Phospholipid composition of Saccharomyces cerevisiae and Zygosaccharomyces bailii and their response to sulphur dioxide

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PHOSPHOLIPID COMPOSITION OF *SACCHAROMYCES CEREVISIAE* AND *ZYGOSACCHAROMYCES BAILII* AND THEIR RESPONSE TO SULPHUR DIOXIDE

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SUMMARY

Sulphite inhibited growth of all four yeasts studied, Zygosaccharomyces bailii NCYC 563 being the most sensitive and Saccharomyces cerevisiae NCYC 431 the least. Vertical Woolf-Eadie plots were obtained for initial velocities of ^{35}S accumulation by all four yeasts suspended in high concentrations of sulphite. Equilibrium levels of ^{35}S accumulation were reached somewhat faster with strains of Sacch. cerevisiae than those with Zygosacch. bailii. With all four yeasts, the greater the extent of ^{35}S accumulation, the larger was the decline in internal pH value. Growth of Sacch. cerevisiae TC8 and Zygosacch. bailii NCYC 563, but to a lesser extent of Sacch. cerevisiae NCYC 431 and Zygosacch. bailii NCYC 1427, was inhibited when mid-exponential phase cultures were supplemented with 1.0 or 2.0 mM-sulphite, the decrease in growth being accompanied by a decline in ethanol and pyruvate production. Unless growth was completely inhibited, the sulphite-induced decline in growth was accompanied by production of acetaldehyde and additional glycerol.

Analyses were made of the total cellular phospholipids from all four yeasts grown aerobically. Fatty-acyl residues of $C_{16:1}$, $C_{18:1}$ and $C_{16:0}$ predominated in phospholipids from Sacch. cerevisiae, while phospholipids from Zygosacch. bailii contained mainly $C_{18:2}$, $C_{18:1}$ and $C_{16:0}$ residues. Strains of Sacch. cerevisiae were found to contain higher contents of phospholipid (mg dry wt organisms)$^{-1}$ compared with strains of Zygosacch. bailii but proportions of phospholipid classes were similar among each strain.

Phosphatidylcholine was the most common class of phospholipid followed by phosphatidylethanolamine and phosphatidylinositol with less than 10% as phosphatidylserine.

Saccharomyces cerevisiae NCYC 431 grown anaerobically in media supplemented with ergosterol and $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$ or $C_{20:1}$ fatty acids contained phospholipids enriched with residues of the exogenously provided acids, to a greater extent with shorter chain than longer chain acids. In these organisms direct correlation between mean fatty-acyl chain lengths and degree of unsaturation (expressed as Δmol^{-1} value) of cellular phospholipids indicated strict control of plasma-membrane synthesis and maintenance of the fluidity and rigidity necessary for normal plasma-membrane function. However, the proportions of each class of phospholipid were not affected significantly by the change in growth conditions. Plots of the permeability coefficient of SO_2 accumulation, derived from Woolf-Eadie plots, against the degree of unsaturation in phospholipids showed that the coefficient was greater the lower the degree of unsaturation in the phospholipids. There was no correlation between the mean fatty-acyl chain lengths and permeability coefficients of SO_2 accumulation in organisms but there was very good correlation between the coefficient and the ratio of mean fatty-acyl chain length and degree of unsaturation of cellular phospholipids. It is concluded that permeability of the yeast plasma membrane to SO_2 is proportional to the thickness and degree of fluidity of the plasma membrane.

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INTRODUCTION

SULPHUR DIOXIDE

Sulphiting agents in various forms have enjoyed a long history as food preservatives dating back to Roman times where wine vessels were apparently sanitised with sulphur dioxide (Roberts and McWeeny, 1972). One of the earliest reports of its use as a food preservative dates to at least 1664 where cider was added to flasks while they still contained sulphur dioxide (Evelyn, 1664). Although no human ailment or untoward effect resulting from such use has been recognised, concern over possible hazard goes back a considerable length of time to an article published by Kionka in 1896 on the possible toxicity of sulphites in foods.

Nowadays sulphiting agents are widely used in foods and beverages and applied in many chemical forms. The principal compound used to generate sulphur dioxide and the related anions in the preservation of foods and beverages is sodium metabisulphite ($Na_2S_2O_5$), designated additive E223 in Directives of the European Economic Community (Hanssen and Marsden, 1984). Other compounds frequently employed as sulphiting agents include gaseous sulphur dioxide (SO_2), potassium bisulphite ($KHSO_3$), potassium metabisulphite ($K_2S_2O_5$), sodium bisulphite ($NaHSO_3$) and sodium sulphite (Na_2SO_3). Their common characteristic is their ability to release free molecular sulphur dioxide and it is this fraction that is believed to be the active food preservative. The antimicrobial activity of each compound varies according to its ability to liberate sulphur dioxide and is expressed in terms of "sulphur

dioxide equivalents", i.e. stoicheiometric amounts of sulphur dioxide available from each sulphiting agent.

Sulphiting agents are very successful preservatives not only because of their antimicrobial properties. They are commonly used to stop enzymic and non-enzymic browning, to act as anti-oxidants and reducing agents, bleaching agents and general aids to food processing. They also fulfil the basic criteria of being water soluble, tasteless, odourless and generally recognised as non-toxic in low concentrations. However, in the interests of the consumer and manufacturers, more efficient and safer alternatives are being sought, but to date none has been found. Possible alternatives usually provide a narrower range of benefits, are often less effective and nearly always more expensive.

Properties of Sulphur Dioxide in Solution

The terminology in this field of research is sometimes confused and needs to be clarified. The terms sulphite, bisulphite and sulphur dioxide are often used interchangeably if not incorrectly. This area is made more complicated because sulphite can become bound to organic molecules so that it is necessary to specify exactly what fraction is being considered. In solution, metabisulphite generates sulphur dioxide, bisulphite and sulphite anions. The proportion of these species present depends on the pH value of the solution. The equilibria are:

$$\underset{\text{sulphur dioxide}}{SO_2} + H_2O \rightleftharpoons \underset{\text{sulphurous acid}}{(H_2SO_3)} \rightleftharpoons \underset{\text{bisulphite}}{HSO_3^-} + H^+ \rightleftharpoons \underset{\text{sulphite}}{SO_3^{2-}} + 2H^+$$

The existence of sulphurous acid is largely unaccepted since ultraviolet and infrared Raman spectroscopy have failed to reveal its presence. Falk and Guiguère (1958) suggested that, in the absence of stable sulphurous acid molecules in solution, SO_2 is dissolved in the molecular state and exists as $SO_2.H_2O$. Dissociation constants for each of the two remaining equilibria have been determined at low sulphite concentrations of the order of those used as food preservatives. The reaction leading to the ionisation of SO_2 has a pKa value of 1.77 at 25°C, while the value for the reaction leading to production of the sulphite ion under the same conditions is 7.20 (King et al., 1981). Using these pKa values, calculations have been made of the proportions of each species present in solution as a function of pH value (Table 1).

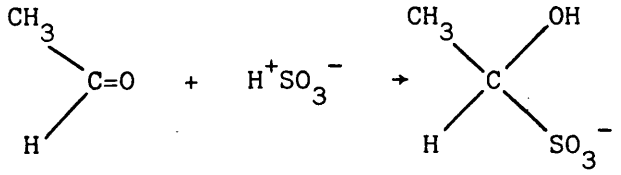
Table 1. Percentage distribution of molecular species of sulphur dioxide as a function of pH values. From King et al. (1981).

pH value	$SO_2.H_2O$	Percentage of HSO_3^-	SO_3^{2-}
2.0	37.03	62.97	0
3.0	5.56	94.43	0.006
4.0	0.59	99.35	0.063
5.0	0.058	99.31	0.63
6.0	0.006	94.15	5.84
7.0	0.0002	61.30	38.70

Although widely different values of pKa for SO_2 were found in the literature, the more recent publication by Wedzicha (1984) supports the values of King et al. (1981) with values of pKa 1.86 (Huss and Eckert, 1977) and pKa 7.18 (Betts and Voss, 1970), respectively. The antimicrobial activity of sulphiting agents increases inversely with pH value where proportionally more molecular SO_2 exists (Macris and Markakis, 1974). Sulphite, like other weak-acid preservatives e.g. benzoic and sorbic acids, exhibits the highest antimicrobial action with the undissociated form of the acid (Eklund, 1983). Ionised species show no significant antimicrobial activity (Ingram, 1959; Carr et al., 1976). From a practical viewpoint, the pKa value of sulphite defines the pH range over which it may be expected to be effective as an antimicrobial agent and this is why sulphur dioxide is the preservative of choice for foods and beverages of a low pH value (Sinskey, 1980).

Reactivity of Sulphur Dioxide

Analysis and control of sulphite residues in foods is made complicated by the rapid reactions between sulphiting agents and a variety of food components. All three species that are found in solutions of sulphite, especially the bisulphite ion, are chemically very reactive. Sulphites react readily with reducing sugars, compounds containing carbonyl groups and proteins to form sulphite addition compounds. Aqueous sulphur dioxide solutions react readily with aldehydes and more slowly with ketones to produce α-hydroxysulphonates (Joslyn and Braverman, 1954):



acetaldehyde sulphite α-hydroxysulphonate

Combination of sulphite with cyclic sugars is slower than with open-chain aldehydes. Ingram and Vas (1950) showed that galactose, mannose and arabinose quickly form addition compounds with sulphite; maltose, lactose and glucose are less active while sucrose and fructose are largely inactive. They prepared a 0.5% (w/v) solution of sodium sulphite containing 1.0% (w/v) citric acid monohydrate. Sugars (5% w/v) were added and allowed to stand at room temperature for 24 hours. After that time, the percentage of combined sulphite in each of the solutions were 88, 68, 63 and 20 for arabinose, mannose, galactose and glucose, respectively. The significant sulphite-binding capacity of glucose has encouraged experimenters to favour using fructose which has a minimal sulphite-binding capacity in physiological investigations (Warth, 1986).

Burroughs and Sparks (1973a) identified 11 different sulphite-binding compounds in cider, but the major portion (59-77%) of the bound SO_2 was attributed to complexes with just three of these, namely acetaldehyde, pyruvate and 2-oxoglutaric acid. The rate of formation of sulphite-binding adducts is dependent on the concentration of binding compound, pH value and temperature (Rehm, 1964; Burroughs and Sparks, 1973c).

In the presence of molecular oxygen sulphite will rapidly oxidise, the stoicheiometric equation for which is:

$$SO_3^{2-} + \tfrac{1}{2}O_2 \rightarrow SO_4^{2-}$$

Bisulphite, however, is much less easily oxidised by oxygen. Data for this reaction are thoroughly reviewed by Wedzicha (1984).

Another reaction of significance is that between bisulphite and disulphide bonds (Means and Feeney, 1971; Ough, 1983):

$$R\text{-}S\text{-}S\text{-}R + SO_3^{2-} \rightleftharpoons R\text{-}S\text{-}S\text{-}O_3^{-} + RS^{-}$$

The products of the reaction are thiosulphonates sometimes known as Bunte salts. Disulphide bonds lying between juxtaposed cysteine residues help to stabilize the tertiary structure of proteins essential for normal enzymic activity. This may be a clue in helping to understand sulphite's antimicrobial properties leading to conformational changes in proteins and causing loss of enzyme function.

A review by Ough (1983) reports on how thiamin pyrophosphate, a required enzymic cofactor in many reactions, can be destroyed by sulphite, and excess SO_2 can, by sulphitolysis of thiamin, destroy the nutritive value of thiamin potentially resulting in vitamin B_1 deficiency (Williams et al., 1935; Gunnison, 1981).

Interactions of sulphiting agents with nucleic acids causing mutagenesis have been reported (Hayatsu and Miura, 1970; Mukai et al., 1970; Shapiro et al., 1973). These and other interactions

with SO_2 are well documented in reviews by Hammond and Carr (1976) and Wedzicha (1984).

Sulphite-Binding Compounds

When sulphite is added as a preservative to fruit juices, wines and ciders etc., part of it combines more or less rapidly with various carbonyl compounds some of which will be present in the extracellular media, food or beverage, and some produced by contaminating organisms or fermentation yeasts. As it is largely accepted that the bound species have little or no antimicrobial activity, the bound preservative is effectively lost and in combination with auto-oxidation of sulphite, serves to lower dramatically the efficiency of sulphiting agents. Identification of such binding compounds is therefore of great practical and commercial interest when considering optimising the effect of SO_2.

Acetaldehyde has long been recognised as the major sulphite-binding compound in most wines with glucose generally having little effect, whereas some wines derived from grapes affected by mould growth have exceptionally high sulphite-binding power due to unidentified substances. Kielhöfer and Würdig (1960) designated the fraction of sulphite bound to compounds other than acetaldehyde or glucose as "Rest" or residual SO_2.

Burroughs and Sparks (1964a) identified and isolated three sugars, namely glucose, xylose and xylosone, responsible for binding most of the sulphite in uncontaminated fruit juice. In cider, the same compounds are accompanied by arabinose and galacturonic acid, derived from the degradation of pectin, and the

products of fermentation, namely acetaldehyde, pyruvate and 2-oxoglutarate. In the presence of spoilage organisms, the list of potential sulphite-binding compounds grows longer with more carbonyl compounds being produced. The very high sulphite-binding power of juices and ciders from damaged fruit has been traced to the combined activities of moulds and acetic-acid bacteria, chiefly Acetomonas species, resulting in high concentrations of sulphite-binding compounds including 5-fructulose, 2-oxogluconic and 2,5-di-oxogluconic acids (Burroughs and Sparks, 1962-1963). All of these observations emphasise the need to minimise the inclusion of potential binding compounds in products in order to maximise the efficiency of sulphiting agents. Burroughs and Sparks (1973a, 1973b) went on to identify and determine dissociation constants for a number of common carbonyl-bisulphite compounds in wines and ciders (Table 2).

Table 2. Apparent equilibrium constants of α-hydroxysulphonates. Adapted from Burroughs and Sparks (1973a)

Carbonyl compound	Concentration (mM) of Carbonyl compound	Total SO_2	Equilibrium constant at pH 3.0	pH 4.0
Acetaldehyde	6.0	4.0	$1.5.10^{-6}$	$1.4.10^{-6}$
2,5-Di-oxogluconic acid	2.0	0.6- 7.2	$4.5.10^{-4}$	$4.3.10^{-4}$
Galacturonic acid	10.0	8.0-20.0	$1.6.10^{-2}$	$2.1.10^{-2}$
2-Oxoglutaric acid	2.0	2.0-10.0	$4.9.10^{-4}$	$7.0.10^{-4}$
5-Fructulose	2.0	1.2- 7.5	$3.4.10^{-4}$	$3.3.10^{-4}$
Pyruvic acid	2.0	0.8- 5.0	$1.4.10^{-4}$	$2.2.10^{-4}$
L-Xylosone	2.0	2.0-10.0	$1.4.10^{-3}$	$1.4.10^{-3}$

Combination of sulphite with carbonyl compounds is reversible to a greater or lesser extent depending upon their respective equilibrium constants; products are therefore essentially buffered with respect to sulphite. Acetaldehyde has a very low dissociation constant and has a strong affinity for sulphite so that, even in the presence of low concentrations of sulphite, nearly all of the acetaldehyde becomes bound whereas other compounds bind progressively as sulphite concentrations increase.

Antimicrobial Activity of Sulphur Dioxide

Commercially sulphiting agents are used in more acidic foods and beverages to prevent the growth of (a) acetic acid-producing and malo-lactic bacteria, (b) fermentation and food-spoilage yeasts, (c) fruit moulds (Joslyn and Braverman, 1954). Sulphites are more effective in inhibiting bacterial and mould contamination than that caused by yeasts, species of which show a considerable range of tolerance to SO_2. The selective nature of SO_2 enhances its value in control of undesirable fermentation and contamination in wine making.

Free molecular SO_2 is the active form of the sulphiting agents in terms of antimicrobial action. Bound forms generally have minimal antimicrobial activity (Rehm, 1964). Molecular SO_2 is more than 1000 times as active as the bisulphite or sulphite ion against Escherichia coli, 500 times more effective against yeasts and 100 times more effective against Aspergillus niger (Rehm and Wittman, 1962). Reports of the antimicrobial properties of bound SO_2, reviewed by Beech and Thomas (1985), suggest that antimicrobial

activity attributed to bound SO_2 probably arises as the bound complex, e.g. pyruvate-sulphite, is metabolised releasing free SO_2, or simply by virtue of the dynamic equilibrium in existence between the bound and free species giving rise to SO_2. Stratford and Rose (1985) showed the former to be true. In Saccharomyces cerevisiae TC8 radiolabelled sulphite derived from a pyruvate-sulphite complex was taken up into organisms more quickly than pyruvate, strongly suggesting that dissociation of the complex takes place before its components are transported by organisms.

Application and Treatment Concentrations of Sulphiting Agents

Concentrations of sulphur dioxide used commercially vary greatly according to the products, ranging between zero and 3000 ppm (SO_2 equivalents) on a dry-weight basis. Dehydrated fruits, such as apples, apricots and peaches, are treated to contain the greatest amount in this range. Dehydrated vegetables and prepared soup mixtures range between a few hundred and 2000 ppm. A World-wide average for wines would be about 100-400 ppm with about 2-8 ppm in beers. It should be noted that concentrations of sulphites used in some products are self-limiting because of organoleptic considerations. Different treatment concentrations are required with various sulphiting agents to yield equivalent doses of active agent (Modderman, 1986). For comparative purposes it is helpful to calculate treatment concentrations on the basis of percentage theoretical yield of SO_2, e.g. for the sulphiting agents sulphur dioxide, sodium bisulphite, sodium metabisulphite, potassium metabisulphite and potassium bisulphite percentage

theoretical yields of SO_2 are 100, 61.56, 67.39, 57.60 and 53.32%, respectively (Green, 1976). It should be noted that these concentrations are rarely achieved and can only be used as a guide. Yields will be dependent upon the solubilities of each species and physical constraints put upon the equilibria by conditions such as temperature, pH value, pressure and, of course, the presence of sulphite-binding compounds.

Sulphite is naturally produced from sulphate during the fermentation process as an intermediate in the biosynthesis of the sulphur-containing amino acids cysteine and methionine in yeasts (Institute of Food Technologist's Expert Panel on Food Safety and Nutrition and the Committee on Public Information, 1975; Brewer and Fenton, 1980; Ough, 1983). Wurdig and Schlotter (1968) reported yeast strains capable of producing up to 130 ppm of SO_2 in fermentation broths.

One associated problem with sulphiting is that concentrations exceeding 50 ppm or 0.8 mM free SO_2 can impart undesirable flavours and odours to the product (Taylor et al., 1986). Since a large proportion of this can be generated by fermentation yeasts before sulphite addition, it is necessary to control sulphite levels (Garza-Ulloa, 1980; Warner et al., 1987). Both free and bound concentrations of SO_2 are measured throughout production and processing of foods, but the concentrations at the point of consumption can only be estimated since little is known of the effects of storage upon sulphites. Generally SO_2 concentrations fall during storage, and rapidly by auto-oxidation if exposed to air. Associated problems of measuring sulphite concentrations while

minimising loss of sulphur dioxide were recorded by Mason and Walsh (1928). Postgate (1963) later observed that a 0.1 M-sulphite solution in physiological saline shaken in air at 37°C fell to 0.07 M after one hour and to 0.022 M after 2.5 hours. Actual concentrations of free and total SO_2 remaining in a particular food product are dictated by the extent of absorption of the sulphites during treatment, the nature of the processing treatment following sulphite addition, and the conditions of storage (Schroeter, 1966).

The efficiency of sulphiting agents can be increased fairly simply and economically. For example, in the cider industry, it is essential to select a fermenting yeast that does not produce excessive amounts of sulphite-binding compound (Burroughs and Whiting, 1961) and is a poor sulphite producer (Eschenbruch and Bonish, 1976; Dott et al., 1976). Growth of bacteria with similar activities must be prevented. Acetaldehyde production by contaminating microflora can be minimised by using sound, clean fruit. Products where possible should be kept in anaerobic conditions and at a low pH value to minimise oxidation of sulphite and to maximise the concentration of active molecular SO_2. Improved factory hygiene and a rigid sanitation programme for the processing of equipment help to minimise the presence of potential sulphite-binding compounds. Sulphur dioxide treatment concentrations must be calculated to give optimal effect according to the pH value and content of sulphite-binding compounds (Beech et al., 1979).

Hazards of Using Sulphiting Agents

Recently the continued large-scale use of SO_2 has been brought

into question for more serious reasons. The Acceptable Daily Intake (ADI) for sulphites set by the Life Science Research Office in 1985 is 42 mg for a 60 kg person. It is estimated that the total intake of sulphites is about 10 mg per person every day although it is not known what proportion of this is in the free molecular form of SO_2. Sulphiting agents are categorised as being Generally Recognised as Safe (GRAS) provided they are not used in meats or other foods recognised as a dietary source of thiamin. However, this GRAS status is presently under review in the light of continuing reports of toxicity apparently caused by SO_2.

The relative toxicity of the free and bound forms of SO_2 is still not known but, by virtue of their relative stabilities, it is thought likely that free SO_2 poses the greater hazard. Numerous cases of sulphite-induced asthma attacks have been reported in medical literature since 1977 (Baker *et al.*, 1981; Bush *et al.*, 1986). Many of these cases were confirmed with positive challenges with capsules or solutions containing inorganic sulphite.

Free sulphite is metabolised principally by sulphite oxidase producing sulphate which is safely excreted in urine. Normally individuals have a tremendous capacity to metabolise sulphite. Profound sulphite oxidase deficiency has been recorded in a very few fatal cases and is characterised by increased urinary excretion of sulphite. Alarm at the widespread usage of SO_2 was heightened by suggestions of its mutagenic effects reported by Mukai *et al.* (1970) who reported mutagenesis of *E. coli* after exposure to sodium bisulphite, but there is no evidence of mutagenesis caused by sulphites in human cells.

Although asthmatic reactions continue to be the most common adverse reaction, individuals have also experienced urticaria, pruritis and swelling of the tongue, while oral challenges produced nausea, flushing and erythema sometimes causing hypertension and anaphylactic-like reactions (Green, 1976; Prenner and Stevens, 1976; Taylor et al., 1986).

Thankfully these rather alarming adverse reactions are relatively uncommon but are certainly undesirable. Pressure is being brought to bear upon manufacturers to lower the permitted levels of sulphite in their products. Unfortunately there is no suitable substitute for sulphiting agents as they have so many desirable properties, but the need for SO_2 can be decreased by minimising contamination, avoiding oxidation, using optimum sulphite concentrations and keeping the pH value as low as possible. Wherever possible formation of sulphite-binding compounds should be prevented and SO_2 conserved by packing products under anaerobic conditions. As Erik Millstone (1985) wrote "Risks which arise from the use of additives are borne almost entirely by the consumer" and he points out that additives are used by industry when their use serves the economic interest of industry. When put in this light it becomes obvious why we must regulate and monitor the use of additives and question the advantages and more importantly the disadvantages of their inclusion in our daily diet.

YEASTS AND FOOD SPOILAGE

Spoilage Yeasts

Products affected by food-spoilage yeasts are generally acidic (pH 2.5 - 4.5) and may contain high concentrations of sugars, ethanol or carbon dioxide. Such yeasts are not known to be toxic or produce serious off flavours, but spoil the product either by producing carbon dioxide causing distortion or explosion of packaging, or by giving a visible haze or sediment which are unacceptable in wines and clear drinks. A list of commonly isolated spoilage yeasts that contaminate preserved acid foods include: Zygosaccharomyces bailii, Zygosaccharomyces bisporus, Zygosaccharomyces rouxii, Pichia membranaefaciens, Candida krusei, Brettanomyces spp., Torulopsis spp. and Schizosaccharomyces pombe (Warth, 1986). Rehm and Wittman (1962) determined inactivation concentrations of SO_2 for a variety of yeast species finding strains of Saccharomyces and Zygosaccharomyces tolerant to concentrations of SO_2 ranging between 0.10 - 20.20 ppm and 7.2 - 8.7 ppm, respectively. Dott and Trüper (1978) found "killer yeasts" which were highly resistant to SO_2 and which, when grown in mixed cultures, cause death of other yeasts by producing sulphite.

Warth (1986) reviewed the relative sensitivities of a number of yeast strains to SO_2, benzoic acid and sorbic acid and found that, generally, a strain tends to be resistant to all three acid preservatives or none (Table 3). He suggested that all three preservatives may have a common mechanism of action. In a previous publication, Warth (1985) highlighted the considerable range of tolerances to sulphite among yeast strains. Kloeckera apiculata, a

yeast found in the early stages of spontaneous fermentation of grape musts (Kunkee and Goswell, 1977) and apple juices (Beech and Carr, 1977), is much more sensitive to sulphite than strains of Zygosacch. bailii which is generally regarded as a resistant strain.

Table 3. Maximum concentrations of preservative tested permitting anaerobic growth of yeasts at pH 3.5. Reproduced from Warth (1985).

Species	Sorbic acid (mM)	Benzoic acid (mM)	Free SO_2 (mM)
Kloekera apiculata	1	1.5	0.05
Saccharomyces cerevisiae 1297	1	0.7	< 0.14
Saccharomyces cerevisiae 1298	2	2	0.51
Candida krusei	3	3	0.48
Saccharomycodes ludwigii	3	3	2.2
Schizosaccharomyces pombe	4	4	1.9
Zygosaccharomyces bailii 2476	2	2	2.8
Zygosaccharomyces bailii 1292	4	4	2.6
Zygosaccharomyces bailii 2227	4	4	2.8

Spoilage yeasts were seen to tolerate a considerable range of concentrations of SO_2 (Balatsouras and Polymenacos, 1963) and Zygosacch. bailii consistently appears as a troublesome food spoiler (Pitt and Richardson, 1973; Rankine and Pilone, 1973; Thomas and Davenport, 1985).

Mechanisms of Action of Sulphur Dioxide on Yeasts

The mechanism of the antimicrobial action of SO_2 is known to be complex, with possible targets in the cell wall, plasma membrane and dispersed throughout the cytoplasm. As the susceptibility of any organism depends upon exposure of target sites to the preservative, it is essential to understand the kinetics of SO_2 transport into the cell. Sulphite may be taken up by an active or passive system which is believed to differ among micro-organisms. Any explanation must take into consideration the molecular composition, organisation and function of the plasma membrane since all of these factors are likely to influence solute transport.

Sulphur Dioxide Transport

Although there has been widespread study of sulphate transport in yeasts (Horák et al., 1981; Benítez et al., 1983; Garcia et al., 1983; Alonso et al., 1984), there is relatively little published material specifically related to sulphite or sulphur dioxide transport. McCready and Din (1974) were the first to propose an active transport system for sulphate in Sacch. cerevisiae which was confirmed in 1977 by Breton and Surdin-Kerjan who found a biphasic transport system involving two distinct permeases. However, the currently accepted mechanism of transport of sulphite into Sacch. cerevisiae and S'codes ludwigii is that of free diffusion of the molecular form of SO_2 (Stratford and Rose, 1986; Stratford et al., 1987) which conflicts with the active transport system previously proposed by Macris and Markakis (1974). Stratford and Rose (1986) presented strong evidence in favour of a protein not being involved

in SO_2 transport in the form of near vertical Woolf-Hofstee plots (referred to in this thesis as Woolf-Eadie plots) at pH 3.0 and 4.0 (Hofstee, 1959). Values for K_T calculated from kinetic plots of $\underline{v}$ against $\underline{v}/\underline{s}$ were 3.2 mM and 0.1 mM at pH 3.0 and 4.0, respectively, where $\underline{v}$ is the initial velocity of sulphite accumulation and s the extracellular SO_2 concentration. These K_T values are far in excess of the concentration of SO_2 required to kill Sacch. cerevisiae suggesting that passive transport predominates under these conditions. This evidence is supported by the inability of carbonylcyanide m-chlorophenylhydrozone (CCCP) and dinitrophenol (DNP) (Borst-Pauwels, 1981) to affect initial velocities of sulphite accumulation. These protonophores are known to dissipate the transmembraneous proton gradient (ΔpH) and to inhibit mediated transport systems. Further evidence for the lack of active transport of SO_2 came from the finding that exclusion of glucose from the reaction mixture had no effect on initial velocities of accumulation. Similarly, inability of the glycolytic inhibitor 2-deoxyglucose to affect SO_2 uptake adds fuel to the theory that energy is not required for SO_2 accumulation. Additional evidence is provided by the absence of an effect of pH value on the process, atypical of protein-mediated transport.

