275.

CONWAY, E.J. and O'MALLEY, E. (1946). The nature of the cation exchanges during yeast fermentations, with formation of 0.02 N - H^+ ion. Biochemical Journal **40**, 59-67.

CONWAY, E.J., BRADY, T.G. and CARTON, E. (1950). Biological production of acid and alkali. II. A redox theory for the process in yeast

with application to the production of gastric acidity. Biochemical Journal **47**, 369-374.

COOPER, T.G. (1982). Transport in Saccharomyces cerevisiae. In Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression, pp. 399-461. Edited by J.N. Strathern, E.W. Jones and J.R. Broach. Cold Spring Harbor Laboratory.

CRABEEL, M. and GRENSON, M. (1970). Regulation of histidine uptake by specific feedback inhibition of two histidine permeases in Saccharomyces cerevisiae. European Journal of Biochemistry **14**, 197-204.

CURTAIN, C.C., ATWELL, J.L., LOONEY, F.D. and ZAJAC-CADE, A. (1984). Membrane mechanism of ethanol tolerance in Saccharomyces cerevisiae: an electron spin resonance study. Proceedings of the Australian Brewing Conference, Adelaide, pp. 1-22.

DANIELLI, J.F. and DAVSON, H. (1935). The permeability of thin lipid films. Journal of Cellular and Comparative Physiology **5**, 495-508.

DANIELS, C.J., BOLE, D.G., QUAY, S.C. and OXENDER, D.L. (1981). Role for membrane potential in the secretion of protein into the periplasm of Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America **78**, 5396-5400.

DARTE, C. and GRENSON, M. (1975). Evidence for three glutamic acid transporting systems with specialized physiological functions in Saccharomyces cerevisiae. Biochemical and Biophysical Research Communications **67**, 1028-1033.

DASARI, G., KESHAVARZ , E., CONNOR, M.A. and PAMMENT, N.B. (1986). Intracellular ethanol concentration and comparative toxicity of endogenously produced and added ethanol in Saccharomyces

cerevisiae. Proceedings of the XIIth International Specialized Symposium on Yeasts, Lisbon, p. 37.

DAVIES, C.W. (1962). Ion Association, pp. 39-43. London and Boston, Butterworth.

DE LA PEÑA, P., BARROS, F., GASCÓN, S., LAZO, P.S. AND RAMOS, S. (1981). Effect of yeast killer toxin on sensitive cells of Saccharomyces cerevisiae. Journal of Biological Chemistry **256**, 10420-10425.

DE LA PEÑA, P., BARROS, F., GASCÓN, S., RAMOS, S. AND LAZO, P.S. (1982). The electrochemical proton gradient of Saccharomyces.The role of potassium. European Journal of Biochemistry **123**, 447-453.

DELHEZ, J., DUFOUR, J.-P, THINES, D. and GOFFEAU, A. (1977). Comparison of the properties of plasma-membrane bound and mitochondria-bound ATPases in the yeast Schizosaccharomyces pombe. European Journal of Biochemistry **79**, 319-328.

DEMAIN, A.L., RICKES, E.L., HENDLIN, D. and BARNES, E.C. (1961). Nutritional studies on Lactobacillus heterohiochii. Journal of Bacteriology **81**, 147-153.

DITTMER, J.C. and LESTER, R.L. (1964). A simple specific spray for the detection of phospholipids on thin-layer chromatograms. Journal of Lipid Research **5**, 126-127.

DIXON, M. (1953). The determination of enzyme inhibition constants. Biochemical Journal **55**, 170-171.

DOMBEK, K.M. and INGRAM, L.O. (1984). Effects of ethanol on the Escherichia coli plasma membrane. Journal of Bacteriology **157**, 233-239.

DUFOUR, J.-P. and GOFFEAU, A. (1978). Solubilization by lysolecithin and purification of the plasma membrane ATPase of the yeast

Schizosaccharomyces pombe. Journal of Biological Chemistry **253**, 7026-7032.

DUFOUR, J.-P. and GOFFEAU, A. (1980). Molecular and kinetic properties of the purified plasma membrane ATPase of the yeast Schizosaccharomyces pombe. European Journal of Biochemistry **105**, 145-154.

DUFOUR, J.-P., GOFFEAU, A. and TSONG, T.Y. (1982). Active proton uptake in lipid vesicles reconstituted with a purified yeast plasma membrane ATPase. Journal of Biological Chemistry **257**, 9365-9371.

EATON, L.C., TEDDER, T.F. and INGRAM, L.O. (1982). Effects of fatty acid composition on the sensitivity of membrane functions to ethanol in Escherichia coli. Substance and Alcohol Actions/Misuse **3**, 77-87.

EDDY, A.A. (1978). Proton-dependent solute transport in micro-organisms. Current Topics in Membranes and Transport **10**, 279-360.

EDDY, A.A. (1982). Mechanism of solute transport into selected eucaryotic micro-organisms. Advances in Microbial Physiology **23**, 1-78.

EDDY, A.A. and NOWACKI, J.A. (1971). Stoichiometrical proton and potassium ion movements accompanying the absorption of amino acids by the yeast Saccharomyces carlsbergensis. Biochemical Journal **122**, 701-711.

EDDY, A.A., BACKEN, K. and WATSON, G. (1970a). The concentration of amino acids by yeast cells depleted of adenosine triphosphate. biochemical Journal **120**, 853-858.

EDDY, A.A., INDGE, K.J., BACKEN, K. and NOWACKI, J.A. (1970b). Interactions between potassium ions and glycine transport in the yeast Saccharomyces carlsbergensis. Biochemical Journal **120**, 845-852.

EILAM, Y. (1983). Membrane effects of phenothiazines in yeasts. I. Stimulation of calcium and potassium fluxes. Biochimica et biophysica acta **733**, 242-248.

EILAM, Y. (1984). Effects of phenothiazines on inhibition of plasma-membrane ATPase and hyperpolarization of cell membranes in the yeast Saccharomyces cerevisiae. Biochimica et biophysica acta **769**, 601-610.

EILAM, Y., LAVI, H. and GROSSOWICZ, N. (1984). Effects of inhibitors of plasma-membrane ATPase on potassium and calcium fluxes, membrane potential and proton motive force in the yeast Saccharomyces cerevisiae. Microbios **41**, 177-189.

ELGAVISH, A. and ELGAVISH, G.A. (1985). In vitro ethanol effects on the transport properties of isolated renal brush-border membrane vesicles. Journal of Membrane Biology **88**, 123-130.

ENEQUIST, H.G., HIRST, T.R., HARAYAMA, S., HARDY, S.J.S. and RANDALL, L.L. (1981). Energy is required for maturation of exported proteins in Escherichia coli. European Journal of Biochemistry **116**, 227-233.

ERASO, P., MAZÓN, M.J. and GANCEDO, J.M. (1984). Pitfalls in the measurement of membrane potential in yeast cells using tetra-phenylphosphonium. Biochimica et biophysica acta **778**, 516-520.

ESFAHANI, M., CAVANAUGH, J.R., PFEFFER, P.E., LUKEN, D.W. and DEVLIN, T.M. (1981). ^{19}F-NMR and fluorescence polarization of yeast plasma membrane and isolated lipids. Biochemical and Biophysical Research Communications **101**, 306-311.

FINK, H. and KÜHLES, R. (1933). Beiträge zur Methylenblaufärbung der Hefezellen und Studien über die Permeabilitat der Hefezellmembran.

II. Mitteilung. Eine verbasserte Färbflüssigkeit zur Erkennung von totan Hefezellen. Hoppe-Seylers Zeitschrift für Physiologische Chemie **218**, 335-349.

FOURY, F., AMORY, A. and GOFFEAU, A. (1981). Large-scale purification and phosphorylation of a detergent-treated adenosine triphosphatase complex from plasma membranes of Saccharomyces cerevisiae. European Journal of Biochemistry **119**, 395-400.

FRIED, V.A. and NOVICK, A. (1973). Organic solvents as probes for the structure and function of the bacterial membrane: effects of ethanol on wild type and ethanol-resistant mutants of Escherichia coli K-12. Journal of Bacteriology **114**, 239-248.

FUHRMANN, G.F. (1973). Dependence of divalent cation transport on ATP in yeast. Experientia **29**, 742.

FUHRMANN, G.F. (1974). Relation between divalent cation transport and divalent cation activated ATPase in yeast plasma membranes. Experientia **30**, 686.

GITS, J.J. and GRENSON, M. (1967). Multiplicity of amino acid permeases in Saccharomyces cerevisiaea. III. Evidence for a specific methionine-transporting system. Biochimica et biophysica acta **135**, 507-516.

GOFFEAU, A. and SLAYMAN, C.W. (1981). The proton translocating ATPase of the fungal plasma membrane. Biochimica et biophysica acta **639**, 197-223.

GOORMAGHTIGH, E., CHADWICK, C. and SCARBOROUGH, G.A. (1986). Monomers of the Neurospora plasma membrane H^+-ATPase catalyze efficient proton translocation. Journal of Biological Chemistry **261**, 7466-7476.

GORDON, L.M., SAUERHEBER, R.D., ESGATE, J.A., DIPPLE, I., MARCHMONT, R.J. and HOUSLAY, M.D. (1980). The increase in bilayer fluidity

of rat liver plasma membranes achieved by the local anaesthetic benzyl alcohol affects the activity of intrinsic membrane enzymes. Journal of Biological Chemistry **255**, 4519-4527.

GRAY, W.D. (1941). Studies on the alcohol tolerance of yeasts. Journal of Bacteriology **42**, 561-574.

GRAY, W.D. (1948). Further studies on the alcohol tolerance of yeasts: its relationship to cell storage products. Journal of Bacteriology **55**, 53-59.

GRAY, W.D. and SOVA, C. (1956). Relation of molecule size and structure to alcohol inhibition of glucose utilization by yeast. Journal of Bacteriology **72**, 349-356.

GREASHAM, R.L. and MOAT, A.G. (1973). Amino acid transport in a polyaromatic amino acid auxotroph of Saccharomyces cerevisiae. Journal of Bacteriology **115**, 975-981.

GREEN, D.E., MURER, E., HULTIN, H.O., RICHARDSON, S.H., SALMON, S.B., BRIERLEY, G.P. and BAUM, H. (1965). Association of integrated metabolic pathways with membranes. I. Glycolytic enzymes of the red blood corpuscle and yeast. Archives of Biochemistry and Biophysics **112**, 635-647.

GREGORY, M.E., KEENAN, M.H.J. and ROSE, A.H. (1982). Accumulation of L-asparagine by Saccharomyces cerevisiae X-2180. Journal of General Microbiology **128**, 2557-2562.

GRENSON, M. (1966). Multiplicity of amino acid permeases in Saccharomyces cerevisiae. II. Evidence for a specific lysine-transporting system. Biochimica et biophysica acta **127**, 339-346.

GRENSON, M. and HOU, C. (1972). Ammonia inhibition of the general amino acid permease and its suppression in NADH-specific glutamate dehydrogenaseless mutants of Saccharomyces cerevisiae. Biochemical and Biophysical Research Communications **48**, 749-756.