Macris and Markakis (1974) studied the kinetics of radiolabelled SO_2 uptake by Sacch. cerevisiae var. ellipsoideus making some valuable observations on SO_2 toxicity and pH dependence. There is a close correlation between accumulation of radiolabel from [^{35}S]sulphite, over the pH range 3.0 - 5.0, and concentration of SO_2 in solution, which is corroborated by Hinze

and Holzer (1985a) and Stratford and Rose (1986). Evidence strongly suggests that, over this pH range, only the molecular form of SO_2 passes into organisms and by inference that Sacch. cerevisiae do not transport sulphite (HSO_3^-). In these organisms, plasma membranes merely act as selective barriers to free diffusion of SO_2. For this reason, the relative structure and fluidity of the plasma membrane most probably affect solute transport, and further investigations are necessary in this area. This aspect will be covered more thoroughly in the following sections.

A slow transport system for HSO_3^- in Sacch. cerevisiae has been tentatively suggested which is evident in the presence of low concentrations of molecular SO_2 (Stratford and Rose, 1986). As sulphite concentrations are increased, this system rapidly becomes saturated and masked by diffusion of higher concentrations of molecular SO_2.

Intracellular Effects of Sulphur Dioxide

Saccharomyces cerevisiae and S'codes ludwigii accumulate SO_2 initially very rapidly reaching a plateau concentration after about five minutes exposure. Intracellular SO_2 concentrations at equilibrium are many times greater than in suspension (Stratford et al., 1987). This can be explained by the dynamic equilibrium between the three forms of sulphur dioxide, sulphite and bisulphite in solution and the presence of sulphite-binding compounds (Burroughs and Sparks, 1964a). Intracellular pH values in Sacch. cerevisiae lie in the region of pH 6.5 where only 0.0015% of free sulphite exists in the molecular form (King et al., 1981). If the

extracellular pH value is below pH 6.5, molecular SO_2 will accumulate and dissociate inside the cell until concentrations of SO_2 are equal on both sides of the plasma membrane resulting in acidification of the cytoplasm. It is conceivable that there may be leakage or active expulsion of anions from the cell resulting in a net flow of protons into the cell which will either equilibrate the cytoplasmic pH value with that of the medium or impose a heavy energy load on the cell in expelling protons. The extent of SO_2 accumulation must depend upon the intracellular pH value in the organisms, so differences in resistance between organisms may be attributed to differences in intracellular pH value or, by implication, their ability to maintain constant intracellular pH values (Sigler et al., 1981a, b; Salmond et al., 1984).

The antimicrobial activity of lipophilic acid food preservatives has been attributed to inhibition of transport mechanisms by lowering the ΔpH component of the proton-motive force (Freese et al., 1973). Salmond et al. (1984) studied the effect of weak acid preservatives on E. coli and concluded that, although accumulation of acid in the cells resulted in a decrease in the intracellular pH value, this was not the primary cause of growth inhibition. It was significant that these workers found the intracellular pH value of organisms was lowered to a greater extent by food preservatives than by weak acids with a similar pK value. They suggested that the inhibitory effect of unidentified metabolic functions by the undissociated acid had a synergistic effect with accumulation of the acid on intracellular pH values. It was suggested by Stratford et al. (1987) that the relative resistance

of S'codes ludwigii may at least be partially attributed to its increased capacity to produce sulphite-binding compounds, specifically acetaldehyde and to a lesser extent pyruvate, compared with Sacch. cerevisiae, and to its decreased capacity to accumulate SO_2. Stratford et al., (1987) also postulate that S'codes ludwigii, having a plasma membrane richer in $C_{18:1}$ phospholipid fatty-acyl residues compared with Sacch. cerevisiae, may have a more fluid membrane thereby facilitating diffusion of SO_2, a theory that will be discussed more fully later in this Introduction.

Sulphur Dioxide Targets

Sulphite will react with a wide variety of cell constituents as suggested earlier and, by implication, is likely to influence the cell at a number of target sites. Outside the cell, SO_2 binds with many compounds rendering them unavailable for yeast nutrition. Portnova (1978) demonstrated that an increase in the concentration of SO_2 added to grape must, from zero to 282 ppm, resulted in a decrease in the lipid content of yeasts, particularly in lipids containing unsaturated fatty-acyl residues. When the SO_2 concentration was increased from 192 to 282 ppm it also caused a decrease in the lipid content of wine particularly in the amount of unsaturated fatty acids present essential to the anaerobic growth of certain yeasts (Andreason and Stier, 1954).

Anacleto and van Uden (1982) proposed that SO_2 acts upon a yeast cell in three stages. Firstly, SO_2 binds to receptors on the cell surface. Next, membrane damage occurs due to a change in activity of the receptor-sulphur dioxide complex. Thirdly, the cell

loses viability. Two distinct receptors for SO_2 in Sacch. cerevisiae are proposed. One is the "sulphur dioxide death site", a membrane protein with a high affinity for SO_2 exposed to the outer surface of the plasma membrane. Combination of this protein with SO_2 causes a lowering of the free energy of activation of the denaturing process resulting in loss of viability. The second receptor is thought to modulate the entropy of activation of the "death site". These workers suggested that the first receptor may be the same target proposed by Schimz and Holzer (1979), and that the receptor was membrane-bound ATPase which, when bound to sulphur dioxide, hydrolyses intracellular ATP in an uncontrolled way, depleting intracellular ATP. However, Hinze and Holzer published data (1985b) showing how concentrations of SO_2 up to 0.5 - 5.0 mM lead to depletion of cellular ATP mainly as a result of inactivation of glyceraldehyde 3-phosphate dehydrogenase, an enzyme intimately involved in degradation of carbohydrates yielding ATP. At the same time a 10 to 100 fold increase in concentration of glyceraldehyde 3-phosphate over the concentration found in the absence of sulphite was observed. This gross depletion of ATP caused by sulphite is probably the major cause of cell death (Schimz, 1980). Prior to cell death, the rapid decrease in the cellular content of ATP was accompanied by an increase in the level of inorganic phosphate while the content of ADP remained reasonably constant (Schimz and Holzer, 1979; Schimz, 1980). Concentrations of other ribonucleoside di- and triphosphates in sulphite-treated cells showed parallel changes to ATP. In addition, Schimz and Holzer (1977) showed that low sulphite concentrations inhibited the

viability of yeast populations.

The extent of the damage imposed on organisms is dependent upon the concentration of sulphite, pH value, physiological condition, density and age of organisms, and on incubation time. If the yeast population was exposed to sulphite for less than one hour, the lethal effect could be prevented and depletion of cellular ATP was reversible. Cultures treated with a sub-lethal dose of SO_2 characteristically showed increased lag times, up to 600 h (Warth, 1985) but, when growth occurred, there was no decrease in growth rate or final yield. In 1986 Hinze and Holzer demonstrated that inhibition of ATP production by SO_2 is confined to inhibition of substrate-level phosphorylation at the level of glyceraldehyde 3-phosphate dehydrogenase and not respiratory-chain phosphorylation. This was confirmed by revealing the same rate of ATP decrease in respiratory-deficient mutants (pet 936), which lack mitochondrial F_1ATPase, as in the wild-type strain of Sacch. cerevisiae X2180. However, in vitro experiments with purified ATPase from yeast mitochondria revealed a sensitivity of this enzyme to sulphite (Maier et al., 1986). Maier et al. (1986) therefore propose that sulphite acts both on glycolysis and on respiratory-chain phosphorylation. Both oxygen consumption and the ATP content of glucose-starved yeast were drastically lowered by sulphite during incubation at pH 3.6. Sulphite may impair respiration by reacting with flavoproteins; for example, cytochrome b_2 (l-lactate dehydrogenase) is known to be competitively inhibited by sulphite (Lederer, 1978).

It is possible that these critical targets in organisms may

vary in their sensitivity to SO_2, or simply that the physical exclusion of SO_2, brought about by variable rates of SO_2 uptake or the mopping up of free SO_2 by binding compounds, will impart a relative degree of resistance to an organism.

In addition to inactivation of glyceraldehyde 3-phosphate dehydrogenase, formation of an acetaldehyde-bisulphite complex with glyceraldehyde 3-phosphate, which slows down the rate of the dehydrogenation by lowering substrate concentration, may also contribute to depletion of ATP. Sulphite also binds glucose and dihydroxyacetone phosphate thereby inhibiting operation of the Embden-Meyerhof-Parnas pathway (Beech and Thomas, 1985). Any activity of the TCA cycle is also decreased since sulphite binds oxaloacetate and glutaric acid, and this may account for the drop in oxygen consumption by sulphited cells (Rehm, 1964). Nicotinamide adenine dinucleotide itself reacts with SO_2 (Johnson and Smith, 1976; Tuazon and Johnson, 1977), and Rehm (1964) has shown that NAD^+-dependent steps of glycolysis in Sacch. cerevisiae were strongly inhibited by sulphite. As a result of sulphite-induced depletion of the intracellular ATP pool and inhibition of ATP production, many ATP-dependent processes are halted, e.g. the sulphite permease (Kleinzeller et al., 1959) and ATP sulphurylase (de Vito and Dreyfuss, 1964).

Intracellular effects are not confined to inhibition of metabolic pathways. Structural damage may also occur due to distortion of structural proteins or peroxidation of membrane lipids (Utsumi et al., 1973).

Stratford (1983) examined the effect of sulphite on initial velocities of accumulation of the amino acids arginine and lysine and of glucose by Sacch. cerevisiae NCYC 366. Accumulation of both amino acids was inhibited after addition of sulphite (0.5 mM) to a cell suspension containing the amino acid (1 - 10 mM), glucose (100 mM) and organisms (0.5 mg dry wt ml^{-1}), but sulphite did not affect the rate of accumulation of glucose. It was concluded that sulphite had caused a dissipation of the proton-motive force that is created across the plasma membrane, thereby inhibiting active transport of solutes. Alternatively, sulphite might cause denaturation of transport proteins exposed on the outer surface of the plasma membrane.

Stimulation of Production of Sulphite-Binding Compounds

The SO_2 resistance of spoilage yeasts has partly been attributed to the variable ability of yeasts to produce sulphite-binding compounds, particularly acetaldehyde, that bind sulphite to form α-hydroxysulphonates. This is especially so when strains are grown in the presence of sulphite (Rankine, 1968; Weeks, 1969), so rendering free SO_2 ineffective (Rankine and Pocock, 1969; Stratford et al., 1987). This ability of SO_2 to stimulate acetaldehyde production has long been recognised as Neuberg's second form of yeast fermentation (Neuberg and Reinfurth, 1918, 1919) resulting in net accumulation of glycerol, compared with Neuberg's first form of fermentation which leads to production of ethanol. Freeman and Donald (1957) summarised Neuberg's second form of fermentation as follows:

$$C_6H_{12}O_6 + NaHSO_3 \rightarrow CH_3CHO.NaHSO_3 + C_3H_8O_3 + CO_2$$

Glucose Bisulphite Acetaldehyde-bisulphite Glycerol

During the course of a normal fermentation NADH, formed during oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid, is re-oxidized when acetaldehyde is reduced to ethanol. In the presence of sulphiting agents, acetaldehyde becomes bound and can no longer serve as the hydrogen acceptor for NADH. Under these conditions, dihydroxyacetone phosphate becomes a substitute hydrogen acceptor for NADH resulting in formation of glycerol 3-phosphate and subsequent accumulation of glycerol (Nord and Weiss, 1958). The steering action of sulphite has been exploited in production of glycerol, notably during World War I where approximately 1,000 tons of glycerol per month were manufactured by the "sulphite process" (Lawrie, 1928). The process was comprehensively reviewed in following years (Prescott and Dunn, 1949; Underkofler, 1954), but there are very little data available in recent publications. Yields of glycerol were found to depend on concentration and type of carbohydrate substrate, concentration of sulphite, yeast strain and size of inocula, surface volume ratio, pH value and temperature (Lees, 1944; Wright *et al.*, 1957; Kalle and Naik, 1985).

Although acetaldehyde is recognised as the primary sulphite-binding compound, pyruvic acid and 2-oxoglutaric acid are known to have significant binding capacities (Rankine and Pocock, 1969; Weeks, 1969). During the fermentation of three grape juices by eight yeasts (*Sacch.* spp.), these constituents resulted in 49 - 83%

of measured sulphite being bound. The maximum range of concentrations of the binding components for individual wines were 10 - 48 ppm for acetaldehyde, 9 - 77 ppm for pyruvic acid and 5 - 63 ppm for 2-oxoglutaric acid, depending on the yeast strain and nature of the grape juice. The amount of acetaldehyde produced was directly related to the total SO_2 present, and both of these factors were related to the strain of yeast used. When a subsequent addition of SO_2 was made after fermentation was complete, the amount bound depended largely on the concentrations of pyruvic and 2-oxoglutaric acids present (Rankine and Pocock, 1969).

It is not clear from these investigations whether production of pyruvate and 2-oxoglutarate is actively stimulated by SO_2. Weeks (1969) reports that pyruvate concentrations are increased in the presence of SO_2, and this has been corroborated more recently by Stratford *et al.* (1987) who recorded production of pyruvate by *Sacch. cerevisiae* TC8 reaching 20 - 40% of the concentration of acetaldehyde in the presence of sulphite. In cultures of *S'codes ludwigii*, however, there were negligible concentrations of pyruvate.

Resistance to Sulphur Dioxide

Tolerance of yeasts to sulphur dioxide falls into two categories, namely inherent tolerance and inducible tolerance. Inherent tolerance of strains like *Zygosacch. bailii* and *S'codes ludwigii* (Ingram, 1960; Reed and Peppler, 1973) is genetically determined (Zambonelli *et al.*, 1972) and transmitted to subsequent generations even under sulphite-free conditions. Opinions vary

regarding the ability of yeasts to acquire SO_2 resistance. Beech and Thomas (1985) showed that a resistant strain of Zygosacch. bailii, if left to acclimatise for 14 days in media containing 3 mg molecular SO_2 l^{-1}, eventually grew even though the concentration of SO_2 when growth occurred exceeded that normally expected to prevent growth. These workers postulated that the organisms had acquired resistance.

The nature of inherent SO_2 resistance may be a reflection of different target sites in different species, for example, in the conformation of the "sulphur death site" receptor or in the rate of uptake of SO_2. In addition, yeasts can detoxify SO_2. Sulphite reductase, which has been detected in yeasts, converts SO_2 to sulphide (Wainwright, 1967) and has an integral role in sulphate metabolism in yeasts and may be involved in SO_2 resistance. Intracellularly, sulphate is converted to adenosine 5'-phosphosulphate which is then converted to the high-energy intermediate 3'-phosphoadenosine 5'-phosphosulphate (PAPS; Robbins and Lipman, 1958); PAPS is then reduced to sulphite which is finally reduced by sulphite reductase to sulphide (Yoshimoto and Sato, 1968a, b, 1970; Prabhakararao and Nicholas, 1969, 1970).

Warth (1977) proposed that the resistance of Zygosacch. bailii to acid preservatives, including sorbic and benzoic acids and SO_2, was primarily from the activity of an inducible energy-requiring pump that transports preservative molecules out of the cell. This explained the enhanced resistance of organisms grown at high concentrations of glucose (Pitt, 1974) in terms of the high energy demands of this resistance mechanism. Support for this theory is

lacking as a mechanism of resistance, because of inability to demonstrate a specific pump and considering the insurmountable task of ejecting the rapidly penetrating acid (Macris, 1975; Cole and Keenan, 1987). Cole and Keenan (1987) investigated the effect of benzoic acid on Zygosacch. bailii NCYC 563 and propose that, by decreasing the protoplast volume and concentrating cellular components, the buffering capacity of organisms may be increased. At the same time, these organisms were able to increase acid efflux either by proton extrusion directly through the plasma membrane ATPase (Peters and Borst-Pauwels, 1979; Serrano, 1980) or by excreting organic acids produced during normal metabolism that do not rapidly re-enter cells (Sigler et al., 1981b; Opekarová and Sigler, 1982).

YEAST PLASMA MEMBRANE: COMPOSITION AND FUNCTION

The yeast plasma membrane has several important functions. Firstly, it acts as a protective barrier enabling the maintenance of a constant internal environment inside the cell. Secondly, by selectively controlling the passage of solutes and metabolites, it allows interaction with the extracellular medium. Finally it serves as an organelle on which enzymic reactions leading to synthesis of wall components may occur.

In general yeast plasma membranes contain, in terms of dry weight, approximately 40% lipid and 60% protein held together by non-covalent interactions. The proportions tend to vary between reports and organisms, largely because of differences in experimental technique (Rank and Robertson, 1983). Some

carbohydrate is also usually present covalently linked to lipid or protein and in the hydrated state, comprising approximately 20% water which is tightly bound and essential for maintenance of structural integrity (Harrison and Lunt, 1980).

Data related to the composition of the plasma membrane in Sacch. cerevisiae are limited and those related to the organelle in Zygosacch. bailii are even more scarce. Detailed analyses of plasma membranes of a strain of Sacch. cerevisiae were first obtained by Longley et al. (1968). The membranes were obtained by osmotic lysis of yeast spheroplasts, and the analyses confirmed in 1971 by Hunter and Rose. About 50% of the dry weight of the membrane was accounted for by protein and approximately 40 - 45% by lipid (Boulton, 1965; Longley et al., 1968; Schibeci et al., 1973), with the remainder probably being carbohydrate.

Although proteins comprise a significant proportion of the plasma membrane in yeasts they have not been fully characterised to date. Perhaps the most extensive contribution to analysis of plasma-membrane proteins of Sacch. cerevisiae has been made by Santos and his colleagues (Santos et al., 1978, 1982). They detected 25 polypeptides and 12 glycoproteins with molecular weights between 10,000 and 300,000 when proteins isolated from plasma membrane of Sacch. cerevisiae were analysed by one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). High molecular-weight proteins were predominant. A similar diversity of polypeptides was observed by Schneider et al. (1979) who isolated 17 - 19 predominantly high molecular-weight proteins from plasma-membrane preparations of

Candida tropicalis. Some individual yeast plasma-membrane proteins have been studied. An example is the general amino-acid permease (GAP) which catalyses the uptake of a wide variety of amino-acids in Sacch. cerevisiae (Woodward and Kornberg, 1980).

The lipid fraction, which is fairly well characterised in the plasma membrane of Sacch. cerevisiae, can be divided into two main classes, namely polar and neutral lipids. Polar lipids in eukaryotic micro-organisms are principally amphipathic glycero-phospholipids, glycolipids and free sterols; neutral lipids comprise triacylglycerols and sterol esters. There are considerable discrepancies in the published literature concerning the relative proportions of each lipid class present in the plasma membranes of Sacch. cerevisiae. Kramer et al. (1978) reported that plasma-membrane phospholipids of Sacch. cerevisiae comprised only 5 - 6% of the total cellular lipid compared to Kaneko et al. (1976) who found that phospholipids constitute over 50% of the total cellular lipid of Sacch. cerevisiae infering a high plasma-membrane phospholipid content. Arnold (1981) surmised that the low values obtained by Kaneko et al. (1976) and Schneider et al. (1979) were artefactual arising from enzymic degradation of phospholipids by non-specific lipase and phospholipases, since both groups of workers, in a similar study on C. tropicalis, reported an abnormally high content of free fatty acids in their plasma-membrane preparations. Rattray (1988), in a general review, reports that cellular phospholipids in 18 different strains of Sacch. cerevisiae contribute between 17 and 66% of the total lipid fraction. This compared with the one strain of Zygosacch. bailii

reported in which the total lipid fraction comprised approximately 15% phospholipid (Malkhas'Yan et al., 1983). Nurminen et al. (1976) reported that Sacch. cerevisiae, grown under glucose-repressed conditions, had over 80% of the total cellular phospholipid and sterol in the plasma-membrane fraction.

Rank and Robertson (1983) reported the relative proportions of lipid classes in yeast plasma-membrane vesicles that were aggregated to remove non-plasma-membrane vesicles. They contained 45% phospholipids, 21% free fatty acids, 16% sterols, 8% sterol esters, 5% tri-acylglycerols and 5% di-acylglycerols, compared with non-aggregated vesicles containing 9% phospholipids, 67% free fatty acids, 20% sterols and minor quantities of tri- and di-acylglycerols and sterol esters. The high concentrations of free fatty acids were again attributed to lipase activity, while phospholipase activity was thought to result in lowering measurable concentrations of phospholipid by the formation of glycerophosphorylcholine from phosphatidylcholine.

With improved purification techniques it seems likely that further studies will show that phospholipids and free sterols constitute the major portion of plasma-membrane lipid in Sacch. cerevisiae, as is the case in plasma membranes derived from other eukaryotic organisms (Harrison and Lunt, 1980). Neutral sterol esters and triacylglycerols usually account for most of the remaining plasma-membrane lipid with minor quantities of free fatty acid and mono- and di-acylglycerols (Rattray, 1988).

Glycerolphospholipid 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. Both the chain length and degree of unsaturation vary in the hydrophobic tail region. Aerobically-grown Sacch. cerevisiae was found to contain $C_{16:1}$ and $C_{18:1}$ residues constituting between 70 and 80% of the total fatty-acyl residues present in plasma-membrane preparations (Longley et al., 1968; Schneider et al., 1979). Cartwright (1986) and Cartwright et al. (1987) found that the relative proportions of fatty-acyl residues within the plasma-membrane phospholipids of Sacch. cerevisiae were similar to those reported by Beavan et al. (1982) for whole-cell phospholipids. The phospholipids from Zygosacch. bailii characteristically contain predominantly $C_{18:1}$ and $C_{18:2}$ fatty-acyl residues which constitute approximately 75% of total phospholipids (Viljoen et al., 1986).

The composition of the hydrophilic head group is also variable, but shows a similar composition in most yeasts. Chemical structures of the four major classes of phospholipid found in yeast are shown in Figure 1. Generally phosphatidylcholine (PC) and phosphatidyl-ethanolamine (PE) predominate comprising between 20 and 50%, and 15 and 40% of total cellular phospholipids respectively, with 10 to 15% phosphatidylinositol (PI) and 5 to 15% phosphatidylserine (PS) (Longley et al., 1968; Rank et al., 1978; Rattray, 1988).

The presence of other minor classes of phospholipid (less than 20% of total phospholipids) has been reported including phosphatidylmonomethylethanolamine (PMME), phosphatidyl-

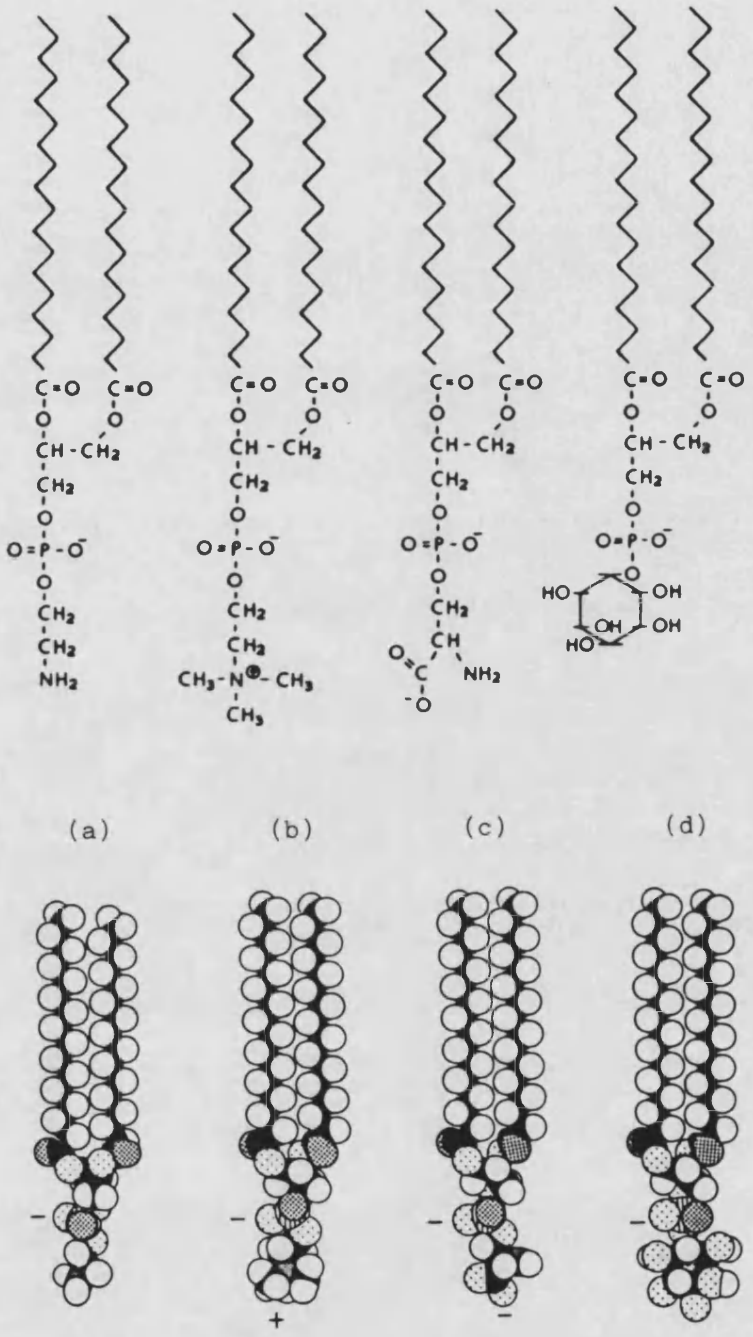
dimethylethanolamine (PDME), phosphatidic acid (PA), lysophosphatidylethanolamine (LPE), diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) (Letters, 1966; Getz et al., 1970; Steiner and Lester, 1972b). It is, however, generally accepted that many of these minor components arise by uncontrolled action of phospholipases during lipid extraction (Ratledge and Evans, 1987). Henry (1982) found the proportions of phospholipid classes found in plasma membranes mirror those found in the whole cell.

Although the relative proportions of phospholipid classes is relatively constant in yeasts, it is significant that phosphatidylserine and phosphatidylinositol are conspicuous by their lack of unsaturated fatty-acyl residues compared to the other phospholipids (Rattray et al., 1975; Watson and Rose, 1980).

Sterols have a fused cyclopentanoperhydrophenathrene ring system forming a rigid backbone with eight to ten carbon atoms in a side chain at C-17 and a hydroxyl head group at C-3. The hydroxyl group represents the polar moiety while the non-polar side chain and steroid skeleton constitute the hydrophobic region of the molecule. Ergosterol is the major sterol component of yeast plasma membranes (Nurminen et al., 1975) and of whole cells (Dulaney et al., 1954; Nes et al., 1978) representing 0.03 to 4.6% of yeasts on a total dry weight basis (El-Refai and El-Kady, 1968). The second most common sterol is the precursor of ergosterol, 24(28)-dehydroergosterol, found by Longley et al. (1968) to appear in roughly equal proportions to ergosterol in Sacch. cerevisiae NCYC 366. Small amounts of zymosterol have also been found in many yeasts (Dulaney et al., 1954; Hossack et al., 1977a; Marriot, 1975).

Figure 1. Chemical structures and space-filling atomic models of (a) phosphatidylethanolamine, (b) phosphatidylcholine, (c) phosphatidylserine and (d) phosphatidylinositol. Carbon atoms are indicated in black, hydrogen atoms in white, oxygen atoms are dotted, double dotted with double bonds, nitrogen atoms are also dotted and phosphorus atoms are striped.

Figure 1.



The lipid composition of yeasts is very sensitive to changes in the extracellular environment (Hunter and Rose, 1971; Rattray et al., 1975). Both physical and chemical factors are important including growth rate, composition of medium, temperature and dissolved oxygen tension (Jollow et al., 1968; Hunter and Rose, 1972; Prasad, 1985). Oxygen has a pronounced effect on the growth, general metabolism and lipid composition of yeasts resulting in specific changes in plasma-membrane composition. This finding has been exploited in a technique developed by Alterthum and Rose (1973). Andreasen and Stier (1953, 1954) discovered that Sacch. cerevisiae has a nutritional requirement for a sterol and an unsaturated fatty acid when grown anaerobically. These compounds cannot be synthesised anaerobically because the fatty acid desaturase enzyme and enzymes involved in the conversion of squalene to ergosterol require molecular oxygen. Other quantitatively minor anaerobically-induced requirements such as nicotinic acid (Suomalainen et al., 1965) are usually supplied by low concentrations of yeast extract (Alterthum and Rose, 1973). Although it is generally believed that the requirements for an unsaturated fatty acid are fairly non-specific (Light et al., 1962) there is evidence that the same is not true for sterols (Nes et al., 1976, 1978; Pinto and Nes, 1983). These workers were able to show that, by comparing pairs of sterols differing in only one component, each structural feature of ergosterol appeared to have some functional significance in the yeast, and the ability of different sterols to support anaerobic growth is not simply an all or nothing phenomenon as had previously been implied (Proudlock

et al., 1968; Hossack and Rose, 1976). The natural yeast sterol, ergosterol, was the most capable of supporting anaerobic growth.