GRENSON, M., HOU, C. and CRABEEL, M. (1970). Multiplicity of amino acid permeases in Saccharomyces cerevisiae. IV. Evidence for a general amino acid permease. Journal of Bacteriology **103**, 770-777.

GRENSON, M., MOUSSET, M., WIAME, J.M. and BECHET, J. (1966). Multiplicity of amino acid permeases in Saccharomyces cerevisiae. I. Evidence for a specific arginine-transporting system. Biochimica et biophysica acta **127**, 325-338.

GRISHAM, C.M. and BARNETT, R.E. (1972). The interrelationship of membrane and protein structure in the functioning of the $[Na^+ + K^+]$-activated ATPase. Biochimica et biophysica acta **266**, 613-624.

GRISHAM, C.M. and BARNETT, R.E. (1973). The effects of long-chain alcohols on membrane lipids and the $[Na^+ + K^+]$-ATPase. Biochimica et biophysica acta **311**, 417-422.

GUIJARRO, J.M. and LAGUNAS, R. (1984). Saccharomyces cerevisiae does not accumulate ethanol against a concentration gradient. Journal of Bacteriology **160**, 874-878.

HANES, C.S. (1932). Studies of plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. Biochemical Journal **26**, 1406-1421.

HAROLD, F.M. (1970). Antimicrobial agents and membrane function. Advances in Microbial Physiology **4**, 45-104.

HARRISON, R. and LUNT, G.G. (1980). In Biological Membranes Their Structure and Function, Glasgow and London, Blackie.

HAUER, R. and HÖFER, M. (1978). Evidence for interactions between the energy-dependent transport of sugars and the membrane potential in the yeast Rhodotorula gracilis (Rhodosporidium toruloides). Journal of Membrane Biology **43**, 335-349.

HAUER, R., UHLEMANN, G., NEUMANN, J. and HÖFER, M. (1981). Proton pumps of the plasmalemma of the yeast Rhodotorula gracilis. Their coupling to fluxes of potassium and other ions. Biochimica et biophysica acta **649**, 680-690.

HAYASHIDA, S. and OHTA, K. (1980). Effects of phosphatidylcholine or ergosterol oleate on physiological properties of Saccharomyces saké. Agricultural and Biological Chemistry **44**, 2561-2567.

HAYASHIDA, S., FENG, D.D. AND HONGO, M. (1974). The mechanism of formation of high concentration alcohol in saké. IX. Function of the high alcohol producing factor. Agricultural and Biological Chemistry **38**, 2001-2006.

HENSCHKE, P.A., THOMAS, D.S., ROSE, A.H. and VEAZEY, F.J. (1983). Association of intracellular low-density vesicles with plasma membranes from Saccharomyces cerevisiae. Journal of General Microbiology **129**, 2927-2938.

HERRERO, A.A. and GOMEZ, R.F. (1980). Development of ethanol tolerance in Clostridium thermocellum. Effect of growth temperature. Applied and Environmental Microbiology **40**, 571-577.

HERRERO, A.A., GOMEZ, R.F. and ROBERTS, M.F. (1982). Ethanol-induced changes in the membrane lipid composition of Clostridium thermocellum. Biochimica et biophysica acta **693**, 195-204.

HÖFER, M. and KÜNEMUND, A. (1984). Tetraphenylphosphonium ion is a true indicator of negative plasma-membrane potential in the yeast Rhodotorula glutinis. Biochemical Journal **225**, 815-819.

HÖFER, M. and MISRA, P.C. (1978). Evidence for a proton/sugar symport in the yeast Rhodotorula gracilis (glutinis). Biochemical Journal **172**, 15-22.

HÖFER, M., HUH, H. and KÜNEMUND, A. (1983). Membrane potential and cation permeability. A study with a nystatin resistant mutant of Rhodotorula gracilis (Rhodosporidium toruloides). Biochimica et biophysica acta **735**, 211-214.

HÖFER, M., NICOLAY, K. and ROBILLARD, G. (1985). The electrochemical H^+ gradient in the yeast Rhodotorula glutinis. Journal of Bioenergetics and Biomembranes **17**, 175-182.

HOFSTEE, B.H.J. (1959). Non-inverted versus inverted plots in enzyme kinetics. Nature **184**, 1296-1298.

HORÁK, J., ŘÍHOVÁ, L. and KOTYK, A. (1981). Energization of sulphate transport in yeast. Biochimica et biophysica acta **649**, 436-440.

HUNTER, C.K., TREANOR, L.L., GRAY, J.P., HALTER, S.A., HOYUMPA, A. and WILSON, F.A. (1983). Effects of ethanol in vitro on rat intestinal brush-border membranes. Biochimica et biophysica acta **732**, 256-265.

INGRAM, L.O., (1976). Adaptation of membrane lipids to alcohols. Journal of Bacteriology **125**, 670-678.

INGRAM, L.O. and BUTTKE, T.M. (1984). Effects of alcohols on micro-organisms. Advances in Microbial Physiology **25**, 253-300.

INGRAM, L.O., DICKENS, B.F. and BUTTKE, T.M. (1980). Reversible effects of ethanol on Escherichia coli. Advances in Experimental Medicine **126**, 299-337.

ISRAEL, Y., SALAZAR, I. and ROSENMANN, E. (1969a). Inhibitory effects of alcohol on intestinal amino-acid transport in vivo and in vitro. Journal of Nutrition **96**, 499-504.

ISRAEL, Y., VALENZUELA, J.E., SALAZAR, I. and UGARTE, G. (1969b). Alcohol and amino-acid transport in the human intestine. Journal of Nutrition **98**, 222-224.

JAIN, M.K. and WU, N.M. (1977). Effect of small molecules on the dipalmitoyl lecithin liposomal bilayer: phase transitions in lipid bilayers. Journal of Membrane Biology **34**, 157-201.

JANOFF, A.S. and MILLER, K.W. (1982). A critical assessment of the lipid theories of general anaesthetic action. In Biological Membranes, pp. 417-476. Edited by D. Chapman. London, Academic Press.

JIMÉNEZ, J. and VAN UDEN, N. (1985). Use of extracellular acidification for the rapid testing of ethanol tolerance in yeasts. Biotechnology and Bioengineering **27**, 1596-1598.

JOHNSON, D.A., LEE, N.M., COOKE, R. and LOH, H.H. (1979). Ethanol-induced fluidization of brain lipid bilayers: required presence of cholesterol in membranes for the expression of tolerance. Molecular Pharmacology **15**, 739-746.

JOHNSON, D.A., LEE, N.M., COOKE, R. and LOH, H.H. (1980). Adaptation to ethanol-induced fluidization of brain lipid bilayers: cross-tolerance and reversibility. Molecular Pharmacology **17**, 52-55.

KAKINUMA, Y., OHSUMI, Y. and ANRAKU, Y. (1981). Properties of H^+-translocating adenosine triphosphate in vacuolar membranes of Saccharomyces cerevisiae. Journal of Biological Chemistry **256**, 10859-10863.

KANEKO, H., HOSAHARA, M., TANAKA, M. AND ITOH, T. (1976). Lipid composition of 30 species of yeast. Lipids **11**, 837-844.

KARNOVSKY, M.J.,KLEINFIELD, A.M., HOOVER, R.L. and KLAUSNER, R.D. (1982). The concept of lipid domains in membranes. Journal of Cell Biology **94**, 1-6.

KEENAN, M.H.J. and ROSE, A.H. (1979). Plasma-membrane lipid unsaturation can affect the kinetics of solute accumulation by Saccharomyces cerevisiae. Federation of European Microbiological Societies Microbiology Letters **6**, 133-137.

KITAMURA, K. and YAMAMOTO, Y. (1981). Lysis of yeast cells showing low susceptibility to Zymolyase. Agricultural and Biological Chemistry **45**, 1761-1766.

KOTYK, A. (1962). The uptake of 2,4-dinitrophenol by the yeast cell. Folia microbiologica **7**, 109-114.

KOTYK, A. and MICHALJANIČOVÁ, D. (1979). Uptake of trehalose by Saccharomyces cerevisiae. Journal of General Microbiology **110**, 323-332.

KOTYK, A., RÍHOVÁ, L. and PONEČ, M. (1971). Uptake of amino acids by actidone-treated yeast cells. I. Specificity of carriers. Folia microbiologica **16**, 432-444.

KOVÁČ, L. and VAREČKA, Ľ. (1981). Membrane potentials in respiring and respiration deficient yeasts monitored by a fluorescent dye. Biochimica et biophysica acta **637**, 209-216.

KRAMER, R., KOPP, F., NIEDERMEYER, W. and FUHRMANN, G.F. (1978). Comparative studies of the structure and composition of the plasmalemma and the tonoplast in Saccharomyces cerevisiae. Biochimica et biophysica acta **507**, 369-380.

LARRIMORE, F.S. and ROON, R.J. (1978). Possible site-specific reagent for the general amino acid transport system of Saccharomyces cerevisiae. Biochemistry **17**, 431-438.

LARUE, F., LAFON-LAFOURCADE, S. and RIBEREAU-GAYON, P. (1984). Relationship between the inhibition of alcoholic fermentations by Saccharomyces cerevisiae and the activities of hexokinase and alcohol dehydrogenase. Biotechnology Letters **6**, 687-692.

LASKO, P.F. and BRANDRISS, M.C. (1981). Proline transport in Saccharomyces cerevisiae. Journal of Bacteriology **148**, 241-247.

LEÃO, C. and VAN UDEN, N. (1982a). Effects of ethanol and other alkanols on the kinetics and the activation parameters of thermal death in Saccharomyces cerevisiae. Biotechnology and Bioengineering **24**, 1581-1590.

LEÃO, C. and VAN UDEN, N. (1982b). Effects of ethanol and other alkanols on the glucose transport system of Saccharomyces cerevisiae. Biotechnology and Bioengineering **24**, 2601-2604.

LEÃO, C. and VAN UDEN, N. (1983). Effect of ethanol and other alkanols on the ammonium transport system of Saccharomyces cerevisiae. Biotechnology and Bioengineering **25**, 2085-2089.

LEÃO, C. and VAN UDEN, N. (1984a). Effects of ethanol and other alkanols on the general amino acid permease of Saccharomyces cerevisiae. Biotechnology and Bioengineering **26**, 403-405.

LEÃO, C. and VAN UDEN, N. (1984b). Effect of ethanol and other alkanols on passive proton influx in the yeast Saccharomyces cerevisiae. Biochimica et biophysica acta **774**, 43-48.

LENARD, J. and SINGER, S.J. (1966). Protein conformation in cell membrane preparations as studied by optical rotatory dispersion and circular dichroism. Proceedings of the National Academy of Sciences of the United States of America **70**, 480-484.

LENAZ, G., BERTOLI, E., CURATOLA, G., MAZZANTI, L. and BIGI, A. (1976). Lipid-protein interactions in mitochondria. Spin

and fluorescence probe studies on the effect of n-alcohols on phospholipid vesicles and mitochondrial membranes. Archives of Biochemistry and Biophysics **172**, 278-288.

LICHKO, L.P. and OKOROKOV, L.A. (1985). H^+-ATPase of yeast vacuoles consists of several sub-units and does not form a phosphorylated intermediate. Biologicheski Membrany **2**, 770-775.

LIN, D.C. (1980). Involvement of the lipid and protein components of $[Na^+ + K^+]$-adenosine triphosphatase in the inhibitory action of alcohol. Biochemical Pharmacology **29**, 771-775.

LONGLEY, R.P., ROSE, A.H. and KNIGHTS, B.A. (1968). Composition of the protoplast membrane from Saccharomyces cerevisiae. Biochemical Journal **108**, 401-412.

LOUREIRO, V. and FERREIRA, H.G. (1983). On the intracellular accumulation of ethanol in yeast. Biotechnology and Bioengineering **25**, 2263-2269.

LOUREIRO-DIAS, M.C. and PEINADO, J.M. (1982). Effect of ethanol and other alkanols on the maltose transport system of Saccharomyces cerevisiae . Biotechnology Letters **4**, 721-724.

LOUREIRO-DIAS, M.C. and PEINADO, J.M. (1984). Transport of maltose in Saccharomyces cerevisiae . Effect of pH and potassium ions. Biochemical Journal **222**, 293-298.

LOVITT, R.W., LONGIN, R. and ZEIKUS, J.G. (1984). Ethanol production by thermophilic bacteria: physiological comparison of solvent effects on parent and alcohol-tolerant strains of Clostridium thermohydrosulphuricum. Applied and Environmental Microbiology **48**, 171-177.

LUCY, J.A. and GLAUERT, A.M. (1964). Structure and assembly of macromolecular lipid complexes composed of globular micelles. Journal of Molecular Biology **8**, 727-748.

LUONG, J.H.T. (1985). Kinetics of ethanol inhibition in alcohol fermentation. Biotechnology and Bioengineering **27**, 280-285.

MALPARTIDA, F. and SERRANO, R. (1980). Purification of the yeast plasma membrane solubilized with a novel zwitterionic detergent. Federation of European Biochemical Societies Letters **111**, 69-72.

MALPARTIDA, F. and SERRANO, R. (1981a). Reconstitution of the proton-translocating ATPase of yeast plasma membranes. Journal of Biological Chemistry **256**, 4175-4177.

MALPARTIDA, F. and SERRANO, R. (1981b). Proton translocation catalyzed by the purified yeast plasma membrane ATPase reconstituted in liposomes. Federation of European Biochemical Societies Letters **131**, 351-354.

MATILE, P., MOOR, H. and MÜHLETAHLER, K. (1967). Isolation and properties of plasmalemma in yeast. Archiv für Mikrobiologie **58**, 201-211.

McDONOUGH, J.P., JAYNES, P.K. and MAHLER, H.R. (1980). Partial characterization of plasma membrane ATPase from a rho° petite strain of Saccharomyces cerevisiae. Journal of Bioenergetics and Biomembranes **12**, 249-264.

McMURCHIE, E.J. and RAISON, J.K. (1979). Membrane lipid fluidity and its effect on the activation energy of membrane-associated enzymes. Biochimica et biophysica acta **554**, 364-374.

MICHAELIS, E.K. and MICHAELIS, M.L. (1983). Physico-chemical interactions between alcohol and biological membranes. Research Advances in Alcohol and Drug Problems **7**, 127-173.

MICHAELSON, D.M., HORWITZ, A.F. and KLEIN, M.P. (1974). Head group modulation of membrane fluidity in sonicated phospholipid

dispersions. Biochemistry **13**, 2605-2612.

MILLAR, D.G., GRIFFITHS-SMITH, K., ALGAR, E. and SCOPES, R.K. (1982). Activity and stability of glycolytic enzymes in the presence of ethanol. Biotechnology Letters **4**, 601-606.

MISRA, P.C. and HÖFER, M. (1975). An energy-linked proton extrusion across the cell membrane of Rhodotorula gracilis. Federation of European Biochemical Societies Letters **52**, 95-99.

MITCHELL, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature **191**, 144-148.

MITCHELL, P. (1963). Molecule, group and electron translocation through natural membranes. Biochemical Society Symposia **22**, 142-168.

MOREIRA, A.R., ULMER, D.C. and LINDEN, J.C. (1981). Butanol toxicity in the butylic fermentation. Biotechnology and Bioengineering Symposia **11**, 567-579.

NAGODAWITHANA, T.W. and STEINKRAUS, K.H. (1976). Influence of the rate of ethanol production and accumulation on the viability of Saccharomyces cerevisiae in "rapid fermentations". Applied and Environmental Microbiology **31**, 158-162.

NAGODAWITHANA, T.W., WHITT, J.T. and CUTAIA, A.J. (1977). Study of the feedback effect of ethanol on selected enzymes of the glycolytic pathway. Journal of the American Society of Brewing Chemists **35**, 179-183.

NANDINI-KISHORE, S.G., MATTOX, S.M., MARTIN, C.E. and THOMPSON, G.A. Jr. (1979). Membrane changes during growth of Tetrahymena in the presence of ethanol. Biochimica et biophysica acta **551**, 315-327.

NAVARRO, J.M. (1980). Fermentation alcoolique: influence des conditions de cultures sur l'inhibition par l'ethanol. Cellular and Molecular Biology **26**, 241-246.

NAVARRO, J.M. and DURAND, G. (1978). Fermentation alcoolique: influence de la temperature sur l'accumulation d'alcool dans les cellules de levure. Annals of Microbiology (Paris) **129B**, 215-224.

NAVON, G., SHULMAN, R.G., YAMANE, T., ECCLESHALL, T.R., LAM, K.-B., BARONOFSKY, J.J. AND MARMUR, J. (1979). Phosphorus-31 nuclear magnetic resonance studies of wild-type and glycolytic pathway mutants of Saccharomyces cerevisiae. Biochemistry **18**, 4487-4499.

NEAL, J.L. (1972). Analysis of Michaelis kinetics for two independent saturable membrane transport functions. Journal of Theoretical Biology **35**, 113-118.

NITSCHMANN, W.H. and PESCHEK, G.A. (1985). Modes of proton translocation across the cell membrane of respiring cyanobacteria. Archives of Microbiology **141**, 330-336.

NORRIS, P.R. and KELLY, D.P. (1977).Accumulation of cadmium and cobalt by Saccharomyces cerevisiae. Journal of General Microbiology **99**, 317-324.

NOVAK, M., STREHAIANO, P., MORENO, M. and GOMA, G. (1981). Alcoholic fermentation: on the inhibitory effect of ethanol. Biotechnology and Bioengineering **23**, 201-211.

NURMINEN, T. (1976). On the isolation, enzymes and lipid composition of the plasma membrane and cell wall of baker's yeast (Saccharomyces cerevisiae). Ph.D. Thesis, University of Helsinki, Finland.

NURMINEN, T., KONTTINEN, K. and SUOMALAINEN, H. (1975). Neutral lipids in the cells and cell envelope fractions of aerobic baker's

and anaerobic brewer's yeast. Chemistry and Physics of Lipids **14**, 15-32.

NURMINEN, T., OURA, E. and SUOMALAINEN, H. (1970). The enzymic composition of the isolated cell wall and plasma membrane of baker's yeast. Biochemical Journal **116**, 61-69.

OKOROKOV, L.A. and LICHKO, L.P. (1983). The identification of a proton pump on vacuoles of the yeast Saccharomyces carlsbergensis. Federation of European Biochemical Societies Letters **155**, 102-106.

OKOROKOV, L.A., KULAKOVSKAYA, T.V. and KULAEV, I.S. (1982). Solubilization and partial purification of vacuolar ATPase of yeast Saccharomyces carlsbergensis. Federation of European Biochemical Societies Letters **145**, 160-162.

OKOROKOV, L.A., KULAKOVSKAYA, T.V., LICHKO, L.P. AND POLOROTOVA, E.V. (1985). H^+/ion antiport as the principal mechanism of transport systems in the vacuolar membrane of the yeast Saccharomyces carlsbergensis. Federation of European Biochemical Societies Letters **192**, 303-306.

OKOROKOV, L.A., LICHKO, L.P., KADOMTSEVA, V.M., KHOLODENKO, V.P., TITOVSKY, V.T. and KULAEV, I.S. (1977). Energy-dependent transport of manganese into yeast cells and distribution of accumulated ions. European Journal of Biochemistry **75**, 373-377.

O'NEILL, B., WEBER, F., HORNIG, D. and SEMENZA, G. (1986). Ethanol selectivity affects Na^+-gradient dependent intestinal transport systems. Federation of European Biochemical Societies Letters **194**, 183-188.

OSMAN, Y.A. and INGRAM, L.O. (1985). Mechanism of ethanol inhibition of fermentation in Zymomonas mobilis CP4. Journal of Bacteriology **164**, 173-180.

OTA, A. (1985). Properties of ATPase activity in intact vegetative cells and sporulating cells of the yeast Saccharomyces cerevisiae. International Journal of Biochemistry **17**, 775-780.

PANG, K.-Y., CHANG T.-L. and MILLER, K.W. (1979). On the coupling between anaesthetic induced membrane fluidisation and cation permeability in lipid vesicles. Molecular Pharmacology **15**, 729-738.

PASCUAL, C., ROMAY, C. and SIGLER, K. (1983). Proton extrusion in Saccharomyces cerevisiae mutants in very dilute suspensions. Folia microbiologica **28**, 353-362.

PEÑA, A. (1975). Studies on the mechanism of K^+ transport in yeast. Archives of Biochemistry and Biophysics **167**, 397-409.

PEÑA, A., GINCO, G., GÓMEZ-PUYOU, A. and TUENA, M. (1969). Studies on the mechanism of the stimulation of glycolysis and respiration by K^+ in Saccharomyces cerevisiae. Biochimica et biophysica acta **180**, 1-8.

PEÑA, A., GINCO, G., GÓMEZ-PUYOU, A. and TUENA, M. (1972). Effect of the pH of the incubation medium on glycolysis and respiration in Saccharomyces cerevisiae. Archives of Biochemistry and Biophysics **153**, 413-425.

PETERS, P.H.J. and BORST-PAUWELS, G.W.F.H. (1979). Properties of plasma membrane ATPase and mitochondrial ATPase of Saccharomyces cerevisiae. Physiologica Plantarum **46**, 330-337.

PETERSON, G.L. and HOKIN, L.E. (1981). Molecular weight and stoichiometry of the sodium- and potassium-activated adenosine triphosphatase subunits. Journal of Biological Chemistry **256**, 3751-3761.

POGGE-VON STRANDMANN, R., KAY, R.R. and DUFOUR, J.-P. (1984). An electrogenic proton pump in plasma membranes from the cellular

slime mould Dictyostelium discoideum. Federation of European Biochemical Societies Letters **175**, 422-428.

RAMOS, E.H., DE BONGIOANNI, L.C. and STOPPANI, A.O.M. (1980). Kinetics of L-[^{14}C]leucine transport in Saccharomyces cerevisiae. Effect of energy coupling inhibitors. Biochimica et biophysica acta **599**, 214-231.