This anaerobic auxotrophy has been exploited by many workers to change the lipid composition of the plasma membrane, particularly the degree of fatty-acyl saturation, to probe basic relationships between composition and function in plasma membranes from Sacch. cerevisiae (Thomas et al., 1978; Thomas and Rose, 1979; Keenan et al., 1982; Calderbank et al., 1984, 1985). The supplemented fatty acid has been shown to account for between 50 and 69% of the residues within the phospholipids, and between 47 and 92% of those in triacylglycerols (Watson and Rose, 1980), depending on the unsaturated fatty acid supplement and strain of Sacch. cerevisiae.

The proportions of phospholipid classes can also be affected by specific supplements (Hossack et al., 1977b). Under aerobic conditions, low concentrations of choline in a chemically defined growth medium induced Sacch cerevisiae to synthesise a greater proportion of phosphatidylcholine resulting in a three-fold increase in this phospholipid (Waechter et al., 1969; Waechter and Lester, 1971). Similarly, phosphatidylethanolamine synthesis could be increased two-fold with the inclusion of ethanolamine in the growth medium (Ratcliffe et al., 1973). Buttke et al. (1982), however, found they were able to modulate the fatty acid composition of phosphatidylethanolamine independently of the other phospholipids in a mutant strain of Sacch. cerevisiae by exploiting the preference to incorporate unsaturated fatty acids into phosphatidylethanolamine. The phospholipid fatty-acyl composition could also be altered in response to different sterols (Wieslander

et al., 1981; Buttke et al., 1982). Mutant strains have also been employed to explore the relationship between membrane fluidity, composition and cell growth (Barber and Lands, 1973; Holub and Lands, 1975; Esfahani et al., 1981a).

Structure of the Plasma Membrane

Danielli and Davson (1935) were among the first to propose a realistic model describing membrane structure and composition. They envisaged a phospholipid bilayer held together by van der Waals forces with the polar head groups aligning on the outer surfaces and the hydrophobic tails of the lipid molecules sandwiched inside the membrane. Proteins were thought to be spread on the surface of the polar head groups, but, at that stage, their role was not understood. Subsequently, additional information was building up about the roles of proteins, and it gradually became clear that proteins are partially or completely embedded on each side of the membrane. This led to the development of more flexible model systems, including the lipoprotein sub-unit model (Lucy and Glauert, 1964), the mosaic model (Lenard and Singer, 1966) and culminated in the suggestion of Singer and Nicolson (1972). Today the Singer and Nicolson (1972) model is regarded as a grossly simplistic and inadequate model but still forms the basis of modern membrane models. It describes a bilayer consisting of oriented lipid molecules similar to the Davson model in which two types of protein are embedded. Firstly, extrinsic proteins, like cytochrome c that are water soluble but function when bound to the membrane surface, are loosely attached to lipid headgroups or other membrane

proteins by ionic or hydrogen bonds; secondly, intrinsic amphipathic globular proteins which are tightly bound and incorporated to various degrees into the fluid lipid bilayer. The essential features of this model are that membranes can exhibit an asymmetric distribution of proteins and lipids, and that lipids in the bilayer exist predominantly in a fluid state. This makes some provision for lateral and rotational movements of lipids and proteins, so that selective exchange of hydrophilic compounds can occur, and from a thermodynamic point of view maximising hydrophobic and hydrophilic interactions. However, the model has subsequently been criticised as it leaves the impression that the only function of membrane lipids is to provide a hospitable environment of proper fluidity and makes no provision for lipid-lipid, protein-lipid (Chapman et al., 1979) and protein-protein interactions which may be important in influencing membrane fluidity and intrinsic protein conformation (Boggs, 1980).

The presence of intrinsic proteins has been shown to affect the conformation of neighbouring lipids (Jost et al., 1973) and the effects of this perturbation usually extend beyond the first boundary lipids but thereafter diminishes (Chapman et al., 1982). The fluid mosaic model also envisaged an entirely fluid lipid matrix where all lipids exist above their transition temperatures. The transition temperature (ΔT) is that which causes hydrocarbon chains to pass from a closely packed ordered crystalline (or gel) state to a disordered liquid-crystalline configuration which is accompanied by an abrupt rise in heat absorption. It is apparent that, for each pure phospholipid, the transition occurs at

characteristic temperatures (T_t). This temperature increases with chain length of the fatty-acyl group in the phospholipid (Michaelson et al., 1974) and with the degree of unsaturation of the fatty-acyl chain. For a phosphatidylcholine bearing two saturated C_{12} chains, T_t is 1.8°C; one with two saturated C_{18} chains has a T_t value of 54.9°C. Similarly with a cis double bond in each chain of the C_{18} chain phospholipid, T_t is lowered to -22°C (Overath and Thilo, 1978). The nature of the phospholipid head group is also important. Phosphatidylcholine has a bulky trimethylammonium terminal head group. If the choline head group is replaced by ethanolamine, which will pack much more closely and in a less fluid conformation, T_t is raised by 26°C (Stein, 1986).

It is generally accepted that the degree of saturation of phospholipids affects the fluidity of membranes. Indeed, this has been supported by experimental data. Membranes rich in saturated fatty-acyl groups are measurably less fluid than those containing proportionally fewer saturated fatty-acyl residues (Yau et al., 1976).

Since each lipid in the bilayer has its own specific transition temperature and the plasma membrane contains a diversity of phospholipids, it is most likely that some will be in a fluid state while others will be in a less mobile rigid formation. Experimental evidence supports this theory. Phospholipid membrane bilayers are not universally fluid, but exist in distinct domains of lipid which are either predominantly in gel or liquid-crystalline form (Israelachvili, 1978; Karnovsky et al., 1982). Indeed, it is most likely that phospholipids are distributed asymmetrically between

the inner and outer surfaces of a membrane although it has yet to be demonstrated in the yeast plasma membrane. Israelachvili (1973) working with artificial membranes comprising phosphatidylglycerol and phosphatidylcholine proposes that the asymmetry reduces electrostatic repulsion between negatively charged phosphatidyl-glycerol molecules when they are concentrated in the outer layer of a curved membrane, and that distribution is affected by the physical shape of the membrane in agreement with data from Michaelson et al. (1973).

Intrinsic proteins are also likely to influence lipid domains as they generally partition into the fluid regions (Cullis and de Kruijff, 1979) and, by influencing lipid-lipid interactions, will affect the fluidity of the lipid bilayer (Esfahani et al., 1981b). Rank et al. (1978) demonstrated the regulating effect of intrinsic proteins on membrane fluidity of plasma membranes isolated from Sacch. cerevisiae. A low molecular-weight protein was found to be associated only in high viscosity plasma-membrane vesicles which were separated from low viscosity vesicles. It was proposed that the protein probably spans only highly viscous domains in the membrane.

Another flaw in the fluid mosaic model is the absence of sterols which are known to contribute to the stability of membranes. Generally they tend to mobilise lipids in the gel state and condense those in the liquid-crystalline state (Finkelstein and Cass, 1967; Demel and de Kruijff, 1976). Sterols have relatively minute head groups compared to phospholipids, these being hydroxyl groups attached to a bulky and rigid ringed portion. The hydroxyl

head group orientates itself on the surface of the membrane and the rigid portion wedges into the hydrophobic region, so that sterols tend to interact specifically with the fatty-acyl chain region of phospholipids with minimal interaction with neighbouring phospholipid headgroups.

The phospholipid head group plays an important role in the packing arrangement and function (Trivedi et al., 1982) of membranes and, like any molecule, will be aligned in its stable conformation. They show a preference towards a highly folded structure with strong intramolecular hydrogen bonds (Pullman and Berthod, 1974). It is also believed that the nature of the polar head group affects the packing of hydrocarbon chains in the body of the membrane. Dipalmitoylphosphatidylcholine (DPPC) will tilt by approximately 30° relative to the normal to the plane of a simple bilayer, whereas hydrocarbon chains of dipalmitoylphosphatidyl-ethanolamine (DPPE) appear to orientate approximately normal to the plane of the bilayer (McIntosh, 1980) because of the size and conformation of the phosphatidylcholine head group (Nagle, 1976).

Plasma Membrane Composition and Diffusion

A considerable amount of literature is concerned with the distribution and packing arrangement of phospholipids in both natural and artificial membranes, but there is little available data on yeasts.

Stratford et al. (1987) suggested that the fluidity of the plasma-membrane lipids may affect the rate of SO_2 uptake arguing that S'codes ludwigii, being richer in unsaturated phospholipid

fatty-acyl residues, will have a more permeable plasma membrane than Sacch. cerevisiae. Konttinen and Suomalainen (1977) found that Sacch. cerevisiae enriched with oleic acid did show increased permeability to pyruvate compared with cells with more saturated membranes, and they presumed this was because of increased mobility of the fatty-acyl groups. Thomas et al. (1978) use a similar argument in discussing the permeability of yeast plasma-membranes to ethanol although this paper was later criticized by Jones and Greenfield (1987). These workers suggest that membrane fluidity cannot be assumed from the relative saturation of membrane phospholipids and that these data in isolation are not reliable. Indeed, this view is supported by Konttinen and Suomalainen (1977) who saw only a 20% increase in passive diffusion of pyruvate with a five-fold increase in membrane unsaturation in Sacch. cerevisiae.

It is reasonable to assume that carbon chain length and the degree of saturation of fatty-acyl residues will affect the geometry of the plasma membrane, but the relative importance of these factors is unknown. With the current understanding of membrane structure and function, if the geometry and by inference the fluidity of the plasma-membrane are altered, then presumably the diffusion of molecules across that membrane will also be influenced. Jones and Greenfield (1987) propose that the relative proportions of the different phospholipids have a considerable influence upon packing of phospholipids in the membrane because of the distinctive configuration of the head groups. The alignment of phospholipid head groups is dependent upon their respective size and charge (Michaelson et al., 1974; Israelachvili et al., 1980;

Stein, 1986). Sterols are also likely to contribute to the packing geometry of the plasma membrane. Experimental data have shown that cholesterol is far more efficient in lowering passive permeability of phospholipid bilayers than is lanosterol (Yeagle, 1985). Thomas et al. (1978) showed that the ability of cells to remain viable in the presence of ethanol shows a marked dependence upon sterol structure, demonstrating that sterols may regulate membrane fluidity.

A number of theories have been proposed to account for the diffusion of small molecules across membranes, and these are comprehensively reviewed by Lee (1975) and Sha'afi (1981). A most useful model appears to be that in which the small diffusing molecule is assumed to dissolve in the bilayer and move across by diffusion (Zwolinski et al., 1949) where the rate of diffusion is a function of the solubility of the diffusing molecule in the lipid bilayer. This is in agreement with "Overton's Rule" (Overton, 1899) which states that the permeability coefficient of a molecule passing through a lipid bilayer correlates with its oil/water partition coefficient. However some very small molecules, e.g. water, formamide and formic acid, permeate lipid bilayer membranes faster than predicted by Overton's Rule (Cohen, 1975; Finkelstein, 1976; Walter and Gutknecht, 1984).

Possible explanations for this behaviour include the "mobile kink" hypothesis where the bilayer is considered to be a slab of hydrocarbon with transient holes or pockets which open up as the hydrocarbon chains rotate about saturated C-C bonds (Lieb and Stein, 1969; Trauble, 1971). Molecules diffuse across the bilayer

by first diffusing into free volumes in the hydrocarbon region provided by "kinks" in the chains. Then it is proposed that thermal fluctuation of the hydrocarbon chains serves to carry diffusing molecules in mobile free volumes across the hydrocarbon phase as kinks move in waves along the chains. Walter and Gutknecht (1986), however, have criticised Trauble's mobile kink mechanism since it does not account for diffusion of larger molecules which tend to show less size dependence than smaller molecules. Fettiplace and Haydon (1980) have also pointed out that the degree of disorder in most bilayers is greater than that assumed in Trauble's model.

Later work (Galey et al., 1973) has shown that there are two barriers to membrane permeation. One is provided by the water-membrane interface and one by the membrane interior. However, the latter is generally regarded as the rate-limiting step. A more attractive model envisaged by Lee et al. (1974) shows small molecules first passing through a transient pore into the fluid part of the hydrocarbon centre and then diffusing through this region in a pocket of free volume. Another possible explanation for the high permeabilities of very small molecules is that "transient aqueous pores" exist in lipid bilayers (Weaver et al., 1984) but this was also rejected by Walter and Gutknecht (1986) because it did not account for the high permeabilities of the smallest molecules.

Walter and Gutknecht (1986) considered the anomalously high permeability coefficients of very small molecules ($M_r < 50$) and found that their permeabilities did not correlate with partition coefficients but were inversely correlated with molecular volumes.

Finkelstein (1976) suggested that size dependency of smaller molecules could be explained by the Stokes-Einstein model for diffusion in a liquid where the diffusion coefficient D is described by:

$$D = kT/(6\pi nr)$$

where r represents the radius of a sphere diffusing in a continuous fluid, k is the Boltzmann constant, T is the absolute temperature, n is the coefficient of viscosity and $6\pi nr$ is the factor describing the frictional drag on a sphere moving through a viscous fluid. However, in the diffusion of molecules across lipid bilayers the rate of diffusion decreases in value very steeply with molecular size and does not obey simple Stokesian fluid-dynamics. The molecular volume dependence of solute permeability suggests that the membrane barrier behaves more like a polymer network than a liquid hydrocarbon. Lieb and Stein (1986) propose that the non-Stokesian movement may be due to the inability of molecules in the membrane to flow around the diffusing molecules, presumably because the hydrocarbon chains are anchored at the membrane water interface. In ideal Stokesian diffusion, membrane lipids would flow freely around the diffusing molecules. Walter and Gutknecht (1986) conclude that only the soft polymer model successfully describes the non-Stokesian diffusion of non-electrolytes. This idea is consistent with the "solubility-diffusion" model, applicable to polymers, which describes diffusion within the hydrocarbon chain region and is represented by the expression:

$$P_{mem} = \frac{K_{mem} D_{mem}}{d_{mem}}$$

where P_{mem} is the permeability coefficient, K_{mem} and D_{mem} are the average partition and diffusion coefficients for the solute in a membrane interior, and d_{mem} is the membrane thickness (Diamond and Katz, 1974). This model takes into account both the hydrophobicity dependence and the molecular volume dependence of non-electrolyte permeability. In keeping with the polymer model, Lieb and Stein (1986), explain non-Stokesian diffusion in terms of free volume or holes between which diffusing molecules jump. Since a suitable hole must have a volume greater than or equal to the diffusing molecule, and since there will always be more small holes than large holes, it follows that small molecules will diffuse much more rapidly than larger ones.

It is assumed that there is a strong correlation between the permeability of a membrane to non-electrolytes and the membrane fluidity, and that permeability is a function of the packing of lipid molecules in the bilayer. Van Zoelen et al. (1978) employed this correlation to estimate membrane fluidity. The maximum number of water molecules than can copermeate with thiourea is a function of packing of the lipids in the bilayer. These workers found that, in multilamellar liposomes containing 4% phosphatidic acid in 20 mM-glucose, the maximum number of molecules (N_{max}) of water that can copermeate with each molecule of solute is dependent on the packing properties of the lipids and the size of cavities in the

bilayer. When cholesterol is included in the membrane, the value of N_{max} is lowered because closer packing of lipids in the presence of cholesterol results in a decrease in the concentration of cavities in the bilayer and lower freedom of motion for the fatty-acyl chains resulting in lower permeability of the bilayers (Bittman and Blau, 1972). This effect has been observed in many other systems including membranes of Acholeplasma laidlawii B (McElhaney et al., 1973).

Some work on natural membranes includes work by Beguinot et al. (1987) using rat thyroid cells. They found a decreased membrane fluidity caused by an absolute increase in membrane cholesterol with an increased cholesterol/phospholipid ratio and an increased ratio of saturated to unsaturated fatty-acyl residues in membrane phospholipids. There is a similar correlation with temperature. The rate of water permeation through lipid bilayers is sharply lowered below the transition temperature (Blok et al., 1976) because of the decrease in cavity size, and permeability is increased when the bilayer is rich in unsaturated phospholipids because of the increase in cavity size.

McElhaney et al. (1973) were able to show similar results in membrane lipids of A. laidlawii B cells and synthesised liposomes (de Gier et al., 1968). These workers also considered the permeability to non-electrolytes and found a marked dependency on chemical structure and chain length of fatty-acyl residues incorporated into lipid membranes. The incorporation of branched-chain or unsaturated fatty acids, or fatty acids with short chain lengths, increased membrane fluidity caused either by

interference with hydrocarbon chain packing or by decreasing chain length both of which lead to increased non-electrolyte permeability.

Other workers (Singh et al., 1978), who were concerned with the effect of altered lipid composition on active transport systems in Candida albicans and Sacch. cerevisiae (Keenan and Rose, 1979), found that the activity of specific amino-acid carrier systems could be influenced by the phospholipid and sterol content of cells. Uratani et al. (1987), working on the leucine transport system of Pseudomonas aeruginosa, found that the mean fatty-acyl chain length of membrane phospholipids was important, and suggest that there exists an optimal bilayer thickness for maximal carrier activity intimating a close relationship between structure and function.

The precise nature of diffusion of molecules in lipid bilayers still needs clarification but it is certain that the specific lipid structures in a membrane will affect the fluidity of a membrane and will also affect diffusion of molecules across the membrane.

The two major aims of this project are firstly to investigate the nature of SO_2 resistance in food-spoilage yeasts and to try to improve our understanding of the mechanisms of this resistance; secondly, to explain the differential rates of diffusion of SO_2 into strains of Sacch. cerevisiae and Zygosacch. bailii with respect to plasma-membrane composition.

METHODS

ORGANISMS

The yeasts used were Saccharomyces cerevisiae NCYC 431, Saccharomyces cerevisiae TC8 (Stratford and Rose, 1985), Zygosaccharomyces bailii NCYC 1427 and Zygosaccharomyces bailii NCYC 563. The strains were maintained at 4°C on slopes containing (l^{-1}): agar (MYGP) 20 g, glucose 10 g, malt extract 3.0 g, yeast extract (Lab M) 3.0 g and mycological peptone 0.5 g (Wickerham, 1951).

EXPERIMENTAL CULTURES

Organisms were grown aerobically in medium containing (l^{-1}): glucose 20 g, $(NH_4)_2SO_4$ 3.0 g, KH_2PO_4 3.0 g, yeast extract (Lab M) 1.0 g, $CaCl_2.2H_2O$ 30 mg and $MgSO_4.7H_2O$ 30 mg (adjusted to pH 4.0 with HCl). This was the medium used by Stratford and Rose (1986) and is referred to as Medium A. It is, however, poorly buffered and, in experiments in which the yeasts were grown in the presence of sulphite, it was replaced by Medium B which differed from Medium A in that KH_2PO_4 was omitted to be replaced by 13.4 g K_2HPO_4 and 12.9 g citric acid (adjusted to pH 4.0 with citric acid). Under the conditions used, the pH value of cultures grown using Medium B did not fall below 4.0. One-litre portions of medium were dispensed into 2 l round flat bottomed flasks which were plugged with cotton wool and sterilized by autoclaving at 6.89×10^4 Pa for 10 min. Starter cultures (100 ml medium in 250 ml conical flasks) were inoculated with a pinhead of yeast from a slant culture and

incubated at 30°C for 24 h on an orbital shaker (200 r.p.m.). One-litre portions of medium were inoculated with portions of starter culture containing 0.05 mg dry wt Sacch. cerevisiae NCYC 431, 0.5 mg dry wt Sacch. cerevisiae TCS or 1.0 mg dry wt of either of the Zygosacch. bailii strains and incubated in a constant temperature (30°C) room with stirring (100 r.p.m.) on a flat-bed stirrer.

Organisms were grown anaerobically by a modification of the method of Alterthum and Rose (1973) in medium containing (l^{-1}): glucose 50 g, KH_2PO_4 4.5 g, $(NH_4)_2SO_4$ 3.0 g, yeast extract (Lab M) 1 g, $CaCl_2.2H_2O$ 25 mg and $MgSO_4.7H_2O$ 25 mg (adjusted to pH 4.0 with HCl). One-litre portions of medium were dispensed into two-litre round flat-bottomed flasks and sterilized as already described. Anaerobic conditions were maintained throughout growth by flushing the flasks with high-purity nitrogen from which the last traces of oxygen had been removed by a column-type Oxy-Trap (Alltech Associates Incorporated, Deerfield, Illinois, U.S.A.). Prior to inoculation, the medium was supplemented with ergosterol (5 mg l^{-1}) and an unsaturated fatty acid (30 mg l^{-1}) either myristoleic acid ($C_{14:1}$ - Δ^9), palmitoleic acid ($C_{16:1}$ - Δ^9), oleic acid ($C_{18:1}$ - Δ^9), linoleic acid ($C_{18:2}$ - $\Delta^{9,12}$), linolenic acid ($C_{18:3}$ - $\Delta^{9,12,15}$) or 11-eicosenoic acid ($C_{20:1}$ - Δ^{11}). Portions of medium were inoculated with 1 mg dry wt organisms from an overnight starter culture grown in medium B and incubated as previously described. Control cultures lacking unsaturated fatty acid were incubated with each batch of experimental cultures. When growth in the control exceeded 0.1 mg dry wt ml^{-1}, experimental

cultures were discarded. Growth was followed by measuring the optical density of portions of culture at 600 nm, measurements being related to dry wt of organism by a standard curve constructed for each strain of yeast. Organisms were harvested from mid-exponential phase cultures, containing 0.5 mg dry wt Sacch. cerevisiae ml^{-1} or 0.25 mg dry wt of Zygosacch. bailii ml^{-1} by filtration through a membrane filter (0.45 µm pore size; 50 mm diam.; Oxoid) and washed twice with 10 ml 30 mM-citrate buffer (pH 3.0), or by centrifugation (6,000 g, 1 min, 4°C) and washed twice with distilled water for phospholipid analysis. All centrifugation regimes were carried out in a Sorvall RC5C refrigerated Superspeed Centrifuge (Du Pont Company, Wilmington, Delaware, U.S.A.) unless otherwise stated.

ASSESSMENT OF SULPHUR DIOXIDE TOLERANCE

The ability of yeasts to grow in Medium B containing different concentrations of sulphite was measured using Dynatech microplates (Dynatech Laboratories Inc., Alexandria, Virginia, U.S.A.). Organisms were harvested from mid-exponential phase cultures by centrifugation (12,000 g for 2 min) and resuspended in fresh medium (pH 4.0) to give 0.1 mg dry wt ml^{-1} suspension. Using a Digital Multichannel Pipette (Flow Laboratories) dilute cell suspension (170 µl) was pipetted into each well of a microtitre plate leaving one well empty to use as a blank. Sodium metabisulphite (30 µl), diluted in fresh medium, was added to each well giving final concentrations of sulphite ranging between zero and 3.3 mM across the plate. The blank well was filled with 200 µl water and the

plate gently shaken for a few seconds on a Titertek shaker (Flow Laboratories), to mix the suspensions. Replicate plates were prepared, covered, sealed in an airtight container with some moist tissue paper to minimize evaporation and incubated at 30°C on an orbital shaker (200 r.p.m.). Using a Dynatech Microplate Reader (MR600), set at 600 nm, optical densities were measured at intervals up to 6 h after adjusting to zero against the blank well. Cells tended to settle to the bottom of the wells so the plates were gently agitated before optical density values were measured.

MEASUREMENT OF SULPHITE ACCUMULATION

To measure initial velocities of sulphite accumulation, organisms grown in Medium A were washed twice with 30 mM-citrate buffer (pH 3.0) containing 100 mM-glucose, suspended in the same buffer at 10 mg dry wt ml^{-1} and the suspension allowed to equilibrate for 5 min at 30°C. A reaction mixture consisting of 30 mM-citrate buffer (pH 3.0) containing 100 mM-glucose and 10-200 μM-[^{35}S]sulphite (0.20 μCi ml^{-1}, 1 μCi = 37 KBq) was prepared in a universal bottle and warmed to 30°C in a water bath. Radiolabelled sulphite was stored at -20°C in 5 mM-EDTA under nitrogen gas in 0.5 ml aliquots (0.1 mCi ml^{-1}) to prevent oxidation. Portions (300 μl) of the suspension of organisms were dispensed into microcentrifuge tubes (Eppendorf). Using a 1.5 ml multi-dispense syringe pipette, 1.25 ml of radiolabelled sulphite reaction mixture was added to the organisms and the suspension quickly mixed by refilling and emptying the syringe. After exactly 4 s, 1.5 ml of the suspension was rapidly filtered through a

membrane filter (0.45 µm pore size; 25 mm diam.; Millipore) which had been washed with 5 ml 10 mM-sulphite in 30 mM-citrate buffer (pH 3.0). After filtration, three 1 ml portions of buffered sulphite solution of the same concentration as employed in the experiment were used quickly to wash the organisms and filter. Filters with organisms were then placed in scintillation vials containing 7 ml Optiphase Safe (Fisons). Radioactivity in the vials was measured in an LKB Rackbeta liquid scintillation spectrometer (model 1217).

To measure the extent of sulphite accumulation, washed organisms grown in Medium A were suspended in glucose-containing citrate buffer as already described. Radiolabelled sulphite was added to a 20 ml suspension containing 2 mg dry wt organisms ml^{-1} giving final concentrations of 0.1 - 5.0 mM-sulphite (0.2 µCi ml^{-1}) and the suspension incubated at 30°C. At appropriate time intervals, three 1 ml portions of suspension were filtered through prewashed filters as already described. The organisms were washed with three 1 ml portions of 30 mM-citrate buffer containing sulphite at the concentrations used in the experiment. Radioactivity was measured as already described. Background activity was estimated by repeating the procedure without organisms to check washing efficiency and to ensure that sulphite was not binding to filters.

MEASUREMENT OF PLASMA-MEMBRANE AREA IN ORGANISMS

Dimensions of organisms were measured by observation in a light microscope fitted with an eyepiece graticule. In calculating membrane areas, it was assumed that organisms of Sacch. cerevisiae

were spheres, those of Zygosacch. bailii were cylinders with rounded ends and that surface areas were equivalent to plasma-membrane areas.

MEASUREMENT OF INTRACELLULAR WATER VOLUME

Volumes of intracellular water in organisms in suspension were calculated by measuring the differential distribution of $^{3}H_2O$, which equilibrates with both extracellular and intracellular water, and D-[1-^{14}C]mannitol which is excluded by the plasma membrane. Initial experiments established that mannitol was not accumulated by any of the yeasts examined. To do this, washed organisms were suspended at 10 mg dry wt ml^{-1} in 30 mM-citrate buffer (pH 3.0) containing 100 mM-glucose and [^{14}C]mannitol at 0.01, 1.0 or 100 mM. The suspensions were incubated for 60 min at 30°C and filtered through filters that had been prewashed with 5 ml 100 mM buffered mannitol (0.45 µm pore size; 25 mm diam.; Millipore).

Membranes and organisms were then washed with non-radioactive mannitol at the concentration used in the experiment, placed in scintillation vials containing 7 ml Optiphase Safe and radioactivity measured as already described. To measure the volume of intracellular water, a suspension of washed organisms (10 mg dry wt ml^{-1}) grown in Medium A was prepared and allowed to equilibrate for 5 min in glucose-containing citrate buffer as already described. To 15 ml of suspension was added [^{14}C]mannitol and tritiated water giving final concentrations of 10 mM- [^{14}C] mannitol (0.02 µCi ml^{-1}) and 0.2 µ Ci $^{3}H_2O$ ml^{-1}. Suspensions were incubated with continuous stirring at 4°C for 10 min. Six 1 ml

portions of suspension were then centrifuged in microcentrifuge tubes (Eppendorf) for 3 min at 12,000 g. Duplicate 200 µl portions of supernatant from each tube were added to scintillation vials containing 7 ml Optiphase Safe and radioactivity measured as previously described. Radioactivity in the suspension of organisms was measured by placing twelve 200 µl portions of suspension in scintillation vials containing 7 ml Optiphase Safe.

To measure the intracellular water volumes of organisms after short exposure to sulphite at least 150 mg dry wt organisms were harvested, washed and suspended in glucose-containing citrate buffer (pH 3.0) as already described. Sulphite was added to a 75 ml suspension containing 2 mg dry wt organisms ml^{-1} giving final concentrations of 1.0 to 5.0 mM-sulphite. After 10 min incubation at 30°C with continuous stirring, organisms were centrifuged (12,000 g for 2 min) and resuspended in 30 mM-citrate buffer (pH 3.0) containing 100 mM-glucose and 1.0 to 5.0 mM-sulphite at 10 mg dry wt ml^{-1}. To 15 ml of this suspension was added [^{14}C] mannitol and tritiated water and intracellular water volumes determined as already described.

MEASUREMENT OF INTRACELLULAR pH VALUES

(a) Use of Propionic Acid

Intracellular pH values of organisms grown in Medium A were calculated by determining the equilibrium distribution of propionic acid across the plasma membrane (Conway and Downey, 1950). Washed organisms, suspended (5 mg dry wt ml^{-1}) in 30 mM-citrate buffer (9 ml) containing 100 mM-glucose (pH 3.0), were allowed to

equilibrate after adding 1 ml 0.1 mM-[2-^{14}C]propionic acid (0.25 μCi ml^{-1}) at 30°C. After 1, 2, 3, 4, 6 and 10 min, duplicate 300 μl portions were taken from the suspension, rapidly filtered through washed membrane filters (0.45 μm pore size; 25 mm diam.; Millipore) and washed with 4 x 1 ml 0.01 mM-propionic acid at 4°C. The filters were transferred, with organisms, to scintillation vials as already described. Once the time for equilibration had been ascertained, replicate measurements were obtained by sampling after 5 min incubation. Intracellular pH values were calculated from the expression derived by Waddell and Butler (1959):

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

where $R = TA_i.V_e/TA_e.V_i$, pH_i and pH_e are the internal and external pH values, TA_i and TA_e the intracellular and extracellular volumes and pK_i and pK_e the dissociation constants for propionic acid in the internal and external environments. The internal and external dissociation constants for propionic acid were calculated from the Davies (1962) simplified version of the Debye-Hückel equations. Values for pK_i and pK_e were calculated to be 4.75 and 4.86, respectively.

The effect of the accumulation of sulphite in organisms upon intracellular pH values was assessed by incubating organisms with propionic acid as described with the addition of sulphite giving final concentrations ranging between zero and 5 mM-sulphite, allowing the sulphite and propionic acid to equilibrate for 10 min, and sampling as already described.

(b) Use of Fluorescein Diacetate as a Fluorescent Probe

This method relies upon the ability of organisms to take up non-fluorescing fluorescein diacetate into the cytoplasm and to enzymically cleave acetate groups through the action of intracellular esterases to produce fluorescein which is trapped inside the cell (Slavik, 1982). Fluorescein has a pH-dependent fluorescence spectrum and so, theoretically, intracellular pH values can be measured by recording the fluorescence intensities at 520 nm after excitation at 435 nm and 490 nm which are the positions of the two major peaks in the fluorescence emission spectrum. A standard curve was constructed by plotting the fluorescence intensities of fluorescein in 0.1 mM-citrate buffer at 520 nm, after excitation at 435 nm and 490 nm, against pH value which was varied between pH 2.5 and pH 7.5 by the addition of HCl. Mid-exponential phase organisms were harvested, washed twice, resuspended in 30 mM-citrate buffer with 100 mM-glucose (pH 3.0; 10 mg dry wt ml^{-1}) and allowed to equilibrate at 30°C. A stock solution of fluorescein diacetate was prepared (10 mM in acetone) and kept in the dark to minimise spontaneous decomposition. Dilutions were prepared only when required. A portion (5 ml) of the cell suspension was left untreated and used as a blank. The rest of the suspension was incubated at 30°C for at least 30 min with 100 µM fluorescein diacetate or until there was visible fluorescence. After incubation, the organisms were thoroughly washed and resuspended in the original volume of buffer. samples (0.5 ml) were placed in a cuvette of an Amico-Bowman Spectro-fluorometer (adapted from right angled illumination to 45° to allow

measurement of a dense cell suspension) and the fluorescence intensity recorded at 520 nm after excitation at 490 nm and 435 nm. The blanks were analysed similarly and their values subtracted from the test results. The final emission ratios were used to calculate intracellular pH values from the standard curve.

VIABILITY MEASUREMENTS

Viability of yeast populations was measured by staining with methylene blue (Fink and Kühles, 1933). Portions of suspensions (0.5 ml) were removed, filtered through membrane filters (0.45 µm pore size; 25 mm diam.; Millipore), washed with 3 x 1 ml distilled water, resuspended in water 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. Viable organisms were colourless.

ANALYTICAL METHODS

(a) Free Sulphite

The method of Burroughs and Sparks (1964b) was used to measure total free sulphur dioxide where:

$$\text{Free } SO_2 = SO_2 + H_2SO_3 + HSO_3^- + SO_3^{2-}$$

and with the assumption that dissociation of bound sulphur dioxide

was minimised by decreasing the pH value to 1.5. Portions (5 ml) of culture filtrate were acidified with 5 ml orthophosphoric acid (25% v/v) followed by removal of free sulphur dioxide under reduced pressure (70-80 mm mercury) in a gentle stream of air for 30 min. Sulphur dioxide was trapped in two absorption tubes each containing 5-10 ml freshly prepared, neutralised 1% (w/v) hydrogen peroxide solution containing 1% (v/v) Tashiro indicator (2 volumes 0.1% methyl red plus 1 volume 0.1% methylene blue both in 95% ethanol) by the reaction:

$$2H^+ + SO_3^{2-} + H_2O_2 \rightarrow H_2SO_4 + H_2O$$

The sulphuric acid was titrated to a grey end point with 0.01 M sodium hydroxide which was standardised with potassium hydrogen iodate. Blank values were obtained by reconnecting two more absorption tubes for a further 30 min and titrating as already described. Titre volumes of blanks were subtracted from the test values and the concentration of sulphur dioxide calculated by the relationship:

1 ml 0.01 M-Sodium Hydroxide ≡ 0.32 mg Sulphur Dioxide.

(b) Pyruvate

Pyruvate concentrations present in culture filtrates were determined using pyruvate test combination kits (Boehringer, Mannheim, West Germany) according to the method of Czok and Lamprecht (1974). This method is based on the enzymic conversion of pyruvate to lactate by lactate dehydrogenase (LDH):

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+$$

Oxidation of NADH is proportional to the amount of substrate converted and is measured spectrophotometrically at 340 nm.

(c) Acetaldehyde

The concentration of acetaldehyde in culture filtrates was determined using the Boehringer, Mannheim UV-method where both free and bound acetaldehyde are oxidised in the presence of acetaldehyde dehydrogenase (Al-DH) by nicotinamide-adenine dinucleotide (NAD^+) to acetic acid:

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{Al-DH}} \text{Acetic Acid} + \text{NADH} + H^+$$

Concentrations of NADH were recorded at 340 nm and the concentrations of total acetaldehyde calculated and compared with standards containing 0.5, 2.5 and 4.5 mM-acetaldehyde. Sequential dilutions of standards were prepared both in the presence and absence of 5 mM-sulphite. The test kit was found to be sensitive to concentrations of acetaldehyde between 0.05 and 5 mM and results were unaffected by the presence of sulphite.

(d) Glycerol

Glycerol concentration in culture filtrates was determined by an assay kit (Boehringer). The kit contained glycerol kinase, which catalysed conversion of glycerol into glycerol 3-phosphate and ADP, pyruvate kinase which catalysed conversion of PEP and ADP to

pyruvate and ATP, and lactate dehydrogenase which calaysed reduction of pyruvate to lactate generating NAD^+. The decline in concentration of NADH was measured spectrophotometrically at 340 nm, and was stoicheiometrically related to the concentration of glycerol. Values obtained were corrected for the concentrations of pyruvate known to be in the culture filtrates.

(e) Ethanol

Ethanol concentrations were determined by gas-liquid chromatography. A portion (3 ml) of culture filtrate was diluted as necessary with water. Portions (0.5 ml) of diluted sample were mixed with equal volumes of 0.2% (v/v) acetone in water, and 1 µl of solution injected onto the column of a Pye GCD gas chromatograph fitted with a flame ionization detector (oven temperature 300°C). The column (1.5 m long, 0.4 cm internal diam.) was packed with Chromosorb 101 (100/120 mesh) and maintained at 150°C. The injection temperature was 250°C, and the nitrogen gas carrier flow rate 40 ml min^{-1}. Standards containing 0.05, 0.10, 0.15 and 0.20% (v/v) ethanol were run with each batch of samples. The value for the peak height multiplied by the retention time for samples was related to ethanol concentration by a standard curve.

LIPID ANALYSIS

(a) Lipid Extraction

Pre-washed organisms (250 mg) were mixed with 10 ml 80% ethanol in a universal bottle and heated at 80°C for 15 min in a water bath to deactivate lipolytic enzymes and to split lipid protein linkages

(Letters, 1967). The extract was filtered through Whatman no. 44 filter paper and the filtrate stored at -20°C while the residue was extracted twice with chloroform/methanol (2:1 v/v) for 2 and 1 h, respectively, as it was stirred magnetically on a flat bed stirrer at room temperature. The three extracts were pooled, washed with 0.25 vol. 0.88% KCl and the mixture left to separate overnight at -20°C. The lower organic phase was removed, taken to dryness using a rotary evaporator, and the residue dissolved in 1 ml light petroleum (b.p. 60-80°C). Extracts, if necessary, were stored under nitrogen gas at -20°C.

Samples were evaporated under a stream of nitrogen gas until approximately 100 µl remained and streaked onto a 20 x 20 cm 0.25 mm Silica Gel 60 TLC plate (Merck) using a 50 µl Terumo Micro Syringe (Terumo Corporation, Tokyo, Japan). On the same plate standards were streaked containing 1 mg phosphatidylethanolamine, ergosterol and palmitic acid ml^{-1} in light petroleum (b.p. 60-80°C). The plate was developed in a light petroleum (b.p. 40-60°C)-diethyl ether-acetic acid (70:30:1, by vol.) solvent mixture, lipids located by spraying with 0.2% (w/v) 2',7'-dichlorofluoroscein in ethanol and the plate viewed under UV (254 nm) radiation. The phospholipid bands were ringed with a pencil and the appropriate areas scrapped off the plate and transferred to 5 ml screw top Reactivials (Pierce Chemical Co., Chester, England). At this stage samples were either methylated for GLC analysis or eluted for quantitation of total phospholipids and separation into individual phospholipid classes.

(b) Fatty-acyl Composition of Total Cellular Phospholipids

To determine the fatty-acyl composition of phospholipids, samples removed from TLC plates were methylated by refluxing with 3 ml borontrifluoride (14% w/v in methanol) for 1 h at 80°C in sealed Reactivials. After cooling, each sample was added to 5 ml of water in stoppered glass tubes, supplemented with 3 ml petroleum ether and shaken vigorously. The fatty acid methyl esters were extracted into the petroleum ether. This extraction procedure was repeated twice more, the extracts pooled, evaporated to dryness using a rotary evaporator, dissolved in 1 ml petroleum ether and stored under nitrogen gas at -20°C until they were analysed by GLC. Fatty acid methyl esters were analysed using a fused capillary column (25 m length; SGE BP 21) in a Pye Unicam GCD chromatograph fitted with an SGE on-column adaptor. The injection temperature was 250°C, and the column maintained at 110°C for the first 5 min, after which the column temperature was raised at the rate of 8°C min^{-1} until it reached 180°C. The carrier gas was hydrogen flowing at 6 ml min^{-1}. Percentage fatty-acyl compositions were calculated using an LDC/Milton Roy integrator.

(c) Fatty-acyl Composition of Individual Phospholipid Classes

For separation of individual phospholipid classes samples were eluted from the gel with 3 x 3 ml of chloroform-methanol-water (5:5:1 v/v), followed by 3 ml methanol and finally 3 ml methanol-acetic acid-water (95:1:5 v/v). The pooled extracts were evaporated to dryness using a rotary evaporator and taken up into 1 ml chloroform-methanol (2:1 v/v). Samples and standards

containing 1 mg phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol ml^{-1} in light petroleum (b.p. 60-80°C) were applied to TLC plates as described and developed in chloroform-methanol-acetic acid-water (120:23:10:4.5 v/v) (Tunbuld-Johansson et al., 1987). Fractions were located as described and compared with standards for identification. Bands containing phospholipid classes were scraped off and transferred to screw top vials. An internal standard of 0.2 mg heptadecanoic acid (1 mg ml^{-1} in methanol) was added to each sample before methylation and GLC analysis as already described.

(d) Analysis of Total Cellular Phospholipids

Total cellular phospholipid was determined by assaying the phosphorus content of the eluted phospholipid band using a modification of the method of Chen et al. (1956). A small portion of silica gel was removed from each plate, eluted and used for a blank while 5 mg, 2.5 mg and 1 mg portions of phosphatidylcholine were used as controls. Samples containing phosphorus were evaporated to dryness in standard Kjeldahl digestion tubes and ashed by adding six drops of concentrated sulphuric acid, and heating in a Kjeldahl digester (Tecator 1007 Digestion System, Sweden) at 250°C until white fumes appeared and the samples blackened. Three drops of 72% perchloric acid were added and digestion continued for 15 min at 250°C or until digestion was complete. After cooling water was added and the samples made up to 25 ml in volumetric flasks. Samples and standard solutions of KH_2PO_4 containing 1-10 µg of phosphorus were placed into pyrex

tubes and the volume adjusted to 4 ml with distilled water. To this 4 ml of colour reagent containing 6 N sulphuric acid - 2.5% ammonium molybdate - 10% ascorbic acid - water (1:1:1:2 v/v, prepared fresh each day) was added, and the tubes covered and incubated at 37°C for 2 h. Absorbance values were measured at 820 nm and compared with reagent blanks, controls and a prepared standard curve. Values for phosphorus contents were multiplied by 25 to give the total phospholipid content.

MATERIALS

All chemicals used were AnalaR grade or of the highest purity available commercially. Boron trifluoride, 2',7'-Dichloro-fluorescein and all lipid standards were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, England. All radioactively labelled compounds were obtained from Amersham International, Amersham, England. 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 ORGANISMS UNDER AEROBIC CONDITIONS

Organisms grown aerobically reached mid-exponential phase after approximately 16 h incubation. The generation time during exponential growth for Sacch. cerevisiae NCYC 431 was 2 h; Sacch. cerevisiae TC8, 2 h 10 min; Zygosacch. bailii NCYC 1427, 2 h 30 min and for Zygosacch. bailii NCYC 563, 2 h 20 min. Final growth yield at stationary phase was approximately 1.7 mg ml^{-1} for strains of Sacch. cerevisiae and 2.5 mg ml^{-1} for Zygosacch. bailii.

Conversion factors used to calculate dry weight of organisms from optical density measurements (OD_{600nm}) of mid-exponential phase aerobically-grown organisms were as follows: Sacch. cerevisiae NCYC 431, 0.58; Sacch. cerevisiae TC8, 0.40; Zygosacch. bailii NCYC 1427, 0.55 and Zygosacch. bailii NCYC 563, 0.58. The conversion factors are equivalent to values of the gradients derived from plots of OD_{600nm} against (mg dry wt organisms)ml^{-1} all of which were linear up to at least OD_{600nm} 0.6.

Values calculated for cell surface area (Table 3) and intracellular water volume (Table 4) were found to vary between different strains of yeast.

EFFECTS OF SULPHITE ON AEROBIC GROWTH

Sulphite inhibited aerobic growth of all four yeasts at concentrations up to and including 3.3 mM as assessed by the microplate method (Fig. 2). Zygosaccharomyces bailii NCYC 563 was the most sensitive and Sacch. cerevisiae NCYC 431 the least.

Table 3. Cell surface areas of aerobically-grown *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* estimated from light-microscope observations. Also indicated are the number of organisms mg^{-1} present in mid-exponential phase cultures from which organisms were taken for cell-surface area estimation. Values quoted for cell number are the mean of at least three independent analyses. Surface areas were calculated from the mean dimensions of at least sixty organisms.

Organism	Number of organisms mg^{-1}	Surface area of organisms $(mm^2(mg\ dry\ wt)^{-1})$
Saccharomyces cerevisiae NCYC 431	5.25×10^7	2600
Saccharomyces cerevisiae TC8	7.89×10^7	5020
Zygosaccharomyces bailii NCYC 1427	3.56×10^7	3770
Zygosaccharomyces bailii NCYC 563	2.73×10^7	3310

Table 4. Intracellular water volumes of aerobically-grown *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* determined as described in the Methods section. Values quoted are the means of at least three independent determinations ± SD.

Organism	Intracellular water volume (μl (mg dry wt)$^{-1}$)	Intracellular water volume (fl)
Saccharomyces cerevisiae NCYC 431	1.55 ± 0.15	29.5 ± 2.9
Saccharomyces cerevisiae TC8	2.74 ± 0.13	34.7 ± 1.6
Zygosaccharomyces bailii NCYC 1427	2.05 ± 0.20	57.6 ± 2.6
Zygosaccharomyces bailii NCYC 563	1.85 ± 0.12	67.6 ± 4.4

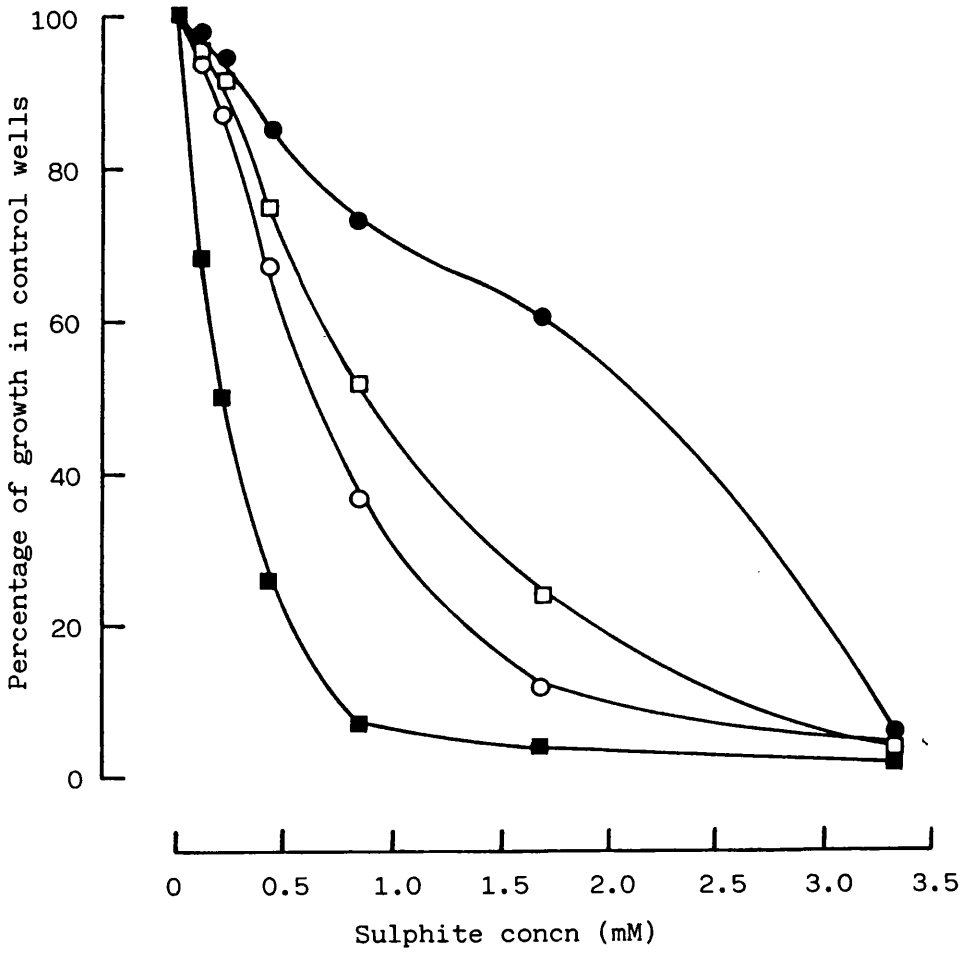


Figure 2. Effect of sulphite concentration on growth of Saccharomyces cerevisiae TC8 (○), Saccharomyces cerevisiae NCYC 431 (●), Zygosaccharomyces bailii NCYC 1427 (□) and Zygosaccharomyces bailii NCYC 563 (■) in Medium B in microtitre wells. Values quoted are the means of measurements on eight separate plates. The maximum variation was ± 10%

ACCUMULATION OF SULPHITE UNDER AEROBIC CONDITIONS

Equilibrium levels for aerobic accumulation of sulphite equivalents were reached somewhat faster with strains of Sacch. cerevisiae (Fig. 3) than those of Zygosacch. bailii (Fig. 4) although all four strains had reached equilibrium levels after 10 min irrespective of the concentration of sulphite. Table 5 lists intracellular water volumes of aerobically-grown yeasts after short term exposure to sulphite. Vertical Woolf-Eadie plots (Hofstee, 1959) were obtained with initial velocities of accumulation by all yeasts suspended in high concentrations of SO_2 (Fig. 5). However, at low concentrations of SO_2 especially with Sacch. cerevisiae NCYC 431, there was considerable deviation from the vertical.

EFFECT OF SULPHITE ON YEAST VIABILITY

Organisms grown aerobically in Medium A, harvested and washed as already described, were allowed to equilibrate in glucose-containing citrate buffer (pH 3.0). Sulphite was added to suspensions containing 2 mg dry wt organisms ml^{-1} giving final concentrations of 0.1 - 5.0 mM-sulphite and the suspensions incubated for 10 min at 30°C. All four yeasts maintained 98% viability after exposure to sulphite concentrations up to and including 5 mM.

EFFECTS OF SULPHITE UPON INTRACELLULAR pH VALUES

Propionic acid accumulated very rapidly in organisms during the first few minutes exposure and in strains of both Sacch. cerevisiae and Zygosacch. bailii equilibrium was reached after 5 min (Fig. 6).

Figure 3. Time-course for accumulation of [^{35}S] sulphite in (a) Saccharomyces cerevisiae NCYC 431 and (b) Saccharomyces cerevisiae TC8 suspended in 30 mM-citrate buffer (pH 3.0) at 30°C containing 100 mM-glucose and 0.1 mM (○), 0.5 mM (●), 1.0 mM (□), 2.0 mM (■) or 5.0 mM (△) sulphite. Values quoted are the means of three independent determinations. The maximum variation was ±15%.

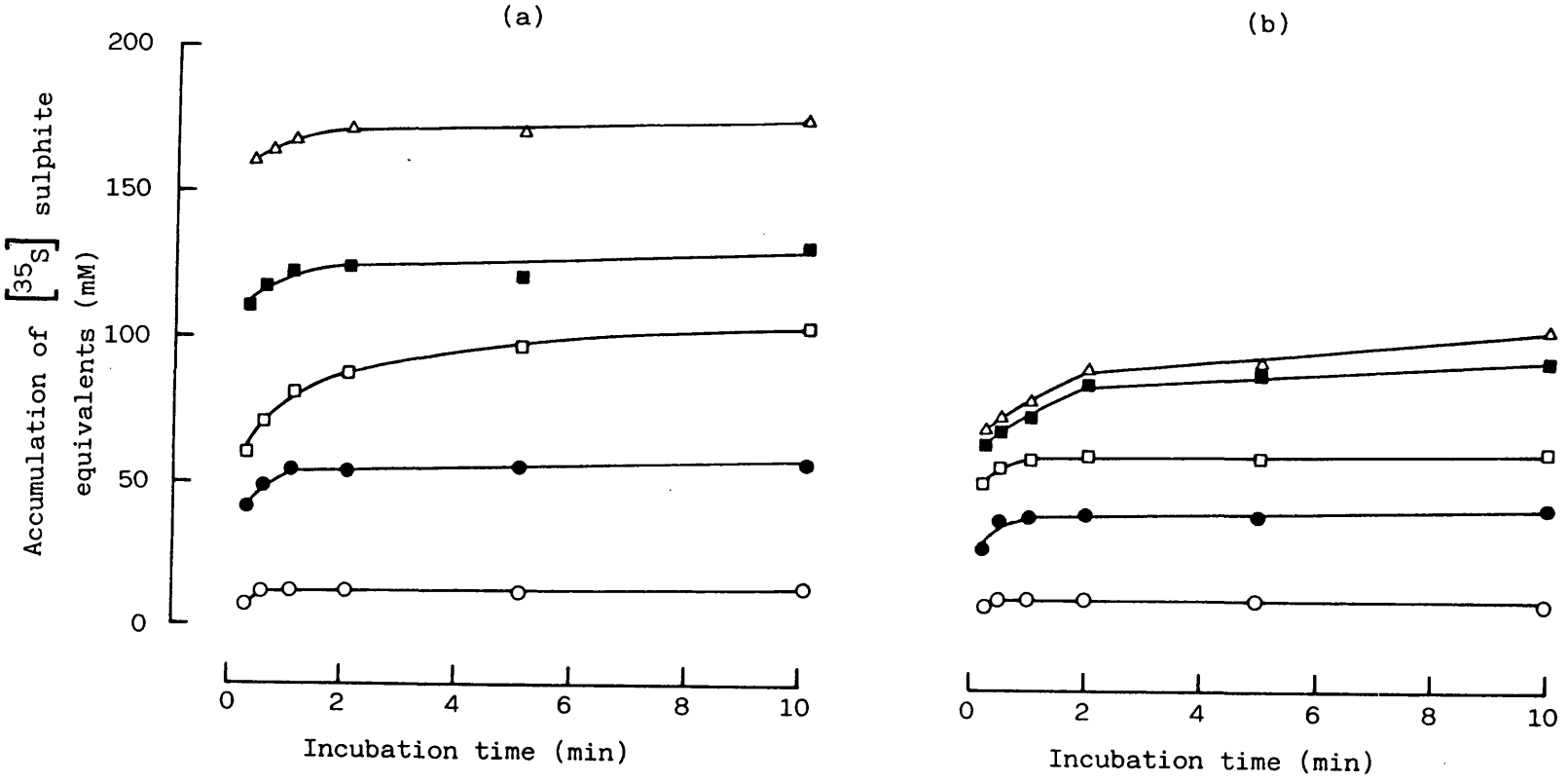

(a)
(b)
Accumulation of [35S] sulphite equivalents (mM)
200
150
100
50
0
0
2
4
6
8
10
Incubation time (min)
Incubation time (min)

Figure 3.

Figure 4. Time-course for accumulation of [^{35}S] sulphite in (a) Zygosaccharomyces bailii NCYC 1427 and (b) Zygosaccharomyces bailii NCYC 563 suspended in 30 mM-citrate buffer (pH 3.0) at 30°C containing 100 mM-glucose and 0.1 mM (○), 0.5 mM (●), 1.0 mM (□), 2.0 mM (■) or 5.0 mM (△) sulphite. Values quoted are the means of three independent determinations. The maximum variation was ±10%.