RANK, G.H. and ROBERTSON, A.J. (1983). Protein and lipid composition of the yeast plasma membrane. In Yeast Genetics. Fundamental and Applied Aspects, pp. 227-241. Edited by J.F.T. Spencer, D.M. Spencer and A.R.W. Smith. New York, Springer-Verlag.

RANK, G.H., GERLACH, J.H., ROBERTSON, A.J. and VAN HOEVEN, R.P. (1978). High viscosity vesicles of yeast separated at pH 4 have surface glycoprotein. Nature **273**, 682-683.

REYNOLDS, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology **17**, 208-212.

RICHARDS, O.W. (1928). Potentially unlimited multiplication of yeast with constant environment, and the limiting of growth by changing environment. Journal of General Physiology **11**, 525-538.

RIEMERSMA, J.C. and ALSBACH, E.J.J. (1974). Proton translocation during anaerobic energy production in Saccharomyces cerevisiae. Biochimica et biophysica acta **339**, 274-284.

RIZZOLO, L.J., LE MAIRE, M., REYNOLDS, J.A. and TANFORD, C. (1976). Molecular weights and hydrophobicity of the polypeptide chain of the sarcoplasmic reticulum calcium (II) adenosine-triphosphatase and of its primary tryptic fragments. Biochemistry **15**, 3433-3437.

ROBERTSON, J.D. (1964). Unit membranes: a review with recent new studies of experimental alterations and a new subunit structure

in synaptic membranes. In Cellular Membranes in Development, pp. 1-81. Edited by M. Locke. New York, Academic Press.

ROOMANS, G.M. and BORST-PAUWELS, G.W.F.H. (1977). Interaction of phosphate with monovalent cation uptake in yeast. Biochimica et biophysica acta **470**, 84-91.

ROOMANS, G.M., KUYPERS, G.A.J., THEUVENET, A.P.R. AND BORST-PAUWELS, G.W.F.H. (1979). Kinetics of sulphate uptake by yeast. Biochimica et biophysica acta **551**, 197-206.

ROON, R.J., EVEN, H.J., DUNLOP, P. and LARRIMORE, F.L. (1975). Methylamine and ammonia transport in Saccharomyces cerevisiae. Journal of Bacteriology **122**, 502-509.

ROSE, A.H. (1980). Recent research on industrially important strains of Saccharomyces cerevisiae. In Biology and Activity of Yeasts, pp. 103-121. Edited by F.A. Skinner, S.M. Passmore and R.R. Davenport. London, Academic Press.

ROTTENBERG, H., ROBERTSON, D.E. and RUBIN, E. (1980). The effect of ethanol on the temperature dependence of respiration and ATPase activities of rat liver mitochondria. Laboratory Investigations **42**, 318-326.

RYAN, J.P. and RYAN, H. (1972). The role of intracellular pH in the regulation of cation exchanges in yeast. Biochemical Journal **128**, 139-146.

RYTKA, J. (1975). Positive selection of general amino acid permease mutants in Saccharomyces cerevisiae. Journal of Bacteriology **121**, 562-570.

SÁ-CORREIA, I. and VAN UDEN, N. (1986). Ethanol-induced death of Saccharomyces cerevisiae at low and intermediate growth temperatures. Biotechnology and Bioengineering **28**, 301-303.

SALHANY, J.M., YAMANE, T., SHULMAN, R.G. and OGAWA, S. (1975). High resolution ^{31}P nuclear magnetic resonance studies of intact yeast cells. Proceedings of the National Academy of Sciences of the United States of America **72**, 4966-4970.

SANDERMANN, H. (1978). Regulation of membrane enzymes by lipids. Biochimica et biophysica acta **515**, 209-237.

SANDERS, D. and SLAYMAN, C.L. (1982). Control of intracellular pH. Predominant role of oxidative metabolism,not proton transport, in the eukaryotic micro-organism Neurospora. Journal of General Physiology **80**, 377-402.

SANDERS, D., HANSEN, U.-P. and SLAYMAN, C.L. (1981). Role of the plasma membrane proton pump in pH regulation in non-animal cells. Proceedings of the National Academy of Sciences of the United States of America **78**, 5903-5907.

SANDERS, D., SLAYMAN, C.L. and PALL, M.L. (1983). Stoichiometry of H^+/amino acid cotransport in Neurospora crassa revealed by current-voltage analysis. Biochimica et biophysica acta **735**, 67-76.

SANTOS, E., LEAL, F. AND SENTANDREU, R. (1982a). The plasma membrane of Saccharomyces cerevisiae. Molecular structure and asymmetry. Biochimica et biophysica acta **685**, 329-339.

SANTOS, E., VILLANEUVA, J.R. and SENTANDREU, R. (1978). The plasma membrane of Saccharomyces cerevisiae. Isolation and some properties. Biochimica et biophysica acta **508**, 39-54.

SANTOS, E., RODRIGUEZ, L., ELORZA, M.V. and SENTANDREU, R. (1982b). Uptake of sucrose by Saccharomyces cerevisiae. Archives of Biochemistry and Biophysics **216**, 652-660.

SCARBOROUGH, G.A. (1976).The Neurospora plasma membrane ATPase is an electrogenic pump. Proceedings of the National Academy of

Sciences of the United States of America **73**, 1485-1488.

SCARBOROUGH, G.A. (1977). Properties of the Neurospora crassa plasma membrane ATPase. Archives of Biochemistry and Biophysics **180**, 384-393.

SCARBOROUGH, G.A. (1980). Proton translocation catalyzed by the electrogenic ATPase in the plasma membrane of Neurospora. Biochemistry **19**, 2925-2931.

SCARBOROUGH, G.A. and ADDISON, R. (1984). On the subunit composition of the Neurospora plasma membrane H^+-ATPase. Journal of Biological Chemistry **259**, 9109-9114.

SCHATZ, G. (1965). Subcellular particles carrying mitochondrial enzymes in anaerobically-grown cells of Saccharomyces cerevisiae. Biochimica et biophysica acta **96**, 342-345.

SCHERER, S. and BÖGER, P. (1984). Vanadate-sensitive proton efflux by filamentous cyanobacteria. Federation of European Microbiological Societies Microbiology Letters **22**, 215-218.

SCHIBECI, A., RATTRAY, J.B.M. and KIDBY, D.K. (1973). Isolation and identification of yeast plasma membrane. Biochimica et biophysica acta **311**, 15-25.

SCHMIDT, R., ACKERMANN, R., KRATZKY, Z., WASSERMAN, B. and JACOBSON, B.S. (1983). Fast and efficient purification of yeast plasma membranes using cationic silica microbeads. Biochimica et biophysica acta **732**, 421-427.

SCHNEIDER, H., FUHRMANN, G.F. and FIECHTER, A. (1979). Plasma membrane from Candida tropicalis grown on glucose or hexadecane. II. Biochemical properties and substrate induced alterations. Biochimica et biophysica acta **554**, 309-322.

SCHOUSBOE, I. (1976). Properties of a triacylglycerol lipase in a mitochondrial fraction from baker's yeast (Saccharomyces cerevisiae). Biochimica et biophysica acta **450**, 165-174.

SEASTON, A., CARR, G. and EDDY, A.A. (1976). The concentration of glycine by preparations of the yeast Saccharomyces cerevisiae depleted of adnosine triphosphate. Biochemical Journal **154**, 669-676.

SEASTON, A., INKSON, C. and EDDY, A.A. (1973). The absorption of protons with specific amino acids and carbohydrates by yeast. Biochemical Journal **134**, 1031-1043.

SEEMAN, P. (1972). The membrane action of anaesthetics and tranquilizers. Pharmacological Reviews **24**, 583-655.

SERRANO, R. (1977). Energy requirements for maltose transport in yeast. European Journal of Biochemistry **80**, 97-102.

SERRANO, R. (1978). Characterization of the plasma membrane ATPase of Saccharomyces cerevisiae. Molecular and Cellular Biochemistry **22**, 51-63.

SERRANO, R. (1980). Effect of ATPase inhibitors on the proton pump of respiratory-deficient yeast. European Journal of Biochemistry **105**, 419-424.

SERRANO, R. (1983). In vivo glucose activation of the yeast plasma membrane ATPase. Federation of European Biochemical Societies Letters **156**, 11-14.

SIGLER, K. and KOTYK, A. (1976). Some properties of the adenosine triphosphatase systems of two yeast species, Saccharomyces cerevisiae and Rhodotorula glutinis. Molecular and Cellular Biochemistry **12**, 73-79.

SIGLER, K., KNOTKOVÁ, A. and KOTYK, A. (1981). Factors governing substrate-induced generation and extrusion of protons in the yeast Saccharomyces cerevisiae. Biochimica et biophysica acta **643**, 572-582.

SINGER, S.J. and NICOLSON, G.L. (1972). The fluid mosaic model of the structure of cell membranes. Science **175**, 720-731.

SLAVIK, J. (1982). Intracellular pH of yeast cells measured with fluorescent probes. Federation of European Biochemical Societies Letters **140**, 22-26.

SLAVIK, J. (1983). Intracellular pH topography: determination by a fluorescent probe. Federation of European Biochemical Societies Letters **156**, 227-230.

SLAVIK, J. and KOTYK, A. (1984). Intracellular pH distribution and transmembrane pH profile of yeast cells. Biochimica et biophysica acta **766**, 679-684.

SLAYMAN, C.L. (1965a). Electrical properties of Neurospora crassa. Effects of external cations on the intracellular potential. Journal of General Physiology **49**, 69-92.

SLAYMAN, C.L. (1965b). Electrical properties of Neurospora crassa. Respiration and the intracellular potential. Journal of General Physiology **49**, 93-116.

SLAYMAN, C.L. (1970). Movement of ions and electrogenesis in micro-organisms. American Zoologist **10**, 377-392.

SLAYMAN, C.L., LONG, W.S. and LU, C.Y.-H. (1973). The relationship between ATP and an electrogenic pump in the plasma membrane of Neurospora crassa. Journal of Membrane Biology **14**, 305-338.

SLAYMAN, C.W. and SLAYMAN, C.L. (1970). Potassium transport in Neurospora. Evidence for a multisite carrier at high pH. Journal of General Physiology **55**, 758-786.

STADTLANDER, K., RADE, S. and AHLERS, J. (1982). Influence of growth conditions on the composition of the plasma membrane from yeast and on kinetic properties of two membrane functions. Journal of Cellular Biochemistry **20**, 369-380.

STROOBANT, P. and SCARBOROUGH, G.A. (1979). Active transport of calcium in Neurospora plasma membrane vesicles. Proceedings of the National Academy of Sciences of the United States of America **76**, 3102-3106.

SULLIVAN, K.H., HEGEMAN, G.D. and CORDES, E.H. (1979). Alteration of the fatty-acid composition of Escherichia coli by growth in the presence of normal alcohols. Journal of Bacteriology **138**, 133-138.

SUOMALAINEN, H. and NURMINEN, T. (1970). The lipid composition of cell wall and plasma membrane of baker's yeast. Chemistry and Physics of Lipids **4**, 247-256.

SWINGS, J. and DE LEY, J. (1977). The biology of Zymomonas. Bacteriological Reviews **41**, 1-46.