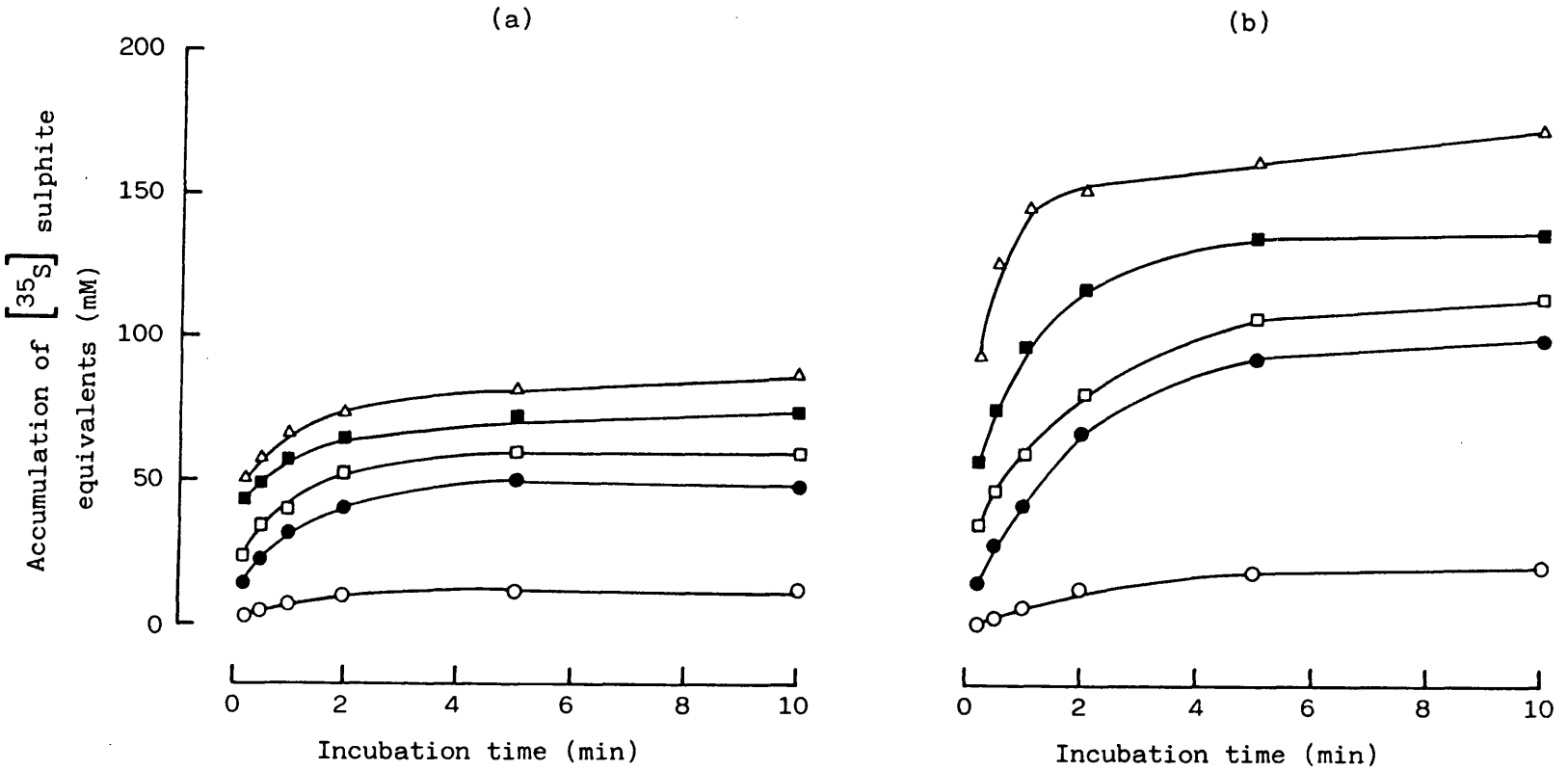

(a)
(b)
Accumulation of [35S] sulphite equivalents (mM)
200
150
100
50
0
0
2
4
6
8
10
Incubation time (min)

Figure 4.

Table 5. Intracellular water volume of organisms grown aerobically calculated from the distribution of radiolabelled [2-^{14}C]propionic acid after 10 min equilibration with sulphite in 30 mM-citrate buffer containing 100 mM-glucose (pH 3.0). Values quoted are the means of three independent determinations ±SD.

Organism	Intracellular water volume (µl (mg dry wt)$^{-1}$) of organisms after 10 min equilibration with:-		
	1 mM-sulphite	2 mM-sulphite	5 mM-sulphite
Saccharomyces cerevisiae NCYC 431	1.45 ± 0.15	1.36 ± 0.29	1.44 ± 0.31
Saccharomyces cerevisiae TC8	2.50 ± 0.29	2.89 ± 0.15	2.57 ± 0.38
Zygosaccharomyces bailii NCYC 1427	1.88 ± 0.12	1.94 ± 0.41	2.07 ± 0.20
Zygosaccharomyces bailii NCYC 563	1.83 ± 0.21	1.92 ± 0.31	2.00 ± 0.15

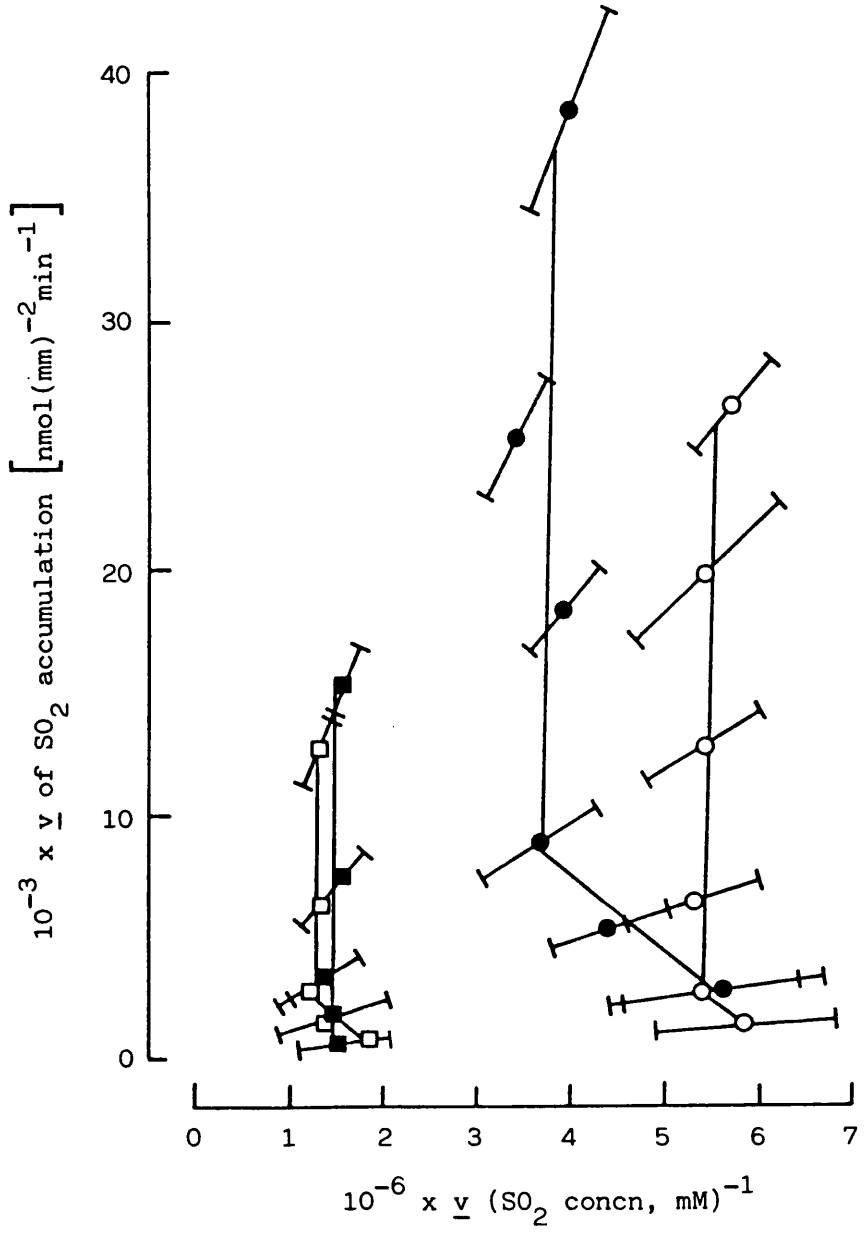


Figure 5. Woolfe-Eadie plots for accumulation of molecular SO_2 by Saccharomyces cerevisiae TC8 (○), Saccharomyces cerevisiae NCYC 431 (●), Zygosaccharomyces bailii NCYC 1427 (□) and Zygosaccharomyces bailii NCYC 563 (■) suspended in 30 mM-citrate buffer (pH 3.0) containing 100 mM-glucose at 30°C. Concentrations of molecular SO_2 were calculated from data of King et al. (1981). Bars indicate SD.

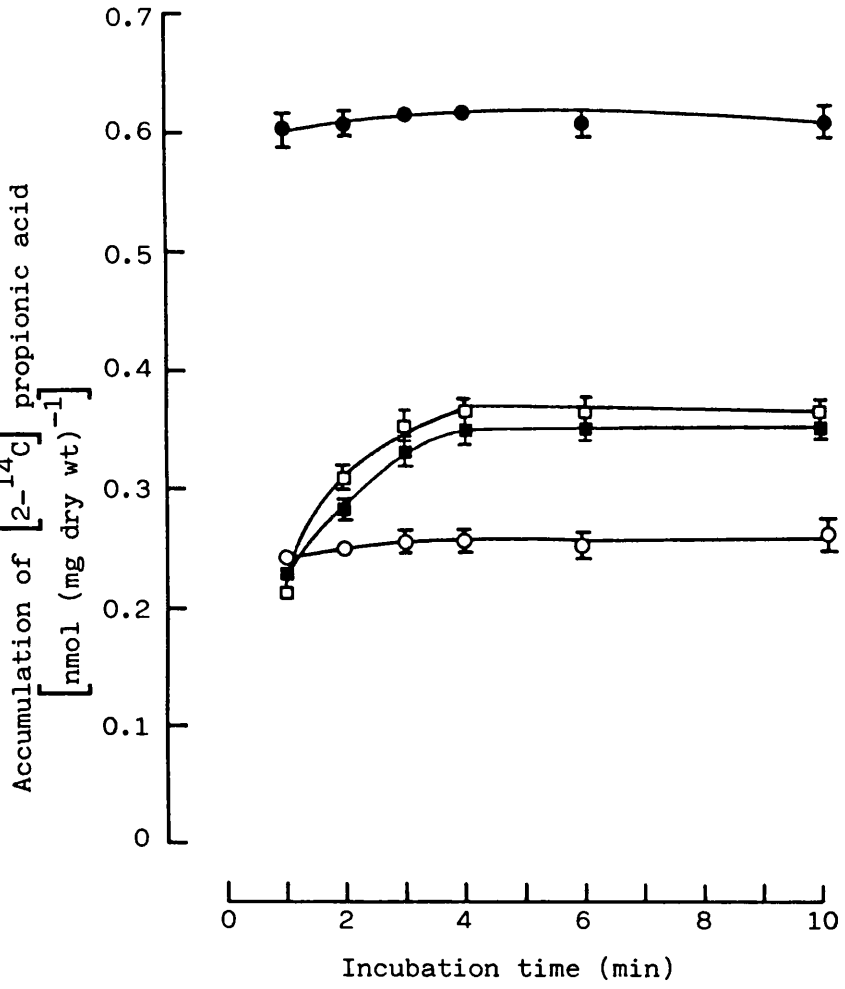


Figure 6. Time-course for accumulation of [2-^{14}C]propionic acid by Saccharomyces cerevisiae NCYC 431 (○), Saccharomyces cerevisiae TC8 (●), Zygosaccharomyces bailii NCYC 1427 (□) and Zygosaccharomyces bailii NCYC 563 (■) suspended in citrate buffer containing 10 μMol [2-^{14}C]propionic acid at pH 3.0. Values quoted are the means of three determinations ± SD.

The greater the extent of accumulation of sulphite equivalents, the larger was the decline in internal pH value (Figs. 7 and 8). Equilibrium accumulation values, and therefore decline in internal pH values, were smallest for Zygosacch. bailii NCYC 1427 (Fig. 8).

Intracellular pH values recorded using the fluorescence probe technique proved unreliable. The mean intracellular pH value of Sacch. cerevisiae TC8 in citrate-glucose buffer (pH 3.0) was found to be pH 5.68, this value being the average of three determinations with a standard deviation of ±0.09. Strains of Zygosacch. bailii either did not take up the fluorescein diacetate or failed to cleave the acetate groups even after prolonged incubation (2 h) with the dye. Intensities of fluorescence recorded were insignificant when compared with blank readings and so it was not possible to assess intracellular pH values of these organisms. Fluorescein was rapidly produced in Sacch. cerevisiae NCYC 431 but equally rapidly leaked from the cells into the surrounding buffer. Consequently, the emission ratio $I_{490/435}$ decreased, essentially measuring the pH value of the extracellular buffer.

PRODUCTION OF BINDING COMPOUNDS BY ORGANISMS GROWN AEROBICALLY IN THE PRESENCE OF SULPHITE

The effect of sulphite on growth of the yeasts in 1 litre cultures (Medium B) was assessed by adding the compound to early/mid-exponential phase cultures giving final concentrations of zero, 1 or 2 mM-sulphite, and measuring the effect on density of organisms and on concentrations in culture filtrates of acetaldehyde, ethanol, glycerol, pyruvate and free sulphite over

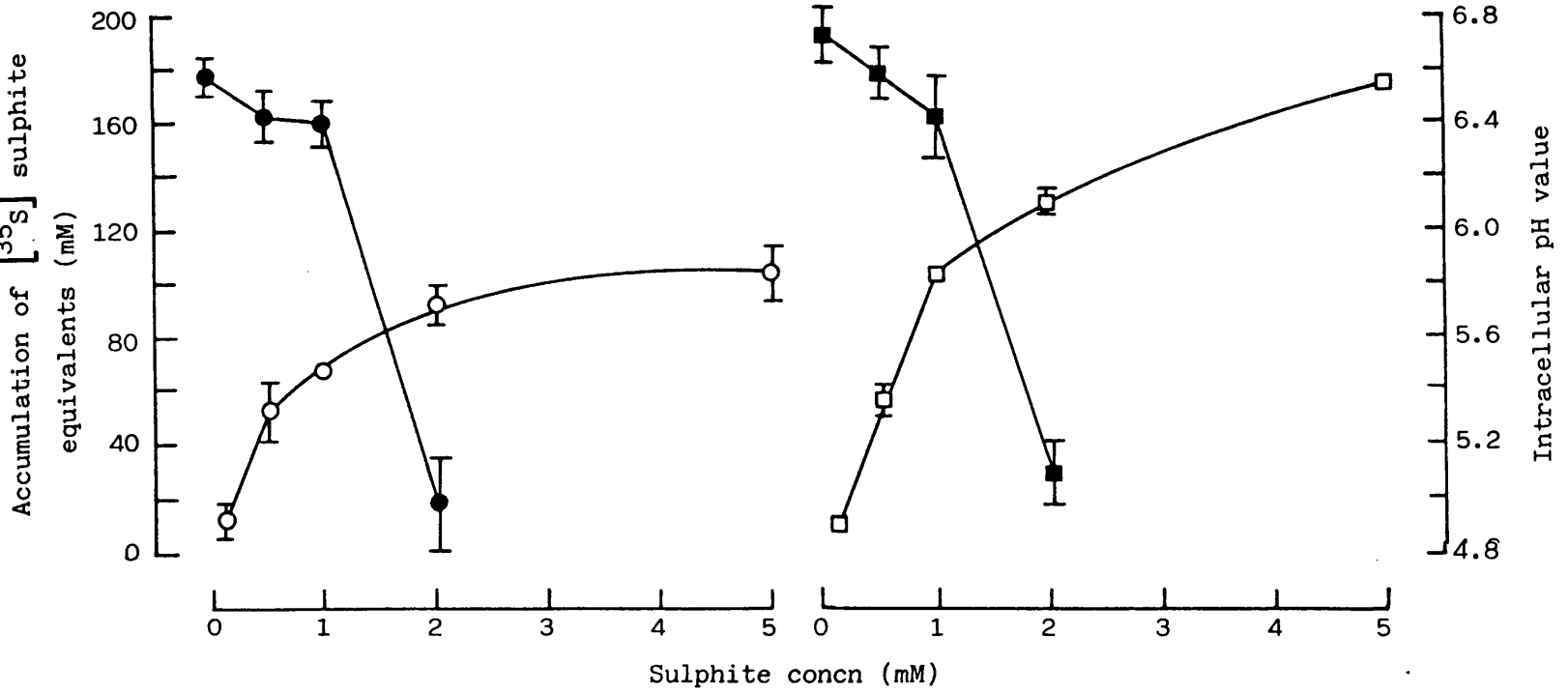


Figure 7. Relationship between extent of accumulation of sulphite equivalents (open symbols) and intracellular pH (closed symbols) in Saccharomyces cerevisiae TC8 (○ and ●), and Saccharomyces cerevisiae NCYC 431 (□ and ■). Measurements were made after organisms had been suspended in buffer for 10 min. Values quoted are means of at least three independent determinations. Bars indicate SD.

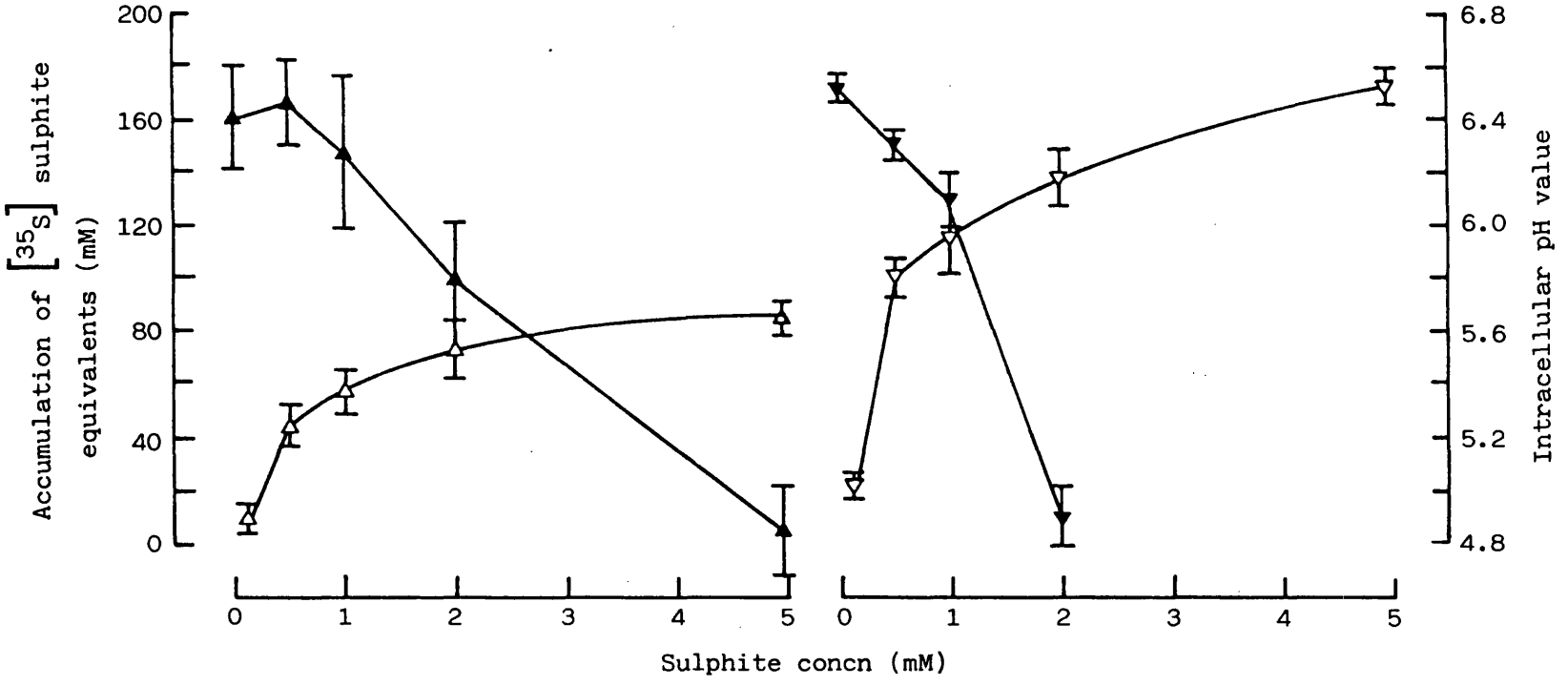


Figure 8. Relationship between extent of accumulation of sulphite equivalents (open symbols) and intracellular pH (closed symbols) in Zygosaccharomyces bailii NCYC 1427 (△ and ▲), and Zygosaccharomyces bailii NCYC 563 (▽ and ▼). Measurements were made after organisms had been suspended in buffer for 10 min. Values quoted are the means of at least three independent determinations. Bars indicate SD.

Figure 9. Effect of supplementing cultures of Saccharomyces cerevisiae NCYC 431 (a), Saccharomyces cerevisiae TC8 (b), Zygosaccharomyces bailii NCYC 1427 (c) and Zygosaccharomyces bailii NCYC 563 (d) with sulphite (■, control; △, 1.0 mM, ▲, 2 mM) on growth and ethanol formation. Also shown are the effects of these supplements on concentrations of acetaldehyde (○), glycerol (●) and free sulphite (□) in culture supernatants. After supplementing cultures with sulphite, they were observed for a further 6 h. Values quoted are the means of three separate determinations. The maximum variation in values for concentrations of acetaldehyde and free sulphite was ±10%; for concentrations of ethanol and glycerol the variation was ±15%.

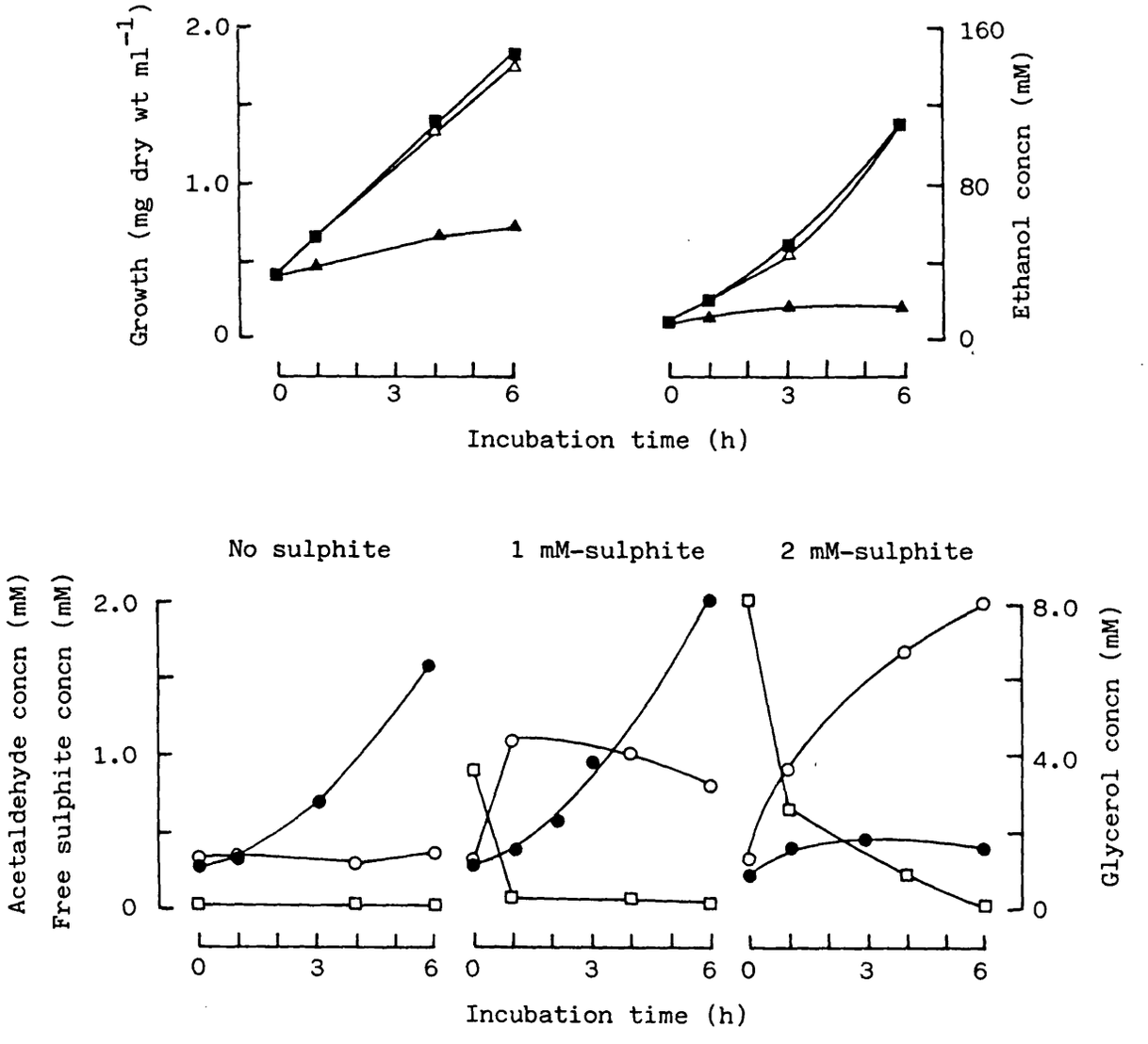
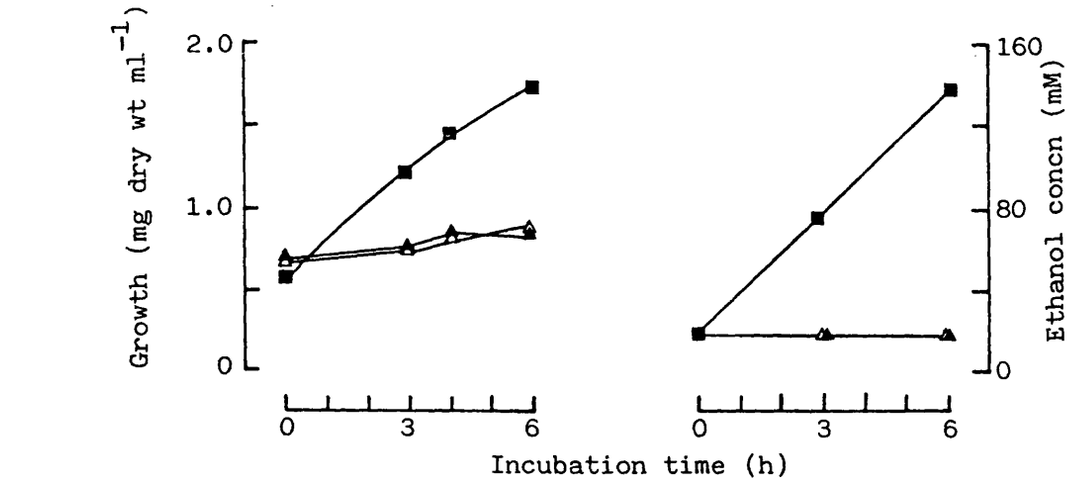

Growth (mg dry wt ml^{-1})
2.0
1.0
0
160
80
0
Ethanol concn (mM)
0
3
6
Incubation time (h)
No sulphite
1 mM-sulphite
2 mM-sulphite
Acetaldehyde concn (mM)
Free sulphite concn (mM)
2.0
1.0
0
8.0
4.0
0
Glycerol concn (mM)
0
3
6
Incubation time (h)

Figure 9a.


Growth (mg dry wt ml^{-1})
2.0
1.0
0
160
80
0
Ethanol concn (mM)
0
3
6
0
3
6
Incubation time (h)

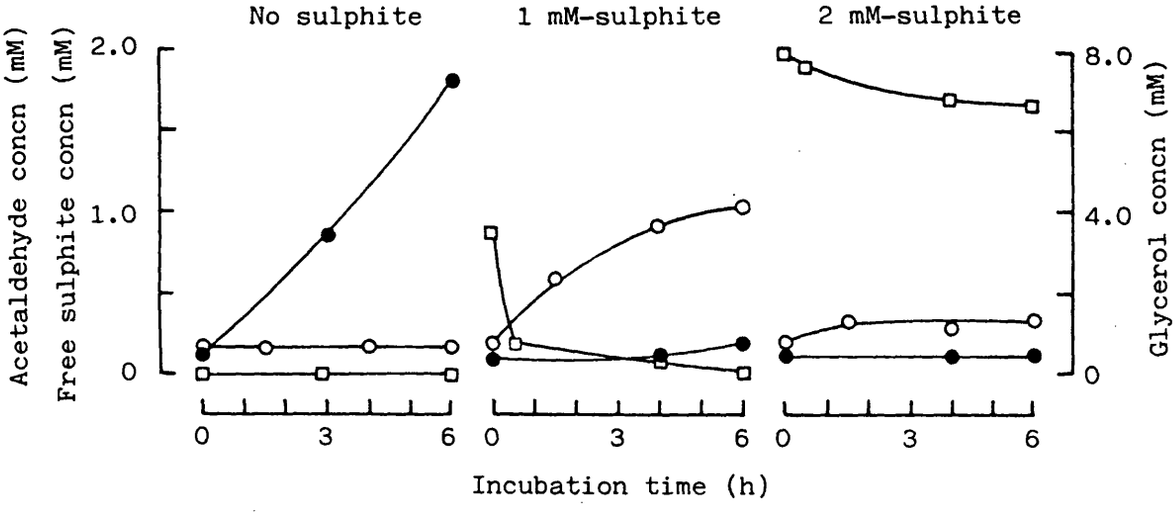

No sulphite
1 mM-sulphite
2 mM-sulphite
Acetaldehyde concn (mM)
Free sulphite concn (mM)
2.0
1.0
0
8.0
4.0
0
Glycerol concn (mM)
0
3
6
0
3
6
0
3
6
Incubation time (h)

Figure 9b.

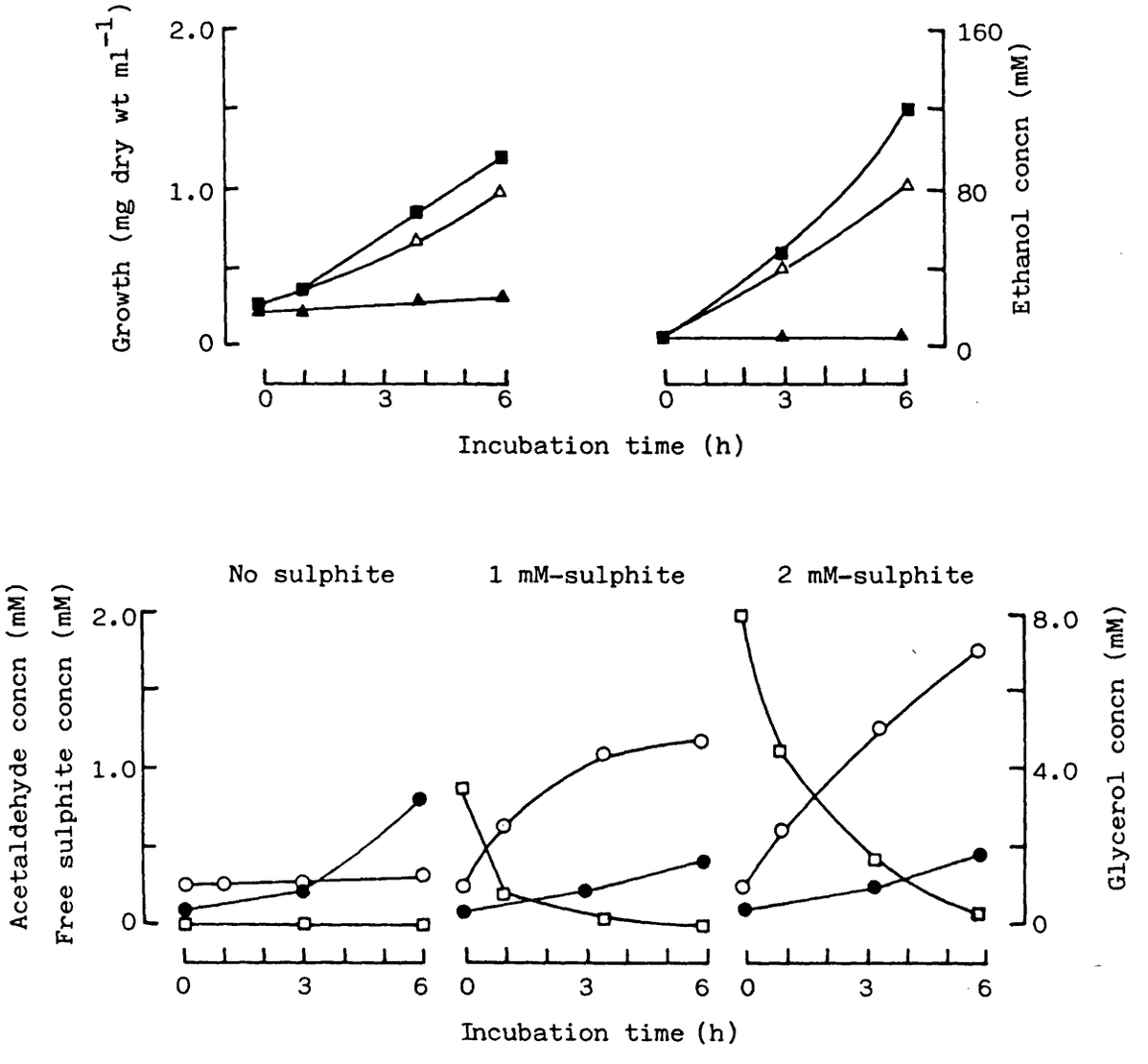

Growth (mg dry wt ml^{-1})
2.0
1.0
0
Ethanol concn (mM)
160
80
0
0
3
6
Incubation time (h)
No sulphite
1 mM-sulphite
2 mM-sulphite
Acetaldehyde concn (mM)
Free sulphite concn (mM)
Glycerol concn (mM)
8.0
4.0

Figure 9c.

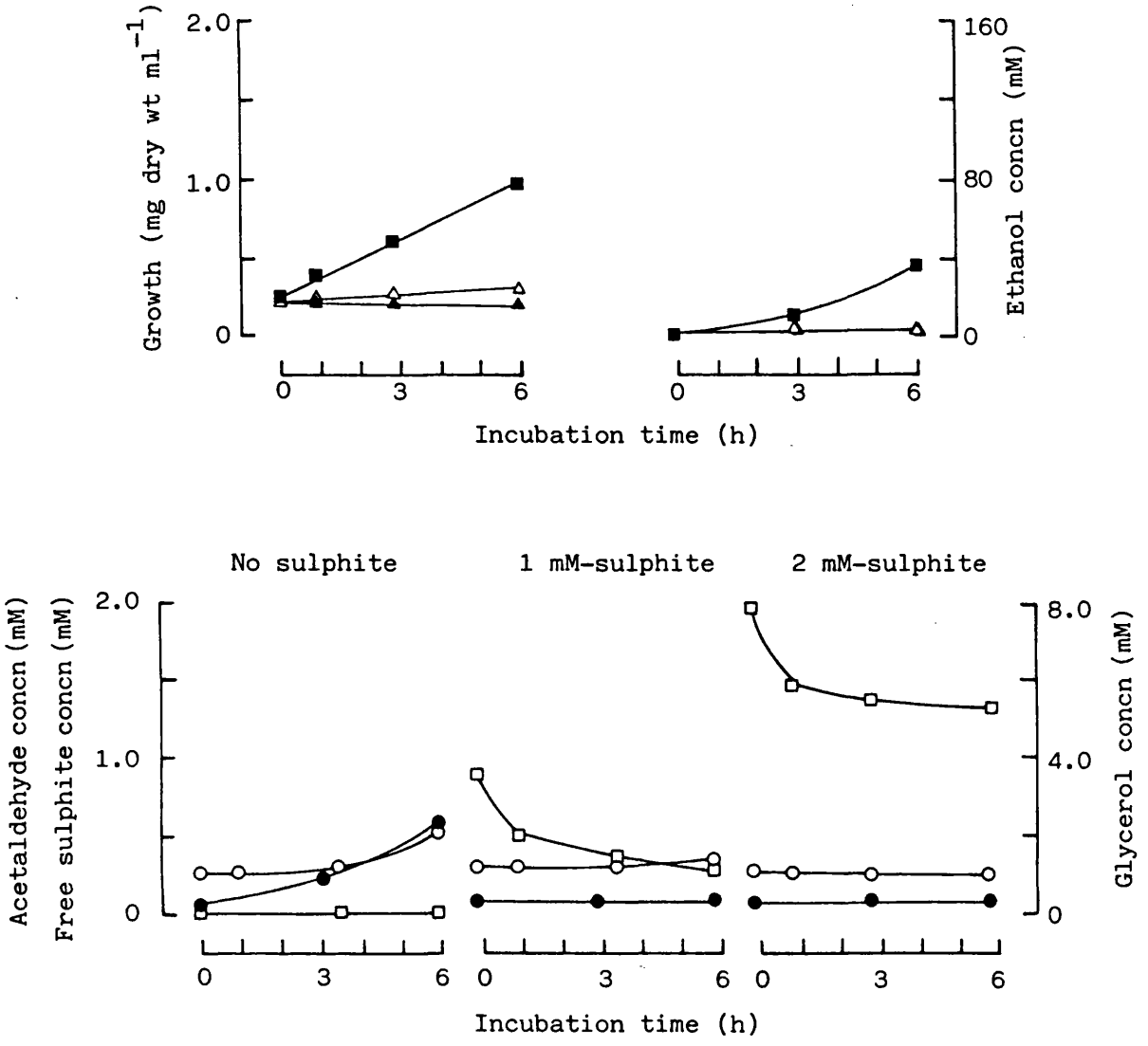

Growth (mg dry wt ml^{-1})
2.0
1.0
0
160
80
0
Ethanol concn (mM)
0
3
6
Incubation time (h)
No sulphite
1 mM-sulphite
2 mM-sulphite
Acetaldehyde concn (mM)
Free sulphite concn (mM)
2.0
1.0
0
8.0
4.0
0
Glycerol concn (mM)
0
3
6
Incubation time (h)

Figure 9d.

Figure 10. Effect of supplementing cultures of Saccharomyces cerevisiae NCYC 431 (a), Saccharomyces cerevisiae TC8 (b), Zygosaccharomyces bailii NCYC 1427 (c) and Zygosaccharomyces bailii NCYC 563 (d) with sulphite (○, control, ●, 1.0 mM, □, 2 mM) on pyruvate concentrations in culture supernatants. After supplementing cultures with sulphite, they were observed for a further 6 h. Values quoted are the means of three separate determinations ± SD.

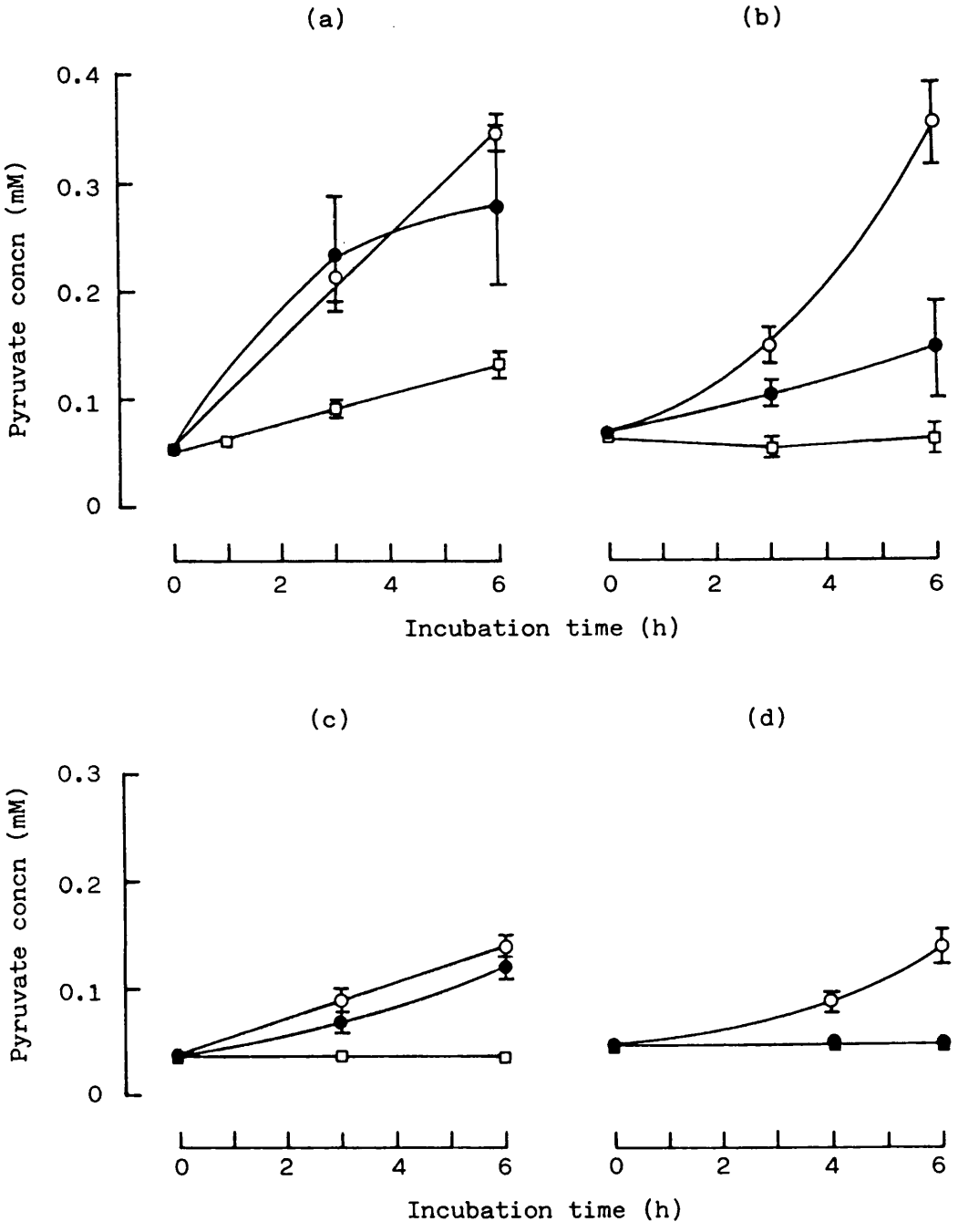


Figure 10.

the following 6 h. Growth of Zygosacch. bailii NCYC 563 was virtually completely inhibited following supplementation of cultures with 1.0 or 2 mM-sulphite (Fig. 9d). Ethanol production was also completely inhibited. Even in the supplemented cultures in which growth was almost completely inhibited, there was a decrease in the concentration of free sulphite despite a lack of production of acetaldehyde. Production of glycerol and pyruvate (Fig. 10d), which was detectable in unsupplemented cultures, was also completely inhibited. A very similar pattern of response was observed in cultures of Sacch. cerevisiae TC8 (Fig. 9b). The much greater production of glycerol by this strain in unsupplemented cultures, which reached a concentration of approximately 7 mM in 6 h cultures, was also completely inhibited by supplementation with 2 mM sulphite. In the presence of 1 mM-sulphite acetaldehyde was produced resulting in a decline in free sulphite concentration, there was very limited glycerol produced and a marked decline in pyruvate production (Fig. 10b). Supplementing cultures of Sacch. cerevisiae NCYC 431 with 1.0 mM sulphite had no effect on growth or ethanol production (Fig. 9a) and little effect on pyruvate production (Fig. 10a). In these cultures, the concentration of free sulphite declined rapidly, while there was an increase in the production of glycerol and a rapid appearance of acetaldehyde in the culture filtrates. When cultures of this yeast were supplemented with 2.0 mM-sulphite, growth was decreased considerably and this was accompanied by decreased production of ethanol, glycerol and pyruvate. However, there was a rapid decline in the concentration of free sulphite, which was accompanied by a greater increase in acetaldehyde concentration than was observed in

cultures supplemented with 1.0 mM-sulphite. Cultures of Zygosacch. bailii NCYC 1427 showed a very similar pattern of responses to those of Sacch. cerevisiae NCYC 431 (Figs. 9c, 10c) except that less glycerol was produced in unsupplemented cultures while supplementation with 1.0 mM-sulphite lowered glycerol production. When cultures were observed 24 h after supplementation with sulphite, only cultures of Zygosacch. bailii NCYC 563 and Sacch. cerevisiae TC8 containing 2 mM-sulphite failed to grow. All of the other cultures, after prolonged lag phases, eventually underwent normal exponential growth.

Sulphite concentrations in control flasks containing Medium B and 1.0 or 2.0 mM-sulphite, after 6 h incubation, decreased by 15.3% and 7.8% respectively (Table 6). Samples analysed immediately after addition of sulphite (T = 0) showed that constituents of Medium B did not bring about significant binding of free sulphite.

FATTY-ACYL COMPOSITION OF PHOSPHOLIPIDS FROM AEROBICALLY GROWN YEASTS

The principal fatty-acyl residue in phospholipids from aerobically-grown strains of Sacch. cerevisiae was $C_{16:1}$, followed by $C_{18:1}$ and $C_{16:0}$ (Table 7). In both strains of Zygosacch. bailii, $C_{18:2}$ was the major fatty-acyl residue in their phospholipids, followed by $C_{18:1}$ and $C_{16:0}$ (Table 7).

Phospholipid classes were separated on TLC plates into distinct bands. The Rf values obtained for standard phospholipids were as follows: phosphatidylethanolamine, 0.64 ± 0.02; phosphatidylserine 0.38 ± 0.05; phosphatidylcholine, 0.27 ± 0.02 and phosphatidyl-inositol, 0.18 ± 0.02. The values quoted are the mean of six

Table 6. Concentration of free sulphite in control flasks containing uninoculated Medium B supplemented with sulphite and sampled over 6 h while being incubated at 30°C and stirred continually. Values represent the mean of three determinations. The maximum variation was ±5%.

Incubation time (h)	Concentration of free sulphite (mM) in media supplemented with:- 1 mM-sulphite	2 mM-sulphite
0	0.98	2.04
3	0.86	1.86
6	0.83	1.88

Table 7. Fatty-acyl composition of phospholipids from aerobically-grown strains of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. Values quoted are the means of three independent determinations ±SD. tr indicates that a trace was detected, - that none was detected.

Fatty-acyl residue	Fatty-acyl residues (percentage of total) in:-			
	Saccharomyces cerevisiae NCYC 431	*Saccharomyces cerevisiae* TC8	*Zygosaccharomyces bailii* NCYC 1427	*Zygosaccharomyces bailii* NCYC 563
10:0	1.3 ± 0.2	tr	-	-
12:0	1.4 ± 0.2	0.7 ± 0.3	-	-
14:0	4.1 ± 0.4	2.2 ± 0.2	tr	-
14:1	1.3 ± 0.3	tr	tr	-
16:0	16.2 ± 0.8	17.3 ± 0.3	14.7 ± 0.7	11.1 ± 3.0
16:1	52.2 ± 1.7	46.4 ± 1.7	12.2 ± 2.2	9.9 ± 1.9
18:0	1.9 ± 0.2	2.7 ± 0.3	6.1 ± 1.1	7.5 ± 1.5
18:1	20.3 ± 1.0	30.0 ± 1.5	29.6 ± 2.6	33.0 ± 1.6
18:2	-	-	41.2 3.6	38.4 ± 2.8

independent experiments ± SD.

Strains of Sacch. cerevisiae were found to contain greater contents of phospholipid (mg dry wt organisms)$^{-1}$ than strains of Zygosacch. bailii (Table 8). The relative proportions of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS) differed only very slightly between the four strains. Phosphatidylcholine was the most abundant phospholipid followed by PE and PI with less than 10% as PS. Saccharomyces cerevisiae had a lower proportion of PI and a higher proportion of PE, compared with strains of Zygosacch. bailii which had approximately equal contents of these phospholipids. In addition, Zygosacch bailii NCYC 563 had a slightly higher proportion of PC than the other three yeasts (Table 8).

Values for Δmol^{-1} for each class of phospholipid in Sacch. cerevisiae NCYC 431 were very similar to those of Sacch. cerevisiae TC8 but much lower than those calculated for the Zygosacch. bailii strains. Both Zygosacch. bailii strains had similar Δmol^{-1} values. For all yeasts the value for Δmol^{-1} for phosphatidylinositol was much lower than those calculated for the other phospholipid classes (Tables 9, 10, 11, 12).

The mean fatty-acyl chain length did not vary between phospholipid classes in strains of Sacch. cerevisiae (Tables 9 and 10). Phospholipids isolated from strains of Zygosacch. bailii contained fatty-acyl residues that were longer and more variable in length compared with Sacch. cerevisiae, where phosphatidylcholine contained the longest fatty-acyl chains and phosphatidylserine the shortest (Tables 11 and 12).

Table 8. Total phospholipid content of aerobically-grown strains of Saccharomyces cerevisiae and Zygosaccharomyces bailii and the relative proportions of each phospholipid class, namely phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). Values quoted are the means of four independent determinations ±SD.

Organism	Total phospholipid content (mg(250 mg dry wt organisms)$^{-1}$)	Percentage of the total phospholipid class			
		PC	PE	PI	PS
Saccharomyces cerevisiae NCYC 431	10.39 ± 0.92	52.2 ± 2.4	28.9 ± 1.6	11.2 ± 1.9	7.4 ± 1.1
Saccharomyces cerevisiae TC8	9.64 ± 0.42	51.0 ± 5.4	31.3 ± 3.5	10.1 ± 3.0	8.4 ± 0.7
Zygosaccharomyces bailii NCYC 1427	7.80 ± 0.64	52.0 ± 1.3	21.0 ± 1.3	20.3 ± 0.7	5.7 ± 0.3
Zygosaccharomyces bailii NCYC 563	8.02 ± 0.33	60.1 ± 1.5	16.2 ± 1.5	17.5 2.7	6.3 ± 0.4

Table 9. Fatty-acyl composition of phospholipid classes in aerobically grown Saccharomyces cerevisiae NCYC 431. Values quoted are the mean of four independent analyses ± SD. tr indicates that a trace was detected, - that none was detected. Values for Δmol^{-1} were calculated as described by Kates and Hagen (1964).

Fatty-acyl residue	Percentage of the total phospholipid class			
	PC	PE	PI	PS
12:0	tr	tr	6.3 ± 1.4	-
14:0	4.0 ± 0.3	3.1 ± 0.5	8.0 ± 1.7	3.7 ± 0.7
14:1	1.6 ± 0.5	tr	tr	tr
16:0	21.7 ± 0.8	15.3 ± 1.6	35.1 ± 3.3	23.3 ± 1.7
16:1	53.0 ± 2.2	57.1 ± 1.9	24.5 ± 2.4	41.4 ± 2.4
18:0	3.1 ± 0.3	tr	6.4 ± 1.5	tr
18:1	15.7 ± 1.1	23.5 ± 0.6	15.7 ± 2.7	29.4 ± 1.2
Δmol^{-1}	0.70 ± 0.01	0.81 ± 0.01	0.40 ± 0.04	0.71 ± 0.02
Mean fatty-acyl chain length	16.26 ± 0.05	16.41 ± 0.05	16.03 ± 0.38	16.53 ± 0.10

Table 10. Fatty-acyl composition of phosholipid classes in aerobically grown Saccharomyces cerevisiae TC8. Values quoted are the mean of four independent analyses ± SD. tr indicates that a trace was detected, - that none was detected.

Fatty-acyl residue	Percentage of the total phospholipid class			
	PC	PE	PI	PS
12:0	-	tr	3.7 ± 1.0	-
14:0	3.3 ± 1.0	4.2 ± 1.3	4.4 ± 1.0	5.9 ± 2.4
14:1	1.8 ± 0.5	tr	tr	-
16:0	19.5 ± 1.8	18.1 ± 1.2	34.5 ± 2.3	28.6 ± 1.9
16:1	53.7 ± 2.2	51.9 ± 1.1	23.1 ± 3.6	33.3 ± 2.6
18:0	3.6 ± 0.4	tr	7.2 ± 1.5	tr
18:1	17.9 ± 2.1	25.9 ± 2.9	25.0 ± 3.6	30.0 ± 2.8
Δmol^{-1}	0.73 ± 0.03	0.78 ± 0.03	0.48 ± 0.03	0.63 ± 0.03
Mean fatty-acyl chain length	16.31 ± 0.20	16.46 ± 0.34	16.41 ± 0.11	16.49 ± 0.22

Table 11. Fatty-acyl composition of phospholipid classes in aerobically-grown Zygosaccharomyces bailii NCYC 1427. Values quoted are the mean of three independent analysis ± SD. tr indicates that a trace was detected.

Fatty-acyl residue	Percentage of the total phospholipid class			
	PC	PE	PI	PS
16:0	9.7 ± 0.5	2.6 ± 1.3	35.3 ± 1.8	8.6 ± 1.5
16:1	13.1 ± 3.5	36.7 ± 4.9	4.6 ± 1.0	45.0 ± 2.9
18:0	4.1 ± 1.3	tr	11.1 ± 1.5	tr
18:1	23.1 ± 1.8	30.3 ± 1.5	31.5 ± 2.3	32.1 ± 1.7
18:2	49.9 ± 2.5	30.0 ± 4.5	17.5 ± 2.4	13.9 ± 2.2
Δmol^{-1}	1.36 ± 0.03	1.27 ± 0.05	0.71 ± 0.04	1.05 ± 0.06
mean fatty-acyl chain length	17.55 ± 0.25	17.13 ± 0.10	17.20 ± 0.05	16.86 ± 0.04

Table 12. Fatty-acyl composition of phospholipid classes in aerobically-grown *Zygosaccharomyces bailii* NCYC 563. Values quoted are the mean of three independent analyses ±SD. tr indicates that a trace was detected.

Fatty-acyl residue	Percentage of the total phospholipid class			
	PC	PE	PI	PS
16:0	10.3 ± 0.5	3.8 ± 0.8	32.1 ± 2.3	11.1 ± 1.3
16:1	7.4 ± 1.2	32.3 ± 1.2	3.6 ± 0.8	40.9 ± 4.3
18:0	6.8 ± 0.8	tr	14.2 ± 1.9	tr
18:1	32.1 ± 1.7	31.4 ± 2.6	36.0 ± 1.8	35.3 ± 2.6
18:2	43.2 ± 1.6	32.4 ± 2.3	14.6 ± 2.0	12.4 ± 2.1
Δmol^{-1}	1.26 ± 0.03	1.29 ± 0.03	0.69 ± 0.04	1.01 ± 0.07
Mean fatty-acyl chain length	17.61 ± 0.04	17.26 ± 0.24	17.21 ± 0.06	16.88 ± 0.15

The overall mean fatty-acyl chain length and Δmol^{-1} values calculated for total phospholipids (Table 13) are higher in strains of Zygosacch. bailii than those of Sacch. cerevisiae and are inversely proportional to the permeability coefficient calculated from the initial rates of diffusion of [^{35}S] sulphite into organisms (Fig. 5).

The permeability coefficient is defined as the rate of flow through a unit area of membrane when the concentration difference across the membrane is 1.0 M. From Fick's first law of diffusion the following relationship is derived:-

$$\underline{v} = \frac{D(C_1 - C_2)}{\ell} \qquad \text{(Laidler, 1977)}$$

where D is the diffusion coefficient, C_1 and C_2 are the extracellular and intracellular solute concentrations and ℓ is the thickness of the membrane. The permeability coefficient is the flux when $C_1 - C_2 = 1$ M so that:-

$$P = \frac{D}{\ell} = \frac{v}{C_1 - C_2}$$

thus the permeability coefficient (P) for SO_2 diffusing across a membrane is equal to $\underline{v}$ (SO_2 concn, M)$^{-1}$ (Fig. 5).

GROWTH OF SACCHAROMYCES CEREVISIAE NCYC 431 UNDER ANAEROBIC CONDITIONS

When media were supplemented with ergosterol (5 mg l^{-1}) and an unsaturated fatty acid (30 mg l^{-1}) the generation time of organisms in the mid-exponential phase of growth was 3 h 30 min reaching a

Table 13. Mean fatty-acyl chain length and degree of unsaturation (Δmol^{-1}) of total phospholipids in yeasts grown aerobically compared with their respective permeability coefficients for SO_2 accumulation calculated from data presented in the Woolf-Eadie plot (Fig. 5). Values for Δmol^{-1} were calculated as described by Kates and Hagen (1964). Values quoted are the means of at least three independent analyses ±SD.

Organism	Mean fatty-acyl chain length of total phospholipid	Value for Δmol^{-1} for total phospholipid	Permeability coefficient ($mm(min)^{-1}$)
Saccharomyces cerevisiae NCYC 431	16.02 ± 0.33	0.74 ± 0.02	3.83 ± 0.42
Saccharomyces cerevisiae TC8	16.55 ± 0.04	0.77 ± 0.04	5.42 ± 0.55
Zygosaccharomyces bailii NCYC 1427	17.50 ± 0.04	1.24 ± 0.02	1.29 ± 0.21
Zygosaccharomyces bailii NCYC 563	17.44 ± 0.16	1.13 ± 0.02	1.51 ± 0.31

final yield at stationary phase of approximately 1.2 mg ml^{-1}. Anaerobic cultures required a much larger inoculum than those grown aerobically. Organisms in media supplemented with myristoleic acid underwent a prolonged lag phase, some 3 h longer than other anaerobically-grown cultures.

Conversion factors used to calculate dry weight of organisms from OD_{600nm} measurements of mid-exponential phase Sacch. cerevisiae NCYC 431 grown anaerobically in media supplemented with ergosterol (5 mg l^{-1}) and an unsaturated fatty acid (30 mg l^{-1}) were as follows: myristoleic acid ($C_{14:1}$) 0.63; palmitoleic acid ($C_{16:1}$), 0.65; oleic acid ($C_{18:1}$), 0.60; linoleic acid ($C_{18:2}$), 0.68; linolenic acid ($C_{18:3}$), 0.62 and 11-eicosenoic acid ($C_{20:1}$), 0.57.

The dimensions of anaerobically-grown Sacch. cerevisiae NCYC 431 were not significantly different from those of organisms of this strain grown aerobically and were not affected by the nature of the fatty-acid supplement. Cell-surface areas calculated for anaerobically-grown Sacch. cerevisiae NCYC 431 using dimensions of aerobically-grown organisms and the number of organisms mg^{-1} present in mid-exponential phase cultures are shown in Table 14. As there is very little variation in the surface areas calculated for organisms grown under different anaerobic conditions a mean surface area of 2150 mm^2 $(mg\ dry\ wt)^{-1}$ is used in subsequent calculations.