SYCHROVÁ, H. and KOTYK, A. (1985). Conditions of activation of yeast plasma membrane ATPase. Federation of European Biochemical Societies Letters **183**, 21-24.

TAKESHIGE, K., HESS, B., BÖHM, M. and ZIMMERMANN-TELSCHOW, H. (1976). Mitochondrial adenosine triphosphatase from yeast, Saccharomyces cerevisiae. Purification, sub-unit structure and kinetics. Hoppe-Seylers Zeitschrift für Physiologische Chemie **357**, 1605-1622.

TANEJA, R. and KHULLER, G.K. (1980). Ethanol-induced alterations in phospholipids and fatty acids of Mycobacterium smegmatis ATCC 607. Federation of European Microbiological Societies Microbiology Letters **8**, 83-85.

TARASCHI, T.F., WU, A. and RUBIN, E. (1985). Phospholipid spin probes measure the effects of ethanol on the molecular order of liver microsomes. Biochemistry **24**, 7096-7101.

THOMAS, D.S. and ROSE, A.H. (1979). Inhibitory effect of ethanol on growth and solute accumulation by Saccharomyces cerevisiae as affected by plasma-membrane lipid composition. Archives of Microbiology **122**, 49-55.

THOMAS, D.S., HOSSACK, J.A. and ROSE, A.H. (1978). Plasma-membrane lipid composition and ethanol tolerance in Saccharomyces cerevisiae. Archives of Microbiology **117**, 239-245.

TRIVEDI, A., SINGHAL, G.S. and PRASAD, R. (1983). Effect of phosphatidylserine enrichment on amino acid transport in yeast. Biochimica et biophysica acta **729**, 85-89.

TRIVEDI, A., KHARE, S., SINGHAL, G.S. and PRASAD, R. (1982). Effect of phosphatidylcholine and phosphatidylethanolamine enrichment on the structure and function of yeast membrane. Biochimica et biophysica acta **692**, 202-209.

TROYER, J.R. (1955). Methanol tolerance of three strains of Saccharomyces cerevisiae Hansen. Ohio Journal of Science **55** 185-187.

TUDURI, P., NSO, E., DUFOUR, J.-P. and GOFFEAU, A. (1985). Decrease of the plasma membrane H^+-ATPase activity during late exponential growth of Saccharomyces cerevisiae. Biochemical and Biophysical Research Communications **133**, 917-922.

UCHIDA, K. (1975). Effects of cultural conditions on the cellular fatty acid composition of Lactobacillus heterohiochii, an alcoholophilic bacterium. Agricultural and Biological Chemistry **39**, 837-842.

VACATA, V., KOTYK, A. and SIGLER, K. (1981). Membrane potential in yeast cells measured by direct and indirect methods. Biochimica et biophysica acta **643**, 265-268.

VIGNE, P., FRELIN, C. and LAZDUNSKI, M. (1984). Intracellular pH measurements using the fluorescence of 9-aminoacridine. Federation of European Biochemical Societies Letters **172**, 275-278.

VILLALOBO, A. (1984). Energy-dependent H^+ and K^+ translocation by the reconstituted yeast plasma membrane ATPase. Canadian Journal of Biochemistry and Cell Biology **62**, 865-877.

VILLALOBO, A., BOUTRY, M. and GOFFEAU, A. (1981). Electrogenic proton translocation coupled to ATP hydrolysis by the plasma membrane Mg^{2+}-dependent ATPase of yeast in reconstituted proteoliposomes. Journal of Biological Chemistry **256**, 12081-12087.

VOLLHERBST-SCHNECK, K., SANDS, J.A. and MONTENECOURT, B.S. (1984). Effect of butanol on lipid composition and fluidity of Clostridium acetylbutylicum ATCC 824. Applied and Environmental Microbiology **47**, 193-194.

WADDELL, W.J. and BUTLER, T.C. (1959). Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxalolidinedione (DMO). Application to skeletal muscle of the dog. Journal of Clinical Investigation **38**, 720-729.

WALKER-CAPRIOGLIO, H.M., RODRIGUEZ, R.J. and PARKS, L.W. (1985). Recovery of Saccharomyces cerevisiae from ethanol-induced growth inhibition. Applied and Environmental Microbiology **50**, 685-689.

WARING, A.J., ROTTENBERG, H., OHNISHI, T. and RUBIN, E. (1981). Membranes and phospholipids of liver mitochondria from chronic alcoholic rats are resistant to membrane disordering by alcohol. Proceedings of the National Academy of Sciences

of the United States of America **78**, 2582-2586.

WICKERHAM, L.J. (1951). Taxonomy of Yeasts. I. Techniques of classification. United States Department of Agriculture Technical Bulletin no. 1029. Washington, D.C., U.S. Department of Agriculture.

WILLSKY, G.R. (1979). Characteristics of the plasma membrane Mg^{2+}-ATPase from the yeast Saccharomyces cerevisiae. Journal of Biological Chemistry **254**, 3326-3332.

WILSON, D.B. (1978). Cellular transport mechanisms. Annual Review of Biochemistry **47**, 933-965.

WOODWARD, J.R. and KORNBERG, H.L. (1980). Membrane proteins associated with amino acid transport by yeast (Saccharomyces cerevisiae). Biochemical Journal **192**, 659-664.

Journal of General Microbiology (1986), **132**, 369–377. *Printed in Great Britain*

Ethanol Dissipates the Proton-motive Force across the Plasma Membrane of *Saccharomyces cerevisiae*

By CHARLES P. CARTWRIGHT, JEAN-RÈGIS JUROSZEK,† MICHAEL J. BEAVAN,‡ FIONA M. S. RUBY,§ SONIA M. F. DE MORAIS‖ AND ANTHONY H. ROSE*

Zymology Laboratory, School of Biological Sciences, University of Bath, Bath BA2 7AY, Avon, UK

(*Received 9 July 1985; revised 4 September 1985*)

Populations of *Saccharomyces cerevisiae* NCYC 431, harvested after 16 h incubation from self-induced anaerobic cultures, were more tolerant to the inhibitory effect of ethanol on fermentation rate and viability than organisms harvested from 8 h cultures. Ethanol increased the rate of passive influx of protons into de-energized organisms at a rate which was greater with organisms from 8 h compared with 16 h cultures. Rates of passive influx of protons into spheroplasts were significantly greater than into intact organisms, although culture age did not affect rates of ethanol-induced influx of protons into spheroplasts. Ethanol retarded both the initial net rate of proton efflux and the final extent of acidification produced by suspensions of energized organisms, both effects being more pronounced with organisms from 8 h as compared with 16 h cultures. The magnitude of the proton-motive force (Δp) was decreased by ethanol in both energized and de-energized organisms. Although culture age did not affect the extent of ethanol-induced decrease in Δp in de-energized organisms, in energized organisms harvested from 8 h cultures ethanol produced a significantly greater decrease in Δp as compared with organisms from 16 h cultures. If the ability of ethanol to decrease the Δp value is important in its inhibitory effect on growth, it is suggested that some phenomenon other than proton uncoupling is involved.

INTRODUCTION

Although *Saccharomyces cerevisiae* is on the whole more tolerant of ethanol than other yeasts, concentrations of this alkanol approaching 2 M are inhibitory to even the most tolerant strains (Rose, 1980). Membranes, and in particular the plasma membrane, are considered to be prime targets for ethanol inhibition in *S. cerevisiae* (Ingram & Buttke, 1984). Evidence that plasma-membrane lipids are targets for the interaction of ethanol with *S. cerevisiae* came from Thomas and her colleagues (Thomas *et al.*, 1978; Thomas & Rose, 1979), who showed that the inhibitory effect of ethanol on viability of, and solute accumulation by, *S. cerevisiae* was less marked when membranes were enriched in linoleyl as compared with oleyl residues. Accumulation of amino acids (Thomas & Rose, 1979; Leão & van Uden, 1984*a*), ammonium ions (Leão & van Uden, 1983), maltose (Loureiro-Dias & Peinado, 1982) and glucose (Thomas & Rose, 1979; Leão & van Uden, 1982) is non-competitively inhibited by ethanol. With the exception of the glucose-transport system, which involves facilitated diffusion (Eddy, 1982), all of these transport

† Present address: École Nationale Supérieure de Biologie Appliquée à la Nutrition et l'Alimentation, Université de Dijon, Dijon, France.
‡ Present address: Weston Research Centre, Toronto, Ontario, Canada.
§ Present address: Beechams Limited, Roehampton, Sussex, UK.
‖ Present address: Laboratorios Joma Ltda, São Paulo, Brazil.

Abbreviations: TPP^+, tetraphenylphosphonium ion; Δp, proton-motive force.

systems are active and therefore probably require a proton-motive force (Δp; Borst-Pauwels, 1981; Serrano, 1977; Seaston *et al.*, 1976). One possibility therefore is that ethanol may affect the formation and maintenance of Δp across the plasma membrane. Evidence to support this notion has recently come from Leão & van Uden (1984*b*) who reported that ethanol and other alkanols accelerated passive re-entry of protons into *S. cerevisiae* in a manner resembling the action of a proton-uncoupling agent. The present paper describes dissipation by ethanol of the Δp across the plasma membrane of *S. cerevisiae*, the magnitude of the dissipation depending on the age of the batch culture from which organisms are harvested.

METHODS

Organisms. The two strains of *S. cerevisiae* used in this study, NCYC 431 (Beavan *et al.*, 1982) and Y185 (Calderbank *et al.*, 1984), were maintained on slopes of malt extract/yeast extract/glucose/mycological peptone (MYGP) medium (Wickerham, 1951) solidified with 2% (w/v) agar.

Experimental cultures. The majority of experiments were done with organisms from self-induced anaerobic cultures of *S. cerevisiae* NCYC 431 grown as described by Beavan *et al.* (1982). Growth was followed by measuring optical density at 600 nm, measurements being related to dry weight of organisms by a standard curve. Organisms were harvested from cultures by centrifugation (3500 *g*; 5 min; 4 °C) after approximately 8 h (0·28 mg dry wt ml^{-1}) or 16 h incubation (2·4 mg dry wt ml^{-1}), and washed twice with water. *S. cerevisiae* Y185 was grown under strict anaerobic conditions in a medium supplemented, as indicated in the text, with a sterol (5 mg l^{-1}) and an unsaturated fatty acid (30 mg l^{-1}) (Alterthum & Rose, 1973). Growth was followed as described previously. Organisms were harvested and washed as described for strain NCYC 431.

Preparation of spheroplasts. Harvested organisms were washed first in water, and then were washed once in buffered sorbitol (20 mM-Tris containing 10 mM-$MgCl_2$ and 1·2 M-sorbitol, adjusted to pH 7·2) and resuspended in the same solution to 10 mg dry wt ml^{-1}. After supplementation with Zymolyase 60 000 [0·1 mg (6 mg dry wt organisms)$^{-1}$], the suspension was incubated at 30 °C with reciprocal shaking (120 r.p.m.). After incubation for 1 h, a check that formation of spheroplasts was complete was made by diluting 0·1 ml portions of the suspension into 2·9 ml of either 1·2 M-sorbitol or water and measuring optical density at 600 nm. Spheroplasts were harvested by centrifugation (1500 *g* for 3 min) and resuspended in 1·2 M-sorbitol to 2·5–5·0 mg dry wt organisms equiv. ml^{-1}.