FATTY-ACYL COMPOSITION OF PHOSPHOLIPIDS FROM ANAEROBICALLY GROWN YEASTS

Neither strain of Zygosacch. bailii grew anaerobically when

Table 14. Cell-surface areas of anaerobically-grown Saccharomyces cerevisiae NCYC 431 grown in media supplemented with ergosterol (5 mg l^{-1}) and an unsaturated fatty acid (30 mg l^{-1}). Also indicated are the number of organisms mg^{-1} present in mid-exponential phase cultures from which organisms were taken for cell-surface area estimation. Values quoted for cell number are the mean of at least three independent analyses while surface areas were calculated from the mean dimensions of at least sixty aerobically grown organisms.

Fatty acid supplement	Number of organisms mg^{-1}	Surface area of organisms (mm^2(mg dry wt)$^{-1}$)
Myristoleic acid ($C_{14:1}$)	4.10×10^7	2030
Palmitoleic acid ($C_{16:1}$)	4.33×10^7	2140
Oleic acid ($C_{18:1}$)	4.70×10^7	2330
Linoleic acid ($C_{18:2}$)	4.41×10^7	2180
Linolenic acid ($C_{18:3}$)	4.47×10^7	2210
11-Eicosenoic acid ($C_{20:1}$)	4.23×10^7	2090

supplemented with ergosterol and oleic acid either singly or together. Both Sacch. cerevisiae NCYC 431 and TC8 grew with both ergosterol and oleic acid, to a lesser extent with just ergosterol and very little in the presence of only oleic acid. Neither strain grew significantly in lipid-free anaerobic medium (Fig. 11). Saccharomyces cerevisiae NCYC 431 was selected to study the manner in which sulphite transport was affected by the composition of the fatty-acyl residues in cellular phospholipids. Organisms grown in the presence of $C_{14:1}$ and $C_{16:1}$ fatty acids led to enrichment in residues of these acids to the greatest extent (Table 15). Enrichment with $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ residues was to a lesser extent, while that with $C_{20:1}$ residues was a mere 13%.

EFFECT OF FATTY-ACYL UNSATURATION AND CHAIN LENGTH ON PERMEATION OF SULPHITE INTO YEASTS

Woolf-Eadie plots of initial rates of sulphite accumulation in anaerobically-grown Sacch. cerevisiae NCYC 431 gave vertical plots (Fig. 12). The permeability coefficients differ between organisms grown in media supplemented with different unsaturated fatty acids. A plot of permeability coefficient against Δmol^{-1} value for permeation of sulphite by all four yeast strains showed that the value for the coefficient was greater the lower the Δmol^{-1} value (Fig. 13). Values for permeability coefficient and Δmol^{-1} were linearly related for Sacch. cerevisiae NCYC 431 enriched in residues of $C_{14:1}$, $C_{16:1}$, $C_{18:1}$ and $C_{20:1}$ and also for this strain enriched in $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ residues (Fig. 14). However, a plot of permeability coefficient against mean fatty-acyl chain

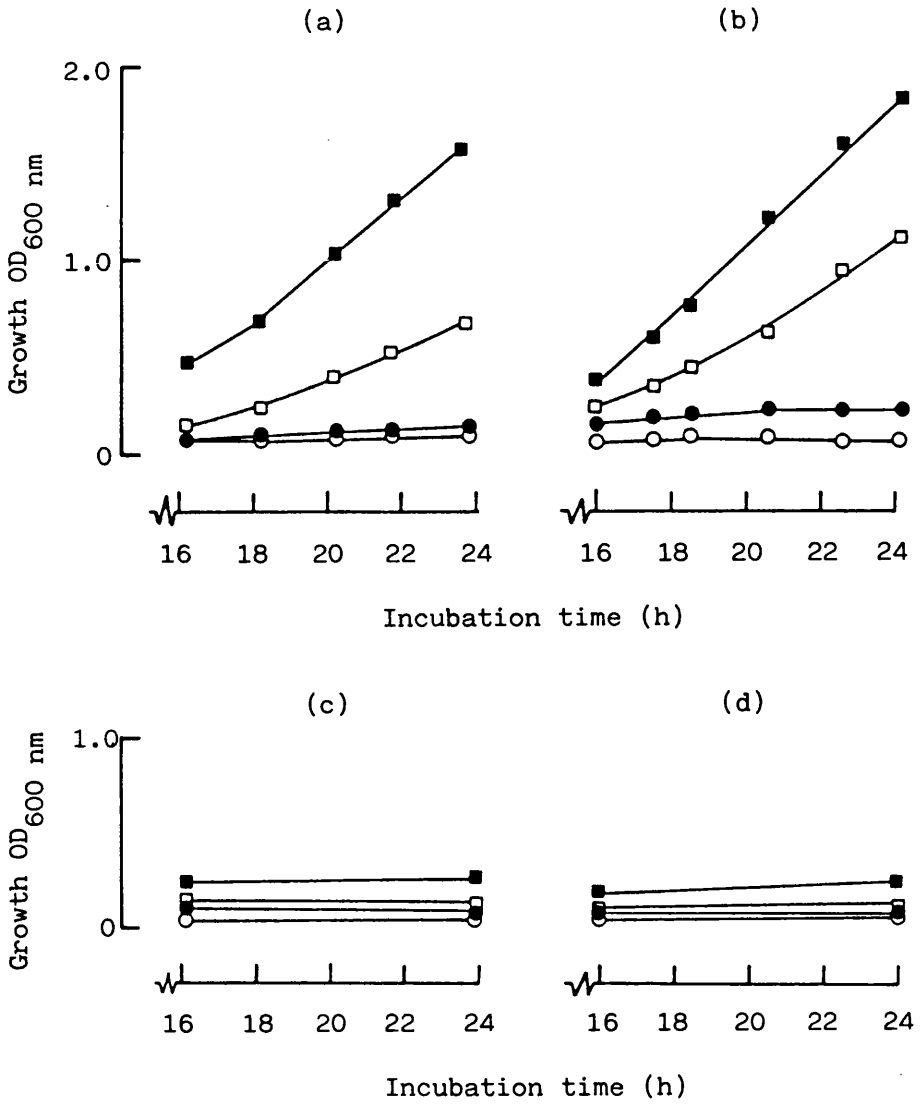


Figure 11. Time-course of growth of Saccharomyces cerevisiae TC8 (a), Saccharomyces cerevisiae NCYC 431 (b), Zygosaccharomyces bailii NCYC 1427 (c) and Zygosaccharomyces bailii NCYC 563 (d) grown anaerobically at 30°C in Medium C only (○) or Medium C supplemented with 30 mg oleic acid l^{-1}, (●), 5 mg ergosterol l^{-1} (□), or with both 5 mg ergosterol l^{-1} and 30 mg oleic acid l^{-1} (■).

Table 15. Fatty-acyl composition of phospholipids from anaerobically-grown Saccharomyces cerevisiae NCYC 431 grown in medium supplemented with ergosterol and an unsaturated fatty acid. Values quoted are the means of three independent determinations ± SD. tr indicates that a trace was detected, - that none was detected.

Fatty-acyl	Percentage composition of fatty-acyl residues in phospholipids from organisms grown anaerobically in media supplemented with:-					
	$C_{14:1}$	$C_{16:1}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$
8:0	-	-	-	-	-	4.0±0.5
10:0	tr	3.1±1.3	7.8±3.8	5.9±3.4	4.6±2.7	16.1±1.6
12:0	tr	4.4±1.3	7.8±2.4	4.7±2.6	4.8±1.9	17.5±1.5
14:0	3.8±1.9	7.2±1.1	15.1±2.4	9.8±2.4	9.8±2.4	13.4±1.4
14:1	52.4±2.0	tr	tr	-	-	2.1±0.6
16:0	34.0±2.0	28.1±1.7	28.0±3.6	32.9±1.1	35.7±1.4	22.9±2.6
16:1	2.1±0.3	52.1±6.1	3.7±1.6	1.1±0.6	0.7±0.4	6.6±2.5
18:0	5.6±1.0	4.7±1.1	tr	4.9±1.6	5.2±1.2	2.8±0.4
18:1	tr	tr	35.6±6.0	-	-	1.5±0.5
18:2	-	-	-	40.9±6.8	-	-
18:3	-	-	-	-	38.2±5.9	-
20:1	-	-	-	-	-	13.1±5.6

Figure 12. Woolfe-Eadie plots for the accumulation of molecular SO_2 by anaerobically-grown Saccharomyces cerevisiae NCYC 431 in medium supplemented with ergosterol (5 mg l^{-1}) and 30 mg myristoleic acid (○), palmitoleic acid (●), oleic acid (□), 11-eicosenoic acid (■), linoleic acid (△) or linolenic acid l^{-1} (▲). Organisms were suspended in 30 mM-citrate buffer (pH 3.0) containing 100 mM-glucose at 30°C and supplemented with 50 µmol, 125 µmol or 250 µmol sulphite. Concentrations of molecular SO_2 were calculated from data of King et al. (1981). Bars indicate SD.

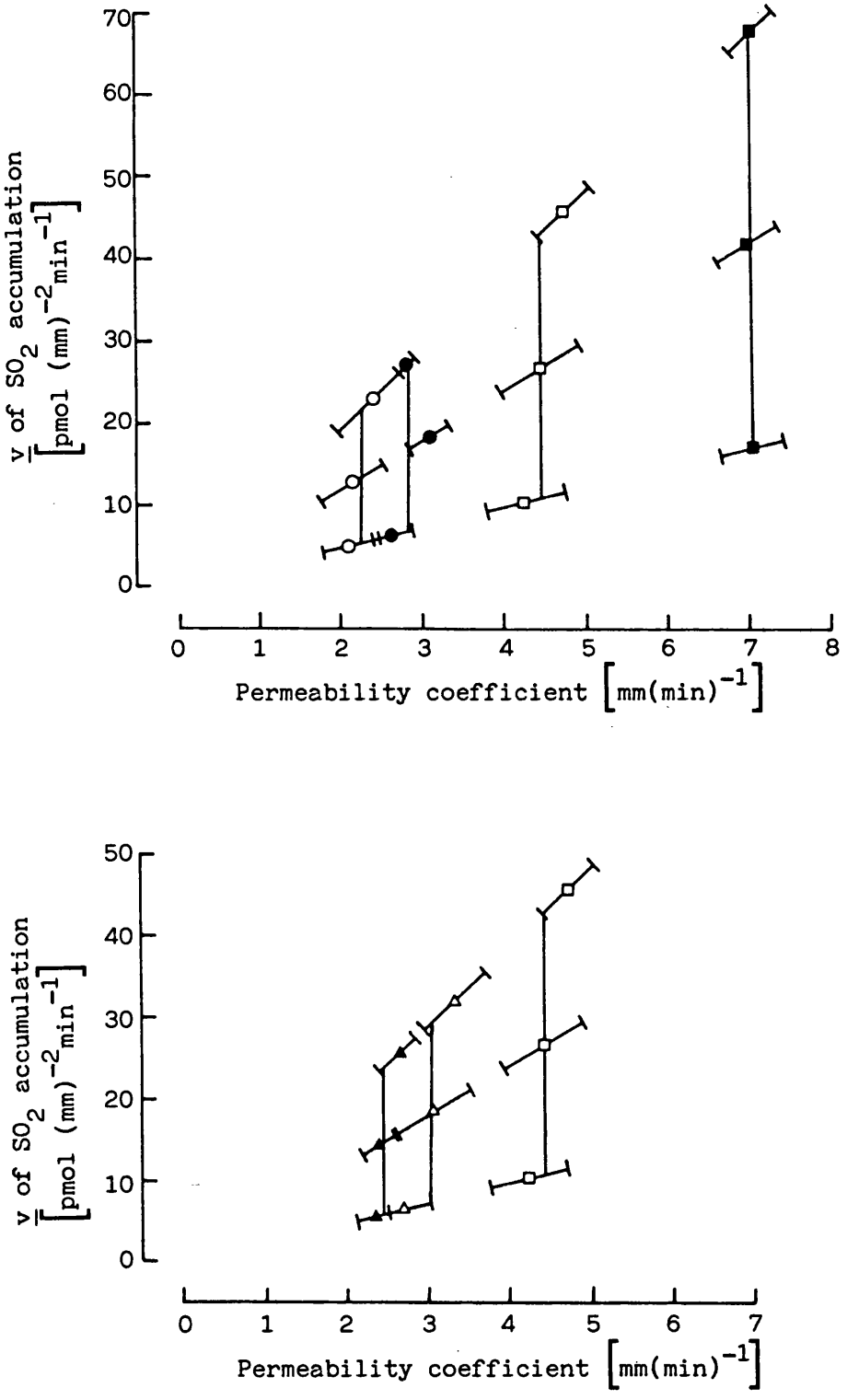

v of SO_2 accumulation [pmol (mm)$^{-2}$min^{-1}]
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8
Permeability coefficient [mm(min)$^{-1}$]
v of SO_2 accumulation [pmol (mm)$^{-2}$min^{-1}]
50
40
30
20
10
0
0 1 2 3 4 5 6 7
Permeability coefficient [mm(min)$^{-1}$]

Figure 12.

Figure 13. Correlation between the permeability coefficient for SO_2 accumulation by organisms and the degree of unsaturation of fatty-acyl residues in phospholipids isolated from aerobically-grown Saccharomyces cerevisiae NCYC 431 (a), Saccharomyces cerevisiae TC8 (b), Zygosaccharomyces bailii NCYC 1427 (c), Zygosaccharomyces bailii NCYC 563 (d) and from anaerobically-grown Saccharomyces cerevisiae NCYC 431 grown in media supplemented with ergosterol and (i) myristoleic acid, (ii) palmitoleic acid, (iii) oleic acid, (iv) linoleic acid, (v) linolenic acid or (vi) 11-eicosenoic acid. Values for Δmol^{-1} were calculated as described by Kates and Hagen (1964). Values quoted are the means of three independent determinations ± SD.

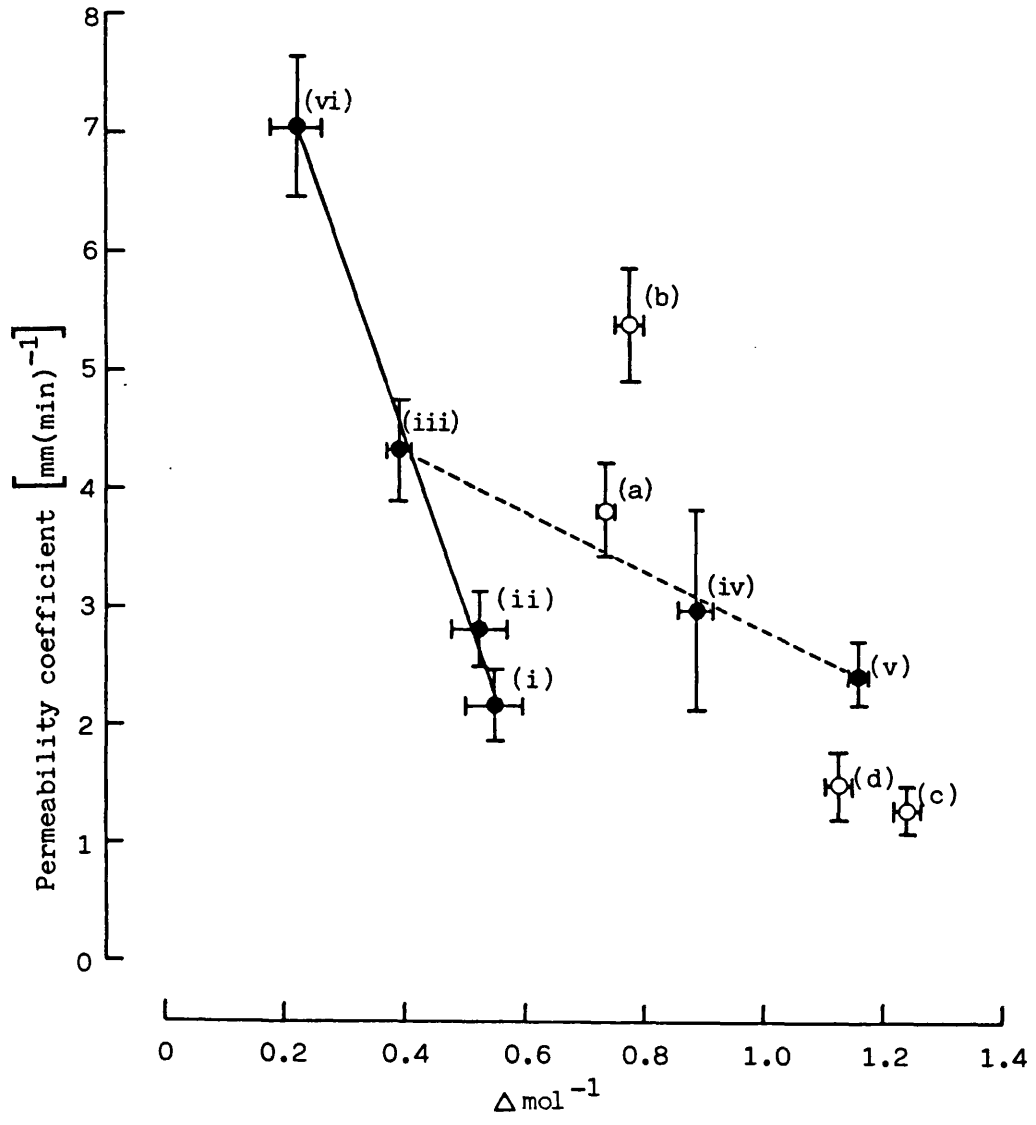

Permeability coefficient [mm(min)$^{-1}$]
8
7
6
5
4
3
2
1
0
(vi)
(b)
(iii)
(a)
(ii)
(iv)
(i)
(v)
(d)
(c)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Δ mol^{-1}

Figure 13.

Figure 14. Relationship between the mean fatty-acyl chain length and degree of unsaturation (Δmol^{-1}) of fatty-acyl residues in phospholipids isolated from aerobically-grown Saccharomyces cerevisiae NCYC 431 (a), Saccharomyces cerevisiae TC8 (b), Zygosaccharomyces bailii NCYC 1427 (c), Zygosaccharomyces bailii NCYC 563 (d) and from anaerobically-grown Saccharomyces cerevisiae NCYC 431 supplemented with ergosterol and (i) myristoleic acid, (ii) palmitoleic acid, (iii) oleic acid, (iv) linoleic acid, (v) linolenic acid or (vi) 11-eicosenoic acid. Values for Δmol^{-1} were calculated as described by Kates and Hagen (1964). Values quoted are the means of three separate determinations $\pm$ SD.

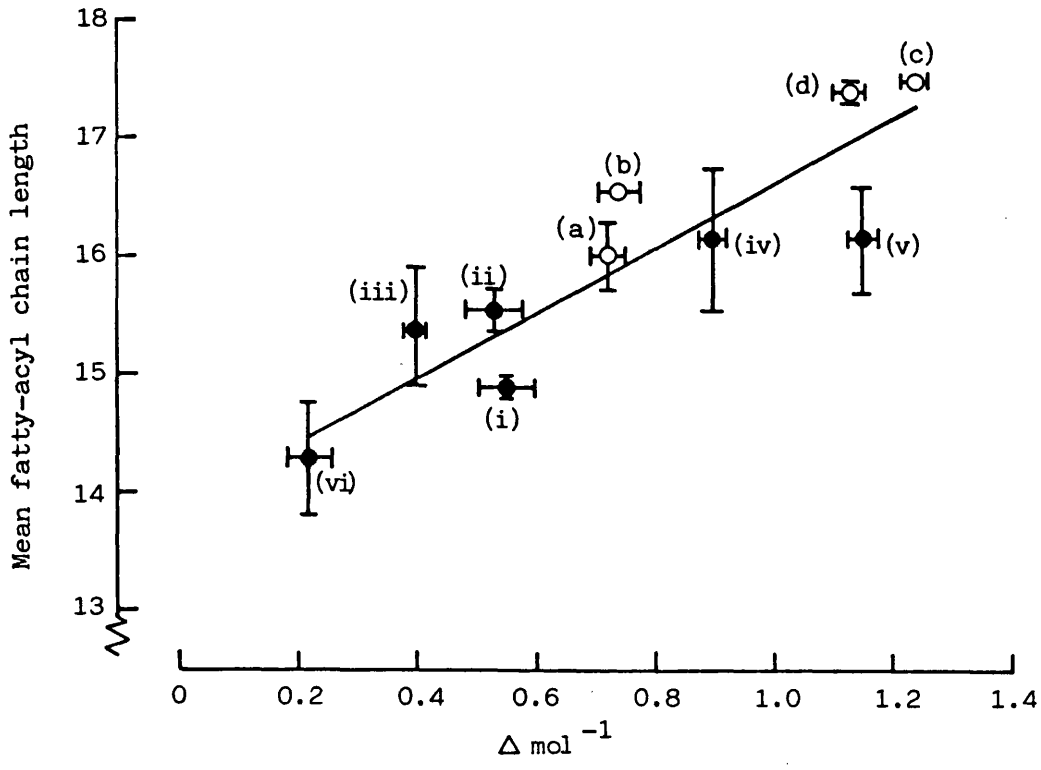

Mean fatty-acyl chain length
18
17
16
15
14
13
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Δ mol $^{-1}$
(a)
(b)
(c)
(d)
(i)
(ii)
(iii)
(iv)
(v)
(vi)

Figure 14.

Figure 15. Plot of the permeability coefficient for accumulation of SO_2 and the mean fatty-acyl chain lengths of phospholipids isolated from aerobically-grown Saccharomyces cerevisiae NCYC 431 (a), Saccharomyces cerevisiae TC8 (b), Zygosaccharomyces bailii NCYC 1427 (c), Zygosaccharomyces bailii NCYC 563 (d) and from anaerobically-grown Saccharomyces cerevisiae NCYC 431 supplemented with ergosterol and (i) myristoleic acid, (ii) palmitoleic acid, (iii) oleic acid, (iv) linoleic acid, (v) linolenic acid or (vi) 11-eicosenoic acid. Values quoted are the means of three independent determinations $\pm$SD.

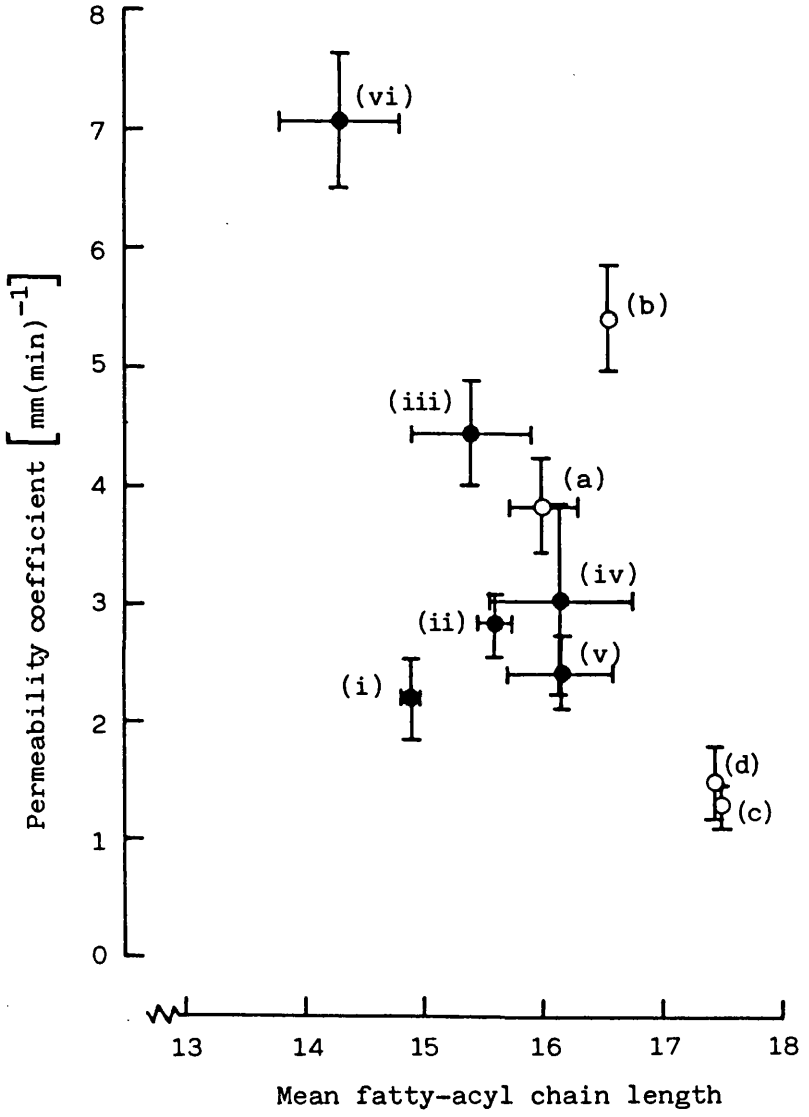

Permeability coefficient [mm(min)$^{-1}$]
0
1
2
3
4
5
6
7
8
13
14
15
16
17
18
Mean fatty-acyl chain length
(i)
(ii)
(iii)
(iv)
(v)
(vi)
(a)
(b)
(c)
(d)

Figure 15.

length in phospholipids showed no significant correlation (Fig. 15).

In all four yeasts there was a very good positive correlation between values for Δmol^{-1} and mean fatty-acyl chain length of phospholipids (Table 16, Fig. 14). The correlation coefficient calculated with eight degrees of freedom was 0.887 which with 99.9% confidence was very highly significant. There was also a very significant correlation between the permeability coefficient for accumulation of SO_2 measured in all four strains and the ratio of the mean fatty-acyl chain lengths and degree of unsaturation (Δmol^{-1}) of total phospholipids (Fig. 16). These data had a highly significant correlation coefficient of 0.791 with 99% confidence limits.

The total phospholipid content of anaerobically grown Sacch. cerevisiae NCYC 431 enriched with an unsaturated fatty-acyl residue was lower than that found in aerobically grown organisms, although the value was not affected by the nature of the supplement (Table 17). Similarly, proportions of each phospholipid class did not vary when organisms were grown with different anaerobic supplements, with one exception. Organisms grown in medium supplemented with myristoleic acid contained a proportionally larger quantity of phosphatidylinositol and less phosphatidylcholine compared with organisms grown with other supplements (Table 17). Only very small differences were observed when the proportions of phospholipid classes were compared between aerobically and anaerobically cultured Sacch. cerevisiae NCYC 431. Aerobically-grown organisms contained a higher proportion of phosphatidylethanolamine and a

Table 16. Mean fatty-acyl chain length and degree of unsaturation (Δmol^{-1}) of total phospolipids in Saccharomyces cerevisiae NCYC 431, grown anaerobically in media supplemented with ergosterol and an unsaturated fatty acid, compared with permeability coefficients calculated from data presented in the Woolf-Eadie plots (Fig. 12). Values for Δmol^{-1} were calculated as described by Kates and Hagen (1964). Values quoted are the means of at least three independent analyses ±SD.

Fatty-acyl supplement	Mean fatty-acyl chain length of total phospholipid	Value for Δmol^{-1} for total phospholipid	Permeability coefficient ($mm(min)^{-1}$)
$C_{14:1}$	14.91 ± 0.10	0.55 ± 0.05	2.23 ± 0.35
$C_{16:1}$	15.59 ± 0.15	0.53 ± 0.05	2.85 ± 0.30
$C_{18:1}$	15.41 ± 0.54	0.40 ± 0.01	4.48 ± 0.43
$C_{18:2}$	16.16 ± 0.67	0.90 ± 0.02	3.07 ± 0.79
$C_{18:3}$	16.14 ± 0.48	1.16 ± 0.02	2.42 ± 0.32
$C_{20:1}$	14.31 ± 0.51	0.22 ± 0.04	7.04 ± 0.61

Figure 16. Correlation between the permeability coefficient for accumulation of SO_2 and the ratio of mean fatty-acyl chain lengths and the degree of unsaturation (mol^{-1}) of total phospholipids in aerobically-grown Saccharomyces cerevisiae NCYC 431 (a), Saccharomyces cerevisiae TC8 (b), Zygosaccharomyces bailii NCYC 1427 (c), Zygosaccharomyces bailii NCYC 563 (d) and from anaerobically-grown Saccharomyces cerevisiae NCYC 431 supplemented with ergosterol and (i) myristoleic acid, (ii) palmitoleic acid, (iii) oleic acid, (iv) linoleic acid, (v) linolenic acid or (vi) 11-eicosenoic acid. Values quoted are the mean of three independent determinations ±SD.