Measurements of rates of proton flux. To measure rates of passive influx of protons, a suspension (50 ml; 2·5–5·0 mg dry wt ml^{-1}) of organisms in water was placed in a 100 ml round-bottomed flask fitted with two ports (1·5 cm diam.), in addition to a Suba-seal port and a short gas-entry port (0·5 cm diam.). The flask was maintained in a water bath at 30 °C and the suspension stirred with a magnetic flea. Changes in pH value in the suspension were measured by two combination pH electrodes (type CMWL; Russell pH Ltd, Auchtermuchty, Fife, UK) inserted through ports. One was connected to a digital read-out pH meter and the other through a second pH meter to a single-pen high-sensitivjty recorder. Initially, the pH value of the suspension steadily rose. Organisms were then de-energized by supplementing the suspension with 2-deoxy-D-glucose (25 mM), a reagent which prevents glycolysis in yeast (Barnett & Sims, 1976; Lobo & Maitra, 1977). The pH value of the suspension continued to rise, but was lowered, after 5 min equilibration time, to 4·0 by addition of 0·1 M-HCl. Proton influx was then followed by measuring the rise in pH value of the suspension for a period of approx. 5 min. To measure the effect of ethanol on proton influx, ethanol (95%, v/v) was added to the suspension, after the pH had been lowered to 4·0, to give final concentrations of 0·5, 1·0, 1·5 or 2·0 M, and changes in extracellular pH value followed over a 5 min period. Rates of passive influx of protons are quoted as nequiv. H^+ (mg dry wt)$^{-1}$ min^{-1}. Rates of proton influx into spheroplasts of *S. cerevisiae* NCYC 431, suspended in 1·2 M-sorbitol, were measured as described for intact organisms except that the suspension was supplemented with ethanol to a maximum concentration of 1·0 M; supplementation with higher concentrations caused spheroplast lysis. After a proton influx rate had been measured on a suspension, the number of spheroplasts present was determined using a haemocytometer. Rates of passive influx of protons into spheroplasts are quoted as nequiv. H^+ (mg dry wt equiv.)$^{-1}$ min^{-1}. With organisms from 8 h cultures, 1 mg dry wt was equivalent to $3·0 \times 10^7$ organisms and with those from 16 h cultures $3·9 \times 10^7$ organisms. Passive influx of protons into *S. cerevisiae* Y185 was followed in a manner similar to that described for *S. cerevisiae* NCYC 431 except that the headspace in the flask was flushed continuously throughout the experiment with oxygen-free nitrogen gas (Alterthum & Rose, 1973) fed through the gas port.

Glucose-stimulated proton efflux from energized *S. cerevisiae* NCYC 431 was followed as already described for proton influx, except that 2-deoxy-D-glucose was not included and, after adjusting the suspension to pH 4·0, glucose (20 mM) was added before proton efflux was measured over a 5 min period. The effect of ethanol on glucose-induced proton efflux was measured by supplementing the suspension with 95% (v/v) ethanol after adjusting its pH value to 4·0, to give final concentrations of 0·5, 1·0, 1·5 or 2·0 M, before addition of glucose (20 mM) and then following proton flux over a 5 min period.

Measurement of intracellular pH values of organisms. Intracellular pH values of energized and de-energized organisms were calculated by determining the equilibrium distribution of a weak acid across the plasma membrane (Conway & Downey, 1950; Seaston *et al.*, 1976). A suspension of washed organisms (2·5–5·0 mg dry wt ml^{-1}) was incubated as already described for measuring rates of proton flux and, after the pH value had been adjusted to 4·0, incubation was continued for a further 5 min. A portion (1·8 ml) of the suspension was then removed, placed in a Bijou bottle and stirred at 30 °C. [2-^{14}C]Propionic acid [sodium salt; 0·1 mM; 0·5 μCi ml^{-1} (18·5 kBq ml^{-1}); 0·2 ml] was added to the suspension and, after 3 min equilibration, triplicate portions (0·3 ml) were rapidly removed and filtered through membrane filters (0·45 μm pore size; 25 mm diam.; Millipore). Organisms on filters were washed with 4 × 1 ml 0·1 mM-propionic acid (4 °C), the filters with organisms transferred to scintillation vials containing 7·5 ml Cocktail T (BDH) and the radioactivity of the contents measured in a LKB Rackbeta liquid scintillation spectrometer (model 1217). When measuring the effect of ethanol on the intracellular pH value of organisms, ethanol (95%, v/v) was added to the suspension after the pH value had been lowered to 4·0, to give a final ethanol concentration of 0·5, 1·0, 1·5 or 2·0 M. In experiments with energized organisms, glucose (20 mM) was incorporated in the suspension 30 s after addition of ethanol. Intracellular pH values were calculated from the expression derived by Waddell & Butler (1959):

$$pH_i = pK_i + \log\left[\boldsymbol{R}(10^{(pH_e - pK_e)} + 1) - 1\right]$$

where $\boldsymbol{R} = TA_i \cdot V_e / TA_e \cdot V_i$ and pH_i and pH_e are, respectively, the internal and external pH values, TA_i and TA_e the internal and external total amounts of propionic acid, V_i and V_e the intracellular and extracellular water volumes, and pK_i and pK_e the dissociation constants for propionic acid in the internal and external environments. Intracellular volumes were calculated from the dry weight of organisms used assuming that, for organisms from 8 h cultures, the cell volume was 1·93 μl (mg dry wt)$^{-1}$ and for organisms from 16 h cultures 1·61 μl (mg dry wt)$^{-1}$ (Beavan *et al.*, 1982). The internal and external dissociation constants for propionic acid were calculated from the Davies' simplified version of the Debye–Huckel equations (Davies, 1962), assuming that the ionic strength within organisms was in the region 0·15–0·25 (Conway & Downey, 1950). Values for pK_i and pK_e were calculated to be 4·86 and 4·75, respectively.

Measurement of the plasma-membrane potential ($\Delta\psi$) of organisms. This value was determined from the equilibrium distribution of tetra[^{3}H]phenylphosphonium (TPP^+) across the yeast plasma membrane (Hauer & Höfer, 1978). Suspensions of energized and de-energized organisms (2·5–5·0 mg dry wt ml^{-1}), prepared as already described, were incubated at pH 4·0 for 5 min. A portion (1·8 ml) was then supplemented with 0·2 ml TPP^+ solution [200 μM; 0·5 μCi ml^{-1} (18·5 kBq ml^{-1})] and, after equilibration for 25 min, triplicate samples (0·3 ml) were taken and rapidly filtered through prewashed (10 ml; 10 mM-TPP^+; 4 °C) membrane filters (0·45 μm pore size; 25 mm diam.; Millipore). Organisms on filters were washed four times with 2 ml portions of 10 μM-TPP^+ at 4 °C after which filters and organisms were transferred to scintillation vials containing 7·5 ml Optiphase Safe (Fisons) and radioactivity was measured in a liquid scintillation spectrometer (LKB Rackbeta, model 1217). When measuring the effect of ethanol on $\Delta\psi$, suspensions after adjustment to pH 4·0 were supplemented with 95% (v/v) ethanol to give the stated concentration. To determine the extent of TPP^+ binding to the yeast wall and plasma membrane, 2,4-DNP (1 mM; Eilam, 1984) was added at the same time as radioactive TPP^+ and the incubation and filtration procedures were done as already described. $\Delta\psi$ in mV was calculated using the equation:

$$\Delta\psi = -2{\cdot}3\,\frac{\boldsymbol{RT}}{F}\cdot\log\frac{[TPP^+]_{inside}}{[TPP^+]_{outside}}$$

Recent evidence suggests that under certain conditions a positive $\Delta\psi$ may exist across the yeast plasma membrane, indicated by accumulation of thiocyanate ions (SCN^-; Höfer & Künemund, 1984). Suspensions (50 ml; 2·5–5·0 mg ml^{-1}) of energized or de-energized organisms from 8 h or 16 h cultures were incubated for 5 min at pH 4·0 with 2·0 M-ethanol. A sample (9 ml) of the suspension was then removed and added to KS^{14}CN [1 ml; 50 μM; 2·5 μCi ml^{-1} (92·5 kBq ml^{-1})]. At 10 min intervals up to 50 min, 0·5 ml samples were removed, filtered through washed (5 mM-KSCN; 10 ml) membrane filters (25 mm diam.; 0·45 μm pore size; Millipore), washed with KSCN (4 × 2 ml; 5 μM) and the radioactivity of filters and organisms measured as described for TPP^+ accumulation. To indicate the extent of non-specific SCN^- binding, de-energized organisms were incubated in the absence of ethanol and filtered as already described. Since, under these conditions, *S. cerevisiae* NCYC 431 has a negative $\Delta\psi$, no SCN^- accumulation should occur.

Viability measurements. Viability of yeast populations was measured by staining with methylene blue (Fink & Kühles, 1933). A portion (0·5 ml) of culture was mixed with an equal volume of methylene blue solution (0·01%, in 2%, w/v, sodium citrate). After 5 min incubation at room temperature, the numbers of live and dead cells were established in a population of at least 500 organisms.

Measurement of fermentation rate. Harvested organisms were washed in citrate buffer (100 mM; pH 4·5) and suspended in the same buffer to 1·0 mg dry wt ml^{-1}. The fermentative activity of organisms was measured by

conventional Warburg manometry (Umbreit *et al.*, 1964). Each flask contained 1·5 ml citrate buffer (100 mM; pH 4·5) containing 300 mM-D-glucose and, where indicated, 1·0 M-ethanol. A suspension containing 1·0 mg dry wt organisms was placed in the side arm. The well contained 20% (w/v) KOH solution or water.

RESULTS

Effect of culture age on the response of organisms to ethanol

Ethanol (1·0 M) completely inhibited fermentation of glucose by organisms from 8 h cultures. The same concentration caused only a 12% decrease in the fermentative activity of organisms from 16 h cultures. Ethanol, up to 3·0 M, also caused a greater loss of viability in populations of organisms from 8 h cultures as compared with those from 16 h cultures (Table 1).

Effect of culture age and ethanol on proton fluxes

Ethanol, up to 2·0 M, accelerated passive influx of protons into de-energized organisms, as reported by Leão & van Uden (1984*b*). However, ethanol-induced acceleration of proton influx was greater with organisms from 8 h cultures compared with 16 h cultures (Fig. 1). With organisms from 8 h cultures, the ethanol-induced increase in rate of proton influx was 11·6 nequiv. H^+ (mg dry wt organisms)$^{-1}$ min^{-1} (ethanol concn; M)$^{-1}$, while with organisms from 16 h cultures the increase in rate was 6·8 nequiv. H^+ (mg dry wt organisms)$^{-1}$ min^{-1} (ethanol concn; M)$^{-1}$. Proton influx was considerably accelerated when spheroplasts were used instead of intact organisms, but the influx rate was only slightly greater with spheroplasts from organisms from 8 h compared with 16 h cultures (Fig. 2). The ethanol-induced increase in the rate of passive influx of protons into spheroplasts from organisms from 8 h cultures was 412·0 nequiv. H^+ (mg dry wt organisms equiv.)$^{-1}$ min^{-1} (ethanol concn; M)$^{-1}$, while that with spheroplasts from organisms from 16 h cultures was 365·8 nequiv. H^+ (mg dry wt equiv.)$^{-1}$ min^{-1} (ethanol concn; M)$^{-1}$.

The extent of extracellular acidification of suspensions of energized organisms was decreased in the presence of ethanol, less so with organisms from 16 h compared with 8 h cultures (Fig. 3). Ethanol-induced decrease in the initial net rate of proton efflux, calculated from the linear portions of the acidification curves (measured over the period approximately 0·5–2·0 min), was greater with organisms from 8 h compared with 16 h cultures. Comparable measurements were not possible with spheroplasts since their incubation in glucose-containing buffer resulted in lysis.

Effect of plasma-membrane lipid composition on ethanol-induced passive influx of protons into de-energized organisms

Ethanol, up to 1·5 M, caused a virtually identical acceleration of proton influx into de-energized *S. cerevisiae* Y185 with plasma membranes enriched in ergosterol and either oleyl or linoleyl residues. This strain of *S. cerevisiae* was used because it is known to incorporate into membranes high proportions of exogenously supplied sterol and unsaturated fatty acids when grown under anaerobic conditions (Calderbank *et al.*, 1984). Higher concentrations of ethanol

Table 1. *Effect of ethanol on the viability of suspensions of S. cerevisiae NCYC 431 harvested from 8 h and 16 h cultures*

Suspensions of washed organisms (2·5–5·0 mg dry wt ml^{-1}) in distilled water, supplemented with ethanol as indicated, were incubated for up to 60 min at 30 °C when portions (0·5 ml) were removed and viability measured as described in Methods.

Age of culture (h)	Viability (% of zero-time value, ±SE) Ethanol concn (M): 0·0	1·0	2·0	3·0
8	98·0 ± 2·0	94·4 ± 1·3	89·0 ± 3·5	81·8 ± 1·3
16	99·4 ± 1·3	97·7 ± 0·7	97·0 ± 1·6	92·4 ± 3·5

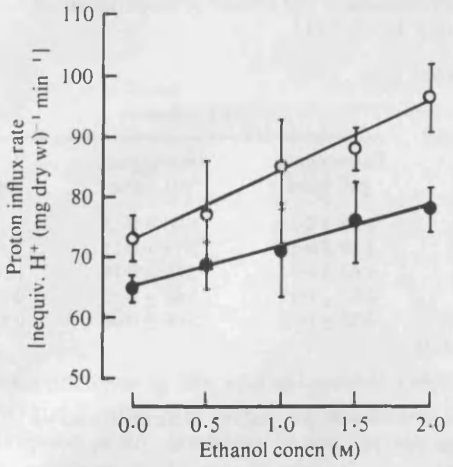


Fig. 1

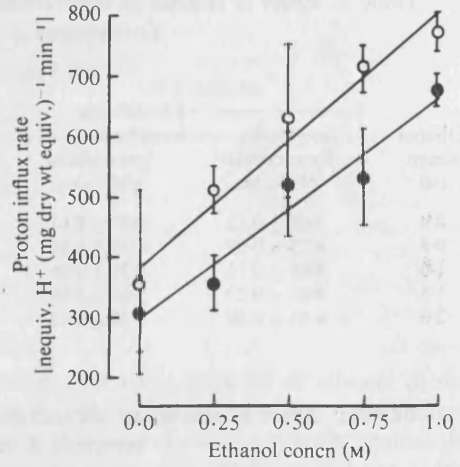


Fig. 2

Fig. 1. Effect of ethanol on influx of protons into de-energized *S. cerevisiae* NCYC 431 harvested from 8 h cultures (○) and 16 h cultures (●). Vertical bars indicate SE.

Fig. 2. Effect of ethanol on influx of protons into spheroplasts from de-energized *S. cerevisiae* NCYC 431 harvested from 8 h cultures (○) and 16 h cultures (●). Vertical bars indicate SE.

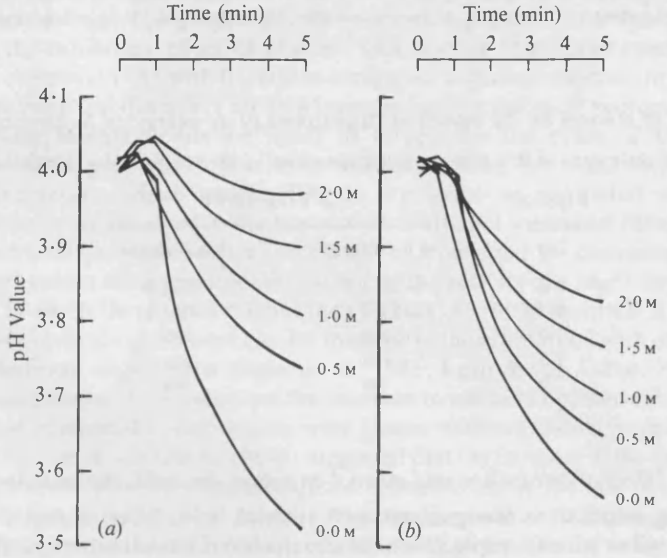


Fig. 3. Time-course of acidification of suspensions of energized *S. cerevisiae* NCYC 431 harvested from 8 h cultures (*a*) and 16 h cultures (*b*). The data are representative of those obtained in five separate experiments.

caused a decrease in viability in suspensions, particularly of organisms enriched in linoleyl residues. Values for rates of proton influx were similar to those obtained with *S. cerevisiae* NCYC 431 from 8 h self-induced anaerobic cultures (Fig. 1). Nor were differences detected in rates of passive influx of protons into *S. cerevisae* Y185 with plasma membranes enriched in oleyl residues and either ergosterol or cholesterol.

Table 2. *Effect of ethanol on intracellular and extracellular pH values of suspensions of de-energized S. cerevisiae NCYC 431*

pH Values are quoted ±SE.

Ethanol concn (M)	8 h cultures			16 h cultures		
	Extracellular pH value	Intracellular pH value	ΔpH	Extracellular pH value	Intracellular pH value	ΔpH
0·0	4·68 ± 0·12	5·85 ± 0·11	1·17	4·55 ± 0·24	5·90 ± 0·18	1·35
0·5	4·73 ± 0·09	5·75 ± 0·06	1·02	4·59 ± 0·16	5·79 ± 0·11	1·20
1·0	4·81 ± 0·15	5·71 ± 0·06	0·90	4·63 ± 0·31	5·69 ± 0·19	1·06
1·5	4·82 ± 0·22	5·63 ± 0·08	0·81	4·67 ± 0·11	5·65 ± 0·17	0·98
2·0	4·85 ± 0·19	5·56 ± 0·12	0·71	4·75 ± 0·20	5·60 ± 0·08	0·85

Table 3. *Effect of ethanol on intracellular and extracellular pH values of suspensions of energized S. cerevisiae NCYC 431*

pH Values are quoted ±SE.

Ethanol concn (M)	8 h cultures			16 h cultures		
	Extracellular pH value	Intracellular pH value	ΔpH	Extracellular pH value	Intracellular pH value	ΔpH
0·0	3·55 ± 0·16	6·36 ± 0·14	2·81	3·65 ± 0·22	6·52 ± 0·12	2·87
0·5	3·66 ± 0·23	6·31 ± 0·14	2·65	3·77 ± 0·30	6·50 ± 0·10	2·73
1·0	3·93 ± 0·18	6·22 ± 0·44	2·29	3·87 ± 0·19	6·44 ± 0·12	2·57
1·5	4·14 ± 0·11	6·10 ± 0·08	1·96	3·99 ± 0·30	6·37 ± 0·14	2·38
2·0	4·20 ± 0·34	5·97 ± 0·12	1·77	4·09 ± 0·27	6·37 ± 0·10	2·28

Table 4. *Effect of ethanol on $\Delta\psi$ values of suspensions of de-energized S. cerevisiae NCYC 431*

Values quoted are means of five separate determinations, ±SE. ND, $\Delta\psi$ value not detectable.

Ethanol concn (M)	$\Delta\psi$ Value (mV)	
	8 h cultures	16 h cultures
0·0	−44 ± 8	−53 ± 8
0·5	−19 ± 12	−41 ± 11
1·0	ND	ND
1·5	ND	ND
2·0	ND	ND

Effect of culture age and ethanol on values for ΔpH, $\Delta\psi$ and Δp

Supplementing suspensions of organisms with ethanol, up to 2·0 M, caused a decrease in the intracellular pH value which, coupled with the simultaneous increase in extracellular pH value, caused a decrease in ΔpH. With de-energized organisms, the decrease in ΔpH was approximately the same in organisms from 8 h and 16 h cultures (Table 2) but, with energized organisms, was proportionately greater with organisms from 8 h compared with 16 h cultures (Table 3). Ethanol supplementation also decreased the magnitude of $\Delta\psi$ in de-energized organisms, 1·0 M-ethanol being sufficient to abolish $\Delta\psi$ in organisms from either 8 h or 16 h cultures (Table 4). However, even in the presence of 2·0 M-ethanol, SCN^- ions were not accumulated. The effect of ethanol on values for ΔpH and $\Delta\psi$ lowered the values for Δp [$= -2{\cdot}3\,(RT/F)\cdot\Delta pH + \Delta\psi$; Table 5]. The ethanol-induced decrease in Δp was about the same for de-energized organisms from 8 h and 16 h cultures, but for energized organisms was greater for those from 8 h compared with 16 h cultures.

Table 5. *Effect of culture age and ethanol on the Δp values in de-energized and energized S. cerevisiae NCYC 431*

Ethanol concn (M)	Δp Value (mV)			
	8 h cultures		16 h cultures	
	De-energized	Energized	De-energized	Energized
0·0	−113	−165	−132	−169
0·5	−79	−155	−111	−161
1·0	−53	−134	−62	−151
1·5	−48	−115	−58	−140
2·0	−42	−104	−50	−134

DISCUSSION

An increase in the rate of passive influx of protons following addition of ethanol to de-energized organisms confirms the report by Leão & van Uden (1984*b*) that this alkanol dissipates proton gradients. However, it is worth noting that rates of passive influx of protons, in the presence or absence of ethanol, were greater than those reported by Leão and van Uden (1984*b*). The considerable increase in the rate of proton influx when spheroplasts rather than intact organisms were examined demonstrated the large proton-buffering capacity of the yeast wall. Since the rate of influx was very similar with spheroplasts from organisms from 8 h and 16 h cultures, it would appear that the different rates observed with intact organisms can be attributed to the greater capacity of the wall of organisms from 16 h cultures to buffer protons.

Leão & van Uden (1984*b*), when describing ethanol-induced passive influx of protons into *S. cerevisiae*, also reported on the effect of other alkanols. They showed that the exponential enhancement constants for different alkanols increased with the lipid solubility of the alkanol, and this led them to suggest that hydrophobic membrane regions, possibly lipid regions, are target sites for the inhibitory effect of ethanol. Our finding that enrichment of the plasma membrane in *S. cerevisiae* Y185 with linoleyl as compared with oleyl residues, or with cholesterol rather than ergosterol, did not affect ethanol-induced passive influx of protons into organisms does not eliminate plasma-membrane lipids as targets for the cause of this influx. It is conceivable that lipid properties other than unsaturation or the magnitude of the sterol–phospholipid interaction, which would differ in ergosterol- as compared with cholesterol-enriched membranes, are involved in the interaction of ethanol with yeast plasma membranes.

The rapid extracellular acidification produced by energized *S. cerevisiae* NCYC 431 is indicative of a net proton efflux, presumably caused by the activity of a Mg^{2+}-dependent proton-translocating ATPase in the plasma membrane (Willsky, 1979; Malpartida & Serrano, 1981). The acidification curves obtained were similar in shape to those obtained with other strains of *S. cerevisiae* in unbuffered suspensions (Sigler *et al.*, 1981; Leão & van Uden, 1984*b*). Both the increase in final extracellular pH value and the decrease in initial net proton efflux rate observed following addition of ethanol to suspensions were greater with organisms from 8 h as compared with 16 h cultures. Leão & van Uden (1984*b*) suggested that the increase in the final extracellular pH value was attributable to the ethanol-induced enhancement of the rate of passive influx of protons. It is possible, however, that other factors are involved in both the ethanol-mediated increase in the final extracellular pH value and the ability of the alcohol to decrease the initial net proton efflux rate. One obvious possibility is that ethanol inhibits the activity of the plasma-membrane ATPase thereby retarding proton extrusion, and this effect augmented by the ethanol-induced increase in passive influx of protons could contribute to the observed decrease in acidification. It was unfortunate that, because of the instability of spheroplasts in glucose-containing buffer, the importance of the cell wall under energized conditions could not be assessed.

With de-energized organisms, ethanol caused a progressive decrease in both components which contribute to the magnitude of Δp, i.e. ΔpH and $\Delta\psi$. This strongly suggests that the proton-uncoupling action of ethanol is alone sufficient to dissipate Δp. However, there was no

significant difference between de-energized organisms from 8 h and 16 h cultures with respect to the effect of ethanol on Δp, supporting the idea that the extent of proton uncoupling in organisms from 8 h and 16 h cultures is very similar. We conclude that, if maintenance of a Δp value is important for *S. cerevisiae* to tolerate ethanol, then the uncoupling effect of ethanol on the proton gradient is not the determining parameter.

Assuming that the extent of proton uncoupling induced by ethanol is the same for energized and de-energized organisms (Leão & van Uden, 1984*b*), then the notion that factors other than simple uncoupling are involved in the effect of ethanol on Δp is supported by data obtained with energized organisms. Our inability to measure a negative $\Delta\psi$ value under these conditions, i.e. with an extracellular pH value of 4·0 or below, is in agreement with previous reports (Hauer *et al.*, 1981). Höfer & Künemund (1984) showed that a positive $\Delta\psi$ value could exist in *Rhodotorula glutinis*. Our inability to demonstrate accumulation of SCN^- ions under conditions where a negative $\Delta\psi$ value could not be measured suggests that either the plasma membrane of *S. cerevisiae* is impermeable to these ions or a positive $\Delta\psi$ cannot exist in this organism. It is of interest that the intracellular pH value in energized organisms was significantly higher than the value for de-energized organisms, which suggests that the activity of the plasma-membrane ATPase may be involved in regulation of intracellular pH (Sanders *et al.*, 1981). Since the inhibitory effect of ethanol on Δp differed in energized organisms from 8 h compared with 16 h cultures, it is possible that ethanol has a greater inhibitory effect on the plasma-membrane ATPase of organisms from 8 h rather than 16 h cultures. Finally, there is the need to comment on the physiological significance to *S. cerevisiae* of an inability to maintain a constant Δp when energized and in the presence of ethanol. There is considerable evidence that a Δp across the plasma membrane is required for energization of solute transport into *S. cerevisiae* (Eddy, 1982). If 1·5 M-ethanol is introduced into cultures of *S. cerevisiae* NCYC 431 after 8 h incubation under self-induced anaerobic conditions, growth ceases immediately (Beavan *et al.*, 1982). We have demonstrated that this ethanol concentration is sufficient also to cause an appreciable decrease in Δp in energized organisms from 8 h cultures. It is conceivable, therefore, that this cessation of growth is a result of an inhibition of solute accumulation.

We are grateful to the Distillers Co. plc for financial support for this research, to Phillipe Thibault for timely assistance in the early stages of the work, and to Jill Calderbank for valuable help in writing the manuscript.

REFERENCES

ALTERTHUM, F. & ROSE, A. H. (1973). Osmotic lysis of spheroplasts from *Saccharomyces cerevisiae* grown anaerobically in media containing different unsaturated fatty acids. *Journal of General Microbiology* **77**, 371–382.

BARNETT, J. A. & SIMS, A. P. (1976). Some physiological observations on the uptake of D-glucose and 2-deoxy-D-glucose by starving and exponentially growing yeasts. *Archives of Microbiology* **111**, 185–192.

BEAVAN, M. J., CHARPENTIER, C. & ROSE, A. H. (1982). Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC 431. *Journal of General Microbiology* **128**, 1447–1455.

BORST-PAUWELS, G. W. F. H. (1981). Ion transport in yeast. *Biochimica et biophysica acta* **650**, 88–127.

CALDERBANK, J., KEENAN, M. H. J., ROSE, A. H. & HOLMAN, G. D. (1984). Accumulation of amino acids by *Saccharomyces cerevisiae* Y185 with phospholipids enriched in different fatty-acyl residues: a statistical analysis of data. *Journal of General Microbiology* **130**, 2817–2824.

CONWAY, E. J. & DOWNEY, M. (1950). pH values of the yeast cell. *Biochemical Journal* **47**, 355–360.

DAVIES, C. W. (1962). *Ion Association*, pp. 39–43. London & Boston: Butterworth.

EDDY, A. A. (1982). Mechanisms of solute transport into selected eukaryotic micro-organisms. *Advances in Microbial Physiology* **23**, 1–78.

EILAM, Y. (1984). Effects of phenothiazines on inhibition of plasma membrane ATPase and hyperpolarization of cell membranes in the yeast *Saccharomyces cerevisiae*. *Biochimica et biophysica acta* **769**, 601–610.

FINK, H. & KÜHLES, R. (1933). Beiträge zur Methylenblaufärburg der Hefezellen und Studien über die Permeabilitat der Hefezellmembran. II. Mitteilung. Eine Verbasserte Färbflüssigkeit zur Erkennung von totan Hefezellen. *Hoppe-Seyler's Zeitschrift für physiologische Chemie* **218**, 65–66.

HAUER, R. & HÖFER, M. (1978). Evidence for interactions between the energy-dependent transport of sugars and the membrane potential in the yeast *Rhodotorula gracilis* (*Rhodosporidium toruloides*). *Journal of Membrane Biology* **43**, 335–349.

HAUER, R., UHLEMANN, G., NEUMANN, J. & HÖFER, M. (1981). Proton pumps of the plasmalemma of the yeast *Rhodotorula gracilis*. Their coupling to fluxes of potassium and other ions. *Biochimica et biophysica acta* **649**, 680–690.

HÖFER, M. & KÜNEMUND, A. (1984). Tetraphenylphosphonium ion is a true indicator of negative plasma-membrane potential in the yeast *Rhodotorula glutinis*. *Biochemical Journal* **225**, 815–819.

INGRAM, L. O. & BUTTKE, T. M. (1984). Effects of alcohols on micro-organisms. *Advances in Microbial Physiology* **25**, 253–300.

LEÃO, C. & VAN UDEN, N. (1982). Effect of ethanol and other alkanols on the glucose transport system of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **24**, 2601–2604.

LEÃO, C. & VAN UDEN, N. (1983). Effect of ethanol and other alkanols on the ammonium transport system of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **25**, 2085–2090.

LEÃO, C. & VAN UDEN, N. (1984*a*). Effect of ethanol and other alkanols on the general amino acid permease of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **26**, 403–405.

LEÃO, C. & VAN UDEN, N. (1984*b*). Effects of ethanol and other alkanols on passive proton influx in the yeast *Saccharomyces cerevisiae*. *Biochimica et biophysica acta* **774**, 43–48.

LOBO, Z. & MAITRA, P. K. (1977). Resistance to 2-deoxyglucose in yeast. A direct selection of mutants lacking glucose-phosphorylating enzymes. *Molecular and General Genetics* **157**, 297–300.

LOUREIRO-DIAS, M. C. & PEINADO, J. M. (1982). Effect of ethanol and other alkanols on the maltose transport system of *Saccharomyces cerevisiae*. *Biotechnology Letters* **4**, 721–724.

MALPARTIDA, F. & SERRANO, R. (1981). Proton translocation catalysed by the purified yeast plasma membrane ATPase reconstituted in liposomes. *FEBS Letters* **131**, 351–354.

ROSE, A. H. (1980). Recent research on industrially important strains of *Saccharomyces cerevisiae*. In *Biology and Activity of Yeasts*, pp. 103–121. Edited by F. A. Skinner, S. M. Passmore & R. R. Davenport. London: Academic Press.

SANDERS, D., HANSEN, U.-P. & SLAYMAN, C. L. (1981). Role of the plasma membrane proton pump in pH regulation in non-animal cells. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 5903–5907.

SEASTON, A., CARR, C. & EDDY, A. A. (1976). The concentration of glycine by preparations of the yeast *Saccharomyces cerevisiae* depleted of adenosine triphosphate. *Biochemical Journal* **154**, 669–676.

SERRANO, R. (1977). Energy requirements for maltose transport in yeast. *European Journal of Biochemistry* **80**, 97–102.

SIGLER, K., KNOTKOVÁ, A. & KOTYK, A. (1981). Factors governing substrate-induced generation and extrusion of protons in the yeast *Saccharomyces cerevisiae*. *Biochimica et biophysica acta* **643**, 572–582.

THOMAS, D. S. & ROSE, A. H. (1979). Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition. *Archives of Microbiology* **122**, 49–55.

THOMAS, D. S., HOSSACK, J. A. & ROSE, A. H. (1978). Plasma-membrane lipid composition and ethanol tolerance in *Saccharomyces cerevisiae*. *Archives of Microbiology* **117**, 239–245.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1964). *Manometric Techniques*, 4th ed. Minneapolis: Burgess Publishing Company.

WADDELL, W. J. & BUTLER, T. C. (1959). Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO). Application to skeletal muscle of the dog. *Journal of Clinical Investigtion* **38**, 720–729.

WICKERHAM, L. J. (1951). Taxonomy of yeasts. I. Techniques of classification. *United States Department of Agriculture Technical Bulletin* no. 1029, Washington, DC: US Department of Agriculture.

WILLSKY, G. R. (1979). Characteristics of the plasma membrane Mg^{2+}-ATPase from the yeast *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **254**, 3326–3332.