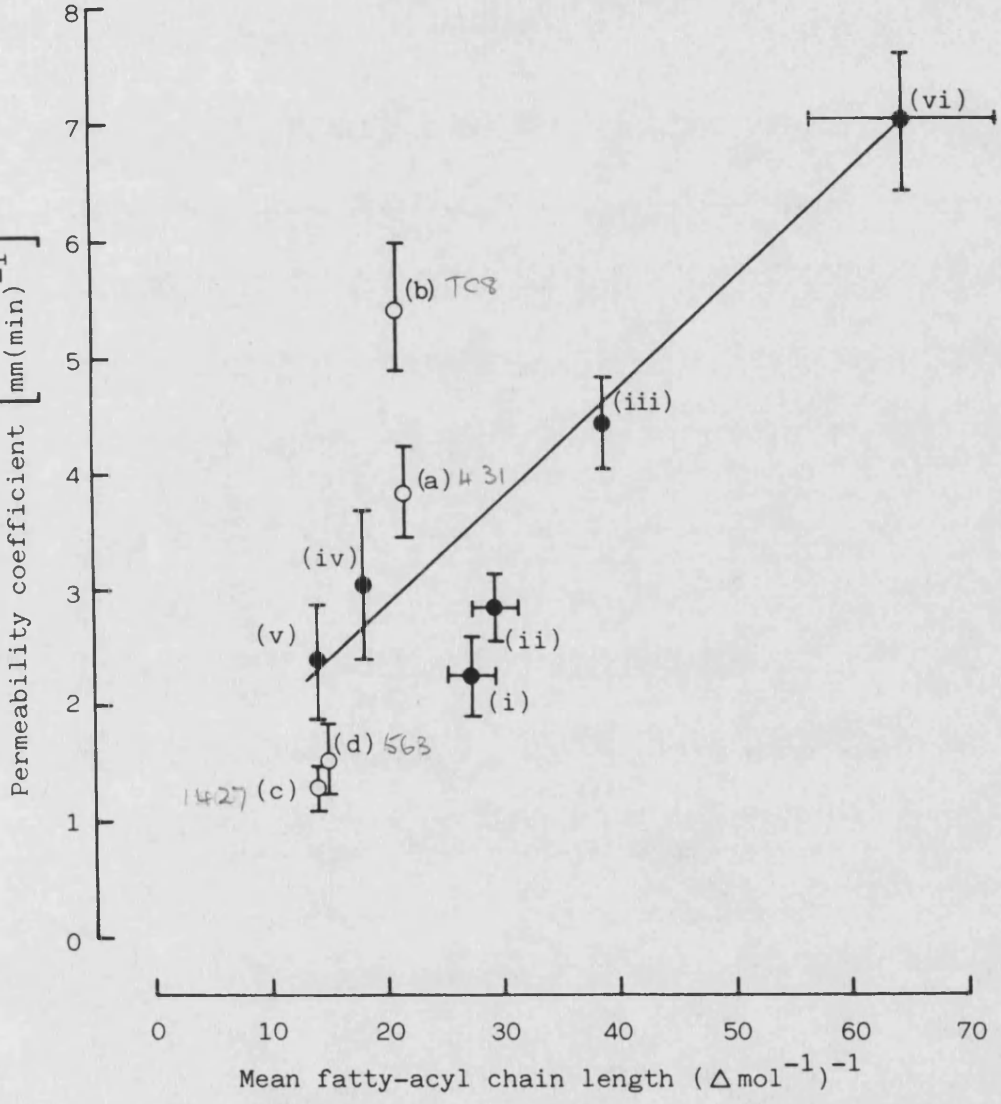

Permeability coefficient [mm(min)$^{-1}$]
Mean fatty-acyl chain length (Δmol^{-1})$^{-1}$
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70
(vi)
(b) TC8
(iii)
(a) 431
(iv)
(v)
(ii)
(i)
(d) 563
1427 (c)

Figure 16.

Table 17. Total phospholipid contents of anaerobically-grown Saccharomyces cerevisiae NCYC 431 in media supplemented with ergosterol and an unsaturated fatty acid and the relative proportions of each of the phospholipid classes. Values quoted are the means of three independent analyses ±SD.

Fatty-acyl supplement	Total phospholipid content (mg(250 mg dry wt organisms)$^{-1}$	Percentage of the total phospholipid classes			
		PC	PE	PI	PS
$C_{14:1}$	7.72 ± 0.90	46.6 ± 1.5	20.8 ± 1.4	29.2 ± 2.2	3.4 ± 1.8
$C_{16:1}$	8.24 ± 0.74	59.2 ± 1.4	21.8 ± 0.4	14.2 ± 2.4	4.9 ± 1.7
$C_{18:1}$	8.58 ± 0.74	56.3 ± 3.7	18.1 ± 1.1	18.2 ± 1.6	7.4 ± 2.9
$C_{18:2}$	8.49 ± 0.46	58.0 ± 1.1	16.9 ± 1.7	19.9 ± 2.1	5.3 ± 1.4
$C_{18:3}$	8.21 ± 0.80	56.5 ± 4.2	16.9 ± 1.3	19.9 ± 3.8	6.7 ± 1.8
$C_{20:1}$	8.80 ± 0.47	51.2 ± 4.2	25.6 ± 4.2	16.5 ± 5.2	6.6 ± 0.7

lower proportion of phosphatidylinositol compared with those grown anaerobically (Tables 8 and 17).

Values for Δmol^{-1} calculated for phospholipids from anaerobically-grown Sacch. cerevisiae NCYC 431 differ according to the nature of the fatty-acid supplement. Within each culture, Δmol^{-1} values for phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine are all very similar. Values for Δmol^{-1} for phosphatidylinositol are all much lower with the exception of phospholipids from organisms grown in media supplemented with myristoleic acid where the Δmol^{-1} values for phosphatidylinositol are not significantly different (Table 18). Mean fatty-acyl chain lengths in phospholipid classes gave a similar relationship. Mean fatty-acyl chain lengths of phospholipids from anaerobically grown Sacch. cerevisiae NCYC 431 also differ according to the nature of the fatty-acid supplement. Within each culture, mean fatty-acyl chain-length values in phospholipid classes are very similar with the exception of phosphatidylinositol which generally has a lower mean fatty-acyl chain length. However, in cultures supplemented with myristoleic acid or palmitoleic acid there was no significant difference between the mean fatty-acyl chain lengths of any of the phospholipid classes (Table 19).

Table 18. Degree of unsaturation (Δmol^{-1}) of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) found in Saccharomyces cerevisiae NCYC 431 grown anaerobically in media supplemented with ergosterol and an unsaturated fatty acid. Values quoted are the means of three independent analyses ± SD. Values for Δmol^{-1} values were calculated as described by Kates and Hagen (1964).

Fatty-acyl supplement	Δmol^{-1} value PC	PE	PI	PS
$C_{14:1}$	0.53 ± 0.03	0.57 ± 0.03	0.47 ± 0.05	0.45 ± 0.06
$C_{16:1}$	0.53 ± 0.06	0.62 ± 0.07	0.31 ± 0.07	0.62 ± 0.07
$C_{18:1}$	0.41 ± 0.04	0.48 ± 0.04	0.17 ± 0.03	0.53 ± 0.07
$C_{18:2}$	0.83 ± 0.05	0.92 ± 0.10	0.40 ± 0.05	0.81 ± 0.09
$C_{18:3}$	1.22 ± 0.13	1.22 ± 0.13	0.52 ± 0.10	1.07 ± 0.04
$C_{20:1}$	0.41 ± 0.08	0.36 ± 0.06	0.12 ± 0.04	0.52 ± 0.03

Table 19. Mean fatty-acyl chain lengths of each of the phospholipid classes found in Saccharomyces cerevisiae NCYC 431 grown anaerobically in media supplemented with ergosterol and an unsaturated fatty acid. Values quoted are the means of three independent analyses ± SD.

Fatty-acyl supplement	Mean fatty-acyl chain length in PC	PE	PI	PS
$C_{14:1}$	15.19 ± 0.40	14.85 ± 0.10	15.21 ± 0.13	15.20 ± 0.31
$C_{16:1}$	15.74 ± 0.20	15.57 ± 0.30	15.77 ± 0.10	15.56 ± 0.48
$C_{18:1}$	15.94 ± 0.08	16.11 ± 0.22	14.81 ± 0.35	16.16 ± 0.45
$C_{18:2}$	16.21 ± 0.22	16.43 ± 0.34	15.39 ± 0.51	16.36 ± 0.26
$C_{18:3}$	16.50 ± 0.09	16.39 ± 0.14	15.46 ± 0.41	16.24 ± 0.18
$C_{20:1}$	15.95 ± 0.22	15.23 ± 0.42	14.29 ± 0.63	16.31 ± 0.13

DISCUSSION

The investigations performed can best be discussed by dividing them into four broad sections. Firstly, there is screening for sulphite tolerance in yeasts; secondly, the short-term effect of exposure of yeasts to sulphite; thirdly, the longer term effects, up to six hours; and finally the contribution of plasma-membrane phospholipid composition in the control of diffusion of SO_2 into yeasts.

SCREENING FOR SULPHITE TOLERANCE IN YEASTS

Initially it was necessary to isolate a limited number of strains that displayed a variety of responses to sulphite; four were selected. Two strains of Sacch. cerevisiae, selected without any knowledge of their reaction to sulphite, were used to compare sulphite resistance with two of Zygosacch. bailii, which have been reported to be extremely resistant to the compound (Thomas and Davenport, 1985; Warth, 1985). The first two were Sacch. cerevisiae NCYC 431, which is a strain originating from a distillery and having a high tolerance of ethanol (Cartwright et al., 1986, 1987) and Sacch. cerevisiae TC8, which is a strain used in cider-making and has been reported to excrete H_2S (Stratford and Rose, 1985). It was surprising, therefore, to find that, of the four strains examined, one of Sacch. cerevisiae was the most tolerant to sulphite while a strain of Zygosacch. bailii was the most sensitive. The availability of authenticated strains of Zygosacch. bailii is limited. Zygosaccharomyces bailii NCYC 563 was included

in the survey because it has been used in research into sulphite resistance of spoilage yeasts (Cole et al., 1987). Significantly, it was the least resistant of the strains examined in the present study.

INITIAL EFFECTS OF SULPHITE ACCUMULATION IN YEASTS

Sulphur dioxide transport

Two yeasts, namely Sacch. cerevisiae (Stratford and Rose, 1986) and S'codes ludwigii (Stratford et al., 1987), have been shown to transport SO_2 by free diffusion, based on evidence from vertical Woolf-Eadie plots. The present report shows that passage of SO_2 into strains of Zygosacch. bailii is also by free diffusion. It was also interesting to note that deviation from the vertical, observed in the present study with strains of Zygosacch. bailii and previously with Sacch. cerevisiae TC8 (Stratford and Rose, 1986) and S'codes ludwigii (Stratford et al., 1987), was very much more pronounced with Sacch. cerevisiae NCYC 431. This suggests that, at low concentrations of SO_2, a facilitated transport system operates, possibly to transport the HSO_3^- ion. This proposal is in agreement with Benítez et al. (1983) and Garcia et al. (1983) who investigated the possibility of there being such an active transport system in strains of C. utilis. Selenate-resistant mutants of C. utilis were shown to have a common transport defect showing an inability to grow in media with either sulphite, sulphate or thiosulphate as the sole source of sulphur whereas the wild type grew with any one of these sources. In addition, the sulphur oxy-anions sulphite, thiosulphate and dithonate were seen

to inhibit competitively active transport of sulphate in wild-type strains. Therefore a possible explanation for the biphasic Woolf-Eadie plots seen in the present study is that the common active transport system observed in C. utilis may well be the same as that intimated by Stratford and Rose (1986) and which predominates at low concentrations of sulphite. As sulphite concentrations are increased, this system rapidly becomes saturated and masked by diffusion of higher concentrations of molecular SO_2.

The importance of diffusion of molecular SO_2 into organisms is often overlooked, especially by experimenters primarily concerned with active transport systems involving sulphite and related anions. Tweedie and Segel (1970) recorded the existence of distinct permeases for sulphite and tetrathionate in Penicillium and Aspergillus species. However, evidence for a sulphite-specific permease is still questionable, for the data could equally be interpreted by simple leakage. All transport studies using multianionic systems are fraught with problems due to oxidation and cross reaction of anions. Tweedie and Segel (1970) clearly recognised these disadvantages but, like Benítez et al. (1983), did not consider the equilibrium position of sulphite. Wherever HSO_3^- ions exist in solution some proportion must be present as molecular SO_2 depending on the pH value. Evidence for the accumulation of sulphite may be misleading in these cases and, in fact, merely reflect molecular SO_2 accumulation. Certainly, in those yeasts that are a major cause of food-spoilage and from the present data, it seems likely that diffusion of molecular SO_2 is common.

Initial rates of accumulation of SO_2 are quoted in this thesis in units of SO_2 accumulated per mm^2 surface area of plasma membrane per minute which takes into account the different sizes of the different species of yeast. Estimated cell-surface areas are assumed to equal plasma-membrane surface areas of organisms. Individual organisms of Zygosacch. bailii have mean plasma-membrane surface areas approximately twice that of either Sacch. cerevisiae strain examined. Therefore, by quoting initial rates of accumulation in this manner, the data have greater physiological significance. Similarly, by using intracellular water volume as an approximation for cytoplasmic volume instead of dry weight, intracellular concentrations of SO_2 are made more meaningful and may be compared between different yeasts. Intracellular water volumes of individual organisms of Zygosacch. bailii have a mean value approximately 90% larger than that of Sacch. cerevisiae.

Intracellular water volumes and intracellular pH values

Intracellular water volumes were not affected by short-term exposure to sulphite, which seems to contradict data put forward by Cole and Keenan (1987) declaring that intracellular water volumes of yeasts decrease in the presence of acid preservatives. Cole and Keenan (1987) found that there is an inverse relationship between protoplast volume and population doubling time, and they proposed that energy is diverted towards maintenance of intracellular pH value, so that less energy is available for biosynthesis, resulting in a slower growth rate and a decrease in protoplast volume. However, these workers found that there was no simple relationship

between intracellular pH value and doubling time. The present investigations show that, during short-term exposure to sulphite in all four yeasts studied, there was no change in protoplast volume despite retardation of growth. It would appear that these yeasts are able to maintain their physical condition in the presence of sulphite in the short term. However, observations were not made on the condition of subsequent generations when retardation of growth was evident.

All of the organisms studied were notably resilient toward sulphite and were able to maintain viability after short-term exposure to 2 mM-sulphite. Indeed, even when growth was arrested and the transmembrane pH gradient severely decreased, organisms were able to recover and undergo normal exponential growth.

On exposure to sulphite, strains of Sacch. cerevisiae and Zygosacch. bailii were seen to attain intracellular concentrations of SO_2 exceeding 100 times that outside organisms. Zygosaccharomyces bailii NCYC 563 concentrated SO_2 by over 200-fold in the presence of 0.5 mM-sulphite. If the influx of SO_2 is governed by the intracellular pH value of yeasts and the dynamic equilibrium between SO_2, HSO_3^- and SO_3^{2-}, then it should be possible to predict intracellular concentrations of SO_2 (Krebs et al., 1983). Taking Zygosacch. bailii NCYC 563 as an example, with an intracellular pH value of approximately 6.4 and an extracellular pH value of 3.0, 0.002 and 5.6% of free sulphite exists in the molecular form respectively (King et al., 1981). If intracellular pH value were the only constraint on influx of SO_2, one would expect to see a 2800-fold concentration of SO_2 in these

organisms. Clearly this is never achieved. Cole and Keenan (1986) found that, in similar experiments with Zygosacch. bailii NCYC 563, the equilibrium distribution of benzoic acid could not be explained by the difference in pH value across the plasma membrane. Warth (1988) observed a similar result when investigating accumulation of benzoic acid by Zygosacch. bailii. It is reasonable to assume that, within the cytoplasm, the pH value is not constant and more probably there exists a complex network of different intracellular pH values and intracellular weak-acid concentrations within different sub-cellular organelles and domains.

Other considerations include the presence of both intracellular and extracellular sulphite-binding compounds. Glucose, in the extracellular buffer, is known to bind sulphite which acts to lower the extracellular concentration of sulphite. Indeed, this effect was seen in control flasks when sulphite (1 mM) was added to medium containing glucose (20 g l^{-1}) with a pH value of 4.0. It resulted in a 15.3% decrease in the concentration of free sulphite. Similarly, Vas (1949) found that, when sulphite (5 mM) was added to buffer (pH 3.97) containing glucose (50 g l^{-1}), 29.2% of sulphite became bound. Over the pH range between 3.0 and 5.5 the value for the equilibrium constant for the sulphite-glucose complex remains practically unchanged (Vas, 1949). Therefore a similar pattern of binding should be observed at a pH value of 3.0. The percentage of sulphite that becomes bound to glucose will naturally depend on the concentrations of both glucose and sulphite present but, in the experimental conditions described, it is unlikely that sulphite-binding by glucose could account for any more than a 30%

decline in free sulphite concentration. Thus, the predicted accumulation of sulphite in the example quoted above is at least 2000 times that in the extracellular buffer, which is still unrealistic. It is difficult to explain this paradox. A number of factors are likely to be involved including the sulphite-binding capacity of intracellular constituents, production and excretion of sulphite-binding compounds and the buffering capacity of organisms.

Warth (1988) explains the non-equilibrium uptake of benzoic acid by postulating an active transport system for the export of anions. But, if the cytoplasmic pH value is maintained, this requires continuous and unreasonable energy expenditure. Recently Cole and Keenan (1987) recorded cytoplasmic pH values of 5.70 and 6.05 for exponential-phase cells of Zygosacch. bailii NCYC 563 where the extracellular media had pH values of 2.8 and 4.5, respectively. Similarly low intracellular pH values were also found by using fluorescein fluorescence (Cole and Keenan, 1987). A very low cytoplasmic pH value would explain the apparently low sulphite concentrations observed and remove the need for active expulsion of anions. However, the validity of these pH values is questioned (Warth, 1988) and is not supported by the present study. The technique using fluorescein diacetate to measure intracellular pH value under the present conditions was found to be wholly unreliable, and was rejected in favour of the method using radiolabelled propionic acid.

Each of the four yeasts examined, on exposure to sulphite, accumulated SO_2 rapidly until equilibrium was achieved. The final intracellular concentrations varied among organisms and are most

likely a function of their intracellular buffering capacities. Intracellular pH values remain fairly constant in the presence of low concentrations of sulphite but decline rapidly once these are raised above 1-mM sulphite. A threshold is reached where organisms can no longer maintain their intracellular pH value. Buffering capacity becomes exhausted, and intracellular pH values decline with the influx and dissociation of more SO_2. Notably, intracellular sulphite concentrations at equilibrium increase linearly with extracellular sulphite concentration. This is in keeping with a system of free diffusion until the threshold is reached when, presumably, buffering capacity is exceeded, intracellular pH control breaks down resulting in a decline in the transmembrane pH gradient and dissipation of the proton-motive force across the plasma-membrane. A result of this would be to retard or inactivate processes, such as active transport of solutes, that require energy from the proton-motive force. These data are consistent with the rapid decrease in the content of ATP in Sacch. cerevisiae when exposed to sulphite (Schimz and Holzer, 1979; Hinze and Holzer, 1986).

Prakash et al. (1986) found that the decreasing effects on the intracellular ATP level are synergistically potentiated when sulphite is added together with either m-chloro-peroxybenzoic acid (CPBA) or nitrite. The mechanisms involved in the synergistic action of these glycolytic enzyme inhibitors are not fully understood, but may prove useful in maximising the antimicrobial effect of sulphite on yeasts.

There is no direct correlation between concentration of sulphite after equilibration and tolerance to this preservative, although Zygosacch. bailii NCYC 1427 is significantly able to maintain a higher intracellular pH value in the presence of sulphite than the other yeasts examined, which may be contributory in its relative resistance. However, this trend does not extend to Sacch. cerevisiae NCYC 431, the other tolerant strain, or to the less tolerant strains studied.

In the absence of sulphite, all four yeast strains maintained intracellular pH values between pH 6.4 and 6.7 when they were allowed to equilibrate under the conditions described. The two more tolerant strains, namely, Sacch. cerevisiae NCYC 431 and Zygosacch. bailii NCYC 1427, maintained intracellular pH values that were highest in this range. When organisms were exposed to low concentrations of sulphite (0.1 - 1.0 mM), the less tolerant strains, Zygosacch. bailii NCYC 563 and Sacch. cerevisiae TC8, showed a greater decline in intracellular pH value than either of the more tolerant strains which indicates that intracellular pH control may be important in sulphite resistance.

The ability of yeasts to grow in the presence of sulphite is primarily a function of their ability to produce acetaldehyde. However, during the first few minutes of exposure to sulphite, it appears that the intracellular buffering capacities of different strains of yeast are important and, in terms of sulphite resistance in yeasts, this may represent a first line of defence.

The buffering capacity of yeast is largely attributed to their ability to actively extrude hydrogen ions. The buffering action of

actively excreted metabolites, e.g. carbon dioxide and organic acids, is thought to contribute only 15 to 40% to the overall buffering capacity (Sigler et al., 1981b). Active transport of charged species requires ATPase activity and the presence of intracellular diffusable anions not only in sufficient quantity but also of sufficiently high plasma-membrane permeability. Consequently, their availability could limit the buffering capacity of the organism. In the future, it would be helpful to find out if the activity of plasma-membrane ATPase is related to sulphite tolerance in yeasts and the importance of its role in the recovery of inhibited yeasts.

LONG-TERM EFFECTS OF SULPHITE

Stimulation of acetaldehyde production

The present study revealed a direct correlation between ability of yeasts to grow in the presence of sulphite and sulphite-induced production of acetaldehyde which suggests that production of this sulphite-binding compound contributes significantly to resistance. It is also noteworthy that the two most sulphite-resistant yeasts examined, namely Sacch. cerevisiae NCYC 431 and Zygosacch. bailii NCYC 1427, are able to produce large amounts of acetaldehyde when growth and ethanol production were almost completely inhibited by 2.0 mM-sulphite. The data are in agreement with the early findings of Neuberg and Reinfurth (1919) where, in the presence of sulphite, acetaldehyde and glycerol were produced in equimolar amounts by strains of Sacch. cerevisiae. Moreover, the data show for the first time that this is true also for strains of Zygosacch. bailii.

Production of glycerol by Zygosacch. acidifaciens (now recognised as Zygosacch. bailii) was reported by Nickerson and Carroll (1945) but this was demonstrated to arise from the existence of a Neuberg type III fermentation without addition of sulphite which had previously only thought to occur under alkaline conditions. The basic fermentation equation (Neuberg type III) from Freeman and Donald (1957) is as follows:

$$2\ \text{Glucose} \rightarrow 2\ \text{Glycerol} + 1\ \text{Acetic acid} + 1\ \text{Ethanol} + 2CO_2$$

With all four yeasts studied, there was significant glycerol production in the absence of sulphite via this fermentation. On addition of sulphite, the switch to Neuberg's second form of fermentation is evidently not complete. Generally the theoretical equimolar production of acetaldehyde and glycerol was not seen. This failure could be attributed to the fact that normal alcoholic fermentation and possibly Neuberg's third form of fermentation continue at decreased rates in the presence of sulphite, particularly evident with more tolerant strains (Sacch. cerevisiae NCYC 431 and Zygosacch. bailii NCYC 1427).

Saccharomyces cerevisiae NCYC 431, the most resistant strain examined, in the presence of 1 mM-sulphite was able to maintain normal growth and ethanol production while simultaneously producing additional equimolar amounts of glycerol and acetaldehyde. All of the other data show that additional acetaldehyde is produced in favour of ethanol. Pyruvate production was not stimulated by sulphite in any of the yeasts studied. Its production, like that of

ethanol, is directly correlated with cell growth.

It is also feasible that acetaldehyde might be produced by yeasts from oxidation of ethanol. Indeed, this has been commercially exploited to produce acetaldehyde (Wecker and Zall, 1987). Acetaldehyde production was induced by sulphite when meat-spoilage yeasts were grown with ethanol and in the absence of glucose. Acetaldehyde did not accumulate in the absence of sulphite (Nychas et al., 1988). Under these conditions, ethanol is oxidised to acetaldehyde and seen to accumulate as an intermediate of substrate catabolism. Free acetaldehyde is subsequently catabolised to acetic acid and the acetic acid to acetyl-CoA. NADH is finally regenerated during oxidative phosphorylation (Pons et al., 1986). In the presence of glucose, oxidative phosphorylation is suppressed and this pathway does not function. Conceivably, NADH could be regenerated with production of glycerol, but there is no evidence of this occurring. None of the data presented in this work show a decrease in ethanol concentration accompanied by glycerol production.

The ability of yeasts to produce acetaldehyde seems to be the most important factor enabling them to tolerate sulphite. It is most likely that the decline in intracellular pH value results when extracellular and intracellular sulphite-binding capacities are exceeded, and that tolerance to sulphite is determined by an organism's ability to withstand both a low intracellular pH value and to produce acetaldehyde. The reason why yeasts show different capacities to produce acetaldehyde in the presence of sulphite, and display different tolerances to this preservative, still remains to be elucidated.

PLASMA MEMBRANE COMPOSITION AND THE DIFFUSION OF SULPHUR DIOXIDE INTO YEASTS

Plasma-membrane composition of aerobically grown yeasts

Aerobically-grown Sacch. cerevisiae was found to contain phospholipids that were rich in $C_{16:1}$ and $C_{18:1}$ residues, with $C_{16:0}$ residues accounting for a minor proportion. Under the same conditions, strains of Zygosacch. bailii contained phospholipids with predominantly $C_{18:1}$ and $C_{18:2}$ fatty-acyl residues. These data are in keeping with those of Rattray (1988) who summarises the fatty-acyl composition of whole-cell lipids as distinct from phospholipids in these yeasts.

Proportions of the four major classes of phospholipid found in each yeast strain are broadly similar, but again, there are striking differences between those of Sacch. cerevisiae and Zygosacch. bailii. The latter have a higher proportion of phosphatidylinositol, a lower proportion of phosphatidylcholine and generally contain less phospholipid compared with strains of Sacch. cerevisiae. In all four yeasts examined, phosphatidylinositol contained fatty-acyl residues that were always more saturated than those found in the other phospholipid classes. This feature is thought to be of importance because phosphatidylinositol is recognised as a precursor involved in recently discovered secondary messenger systems controlling transduction in mammalian cells. In these cells, phosphatidylinositol is initially phosphorylated to phosphatidylinositol 4-phosphate and then to phosphatidylinositol 4,5-bisphosphate. Growth factors then, acting via a GTP-binding protein, stimulate a phosphodiesterase which cleaves phospha-

tidylinositol 4,5-biphosphate to diacylglycerol and inositol 1,4,5-triphosphate. The latter acts to release calcium, while diacylglycerol stimulates protein kinase C activity and it appears that both pathways act to control DNA synthesis (Berridge, 1987). Presently, similar evidence is accumulating for the existence of such systems in yeasts. The active secondary messenger, inositol 1,4,5-triphosphate, has already been detected in Sacch. cerevisiae (Kaibuchi et al., 1986). Moreover, the loss of radioactivity from pulse-labelled di- and tri-phosphoinositides in these organisms demonstrates rapid turnover of these intermediary compounds (Steiner and Lester, 1972a,b) reinforcing their potential role in a messenger system.

Permeability coefficients derived from the vertical Woolf-Eadie plots show the two strains of Zygosacch. bailii to have lower coefficients of SO_2 accumulation than either of the Sacch. cerevisiae strains which focuses ones thoughts on the specific plasma-membrane composition of each yeast and its contribution in the regulation of SO_2 diffusion. Both strains of Zygosacch. bailii show a slower rate of accumulation of propionic acid compared to either strain of Sacch. cerevisiae and, notably, do not accumulate fluorescein diacetate whereas both Sacch. cerevisiae NCYC 431 and TC8 readily take up this dye. All of these observations suggest that the plasma membranes of strains of Zygosacch. bailii and Sacch. cerevisiae have distinctive properties which allow them to act as selectively permeable barriers to diffusing molecules.

It has been suggested (Stratford et al., 1987) that the degree of phospholipid unsaturation within a plasma membrane will affect

the degree of fluidity and consequently the permeability coefficient of SO_2 accumulation. However, with the yeast strains used in the present study this did not prove to be true. Plasma membranes of the two strains of Zygosacch. bailii were less permeable to SO_2 despite having a much higher Δmol^{-1} value for cellular phospholipids compared to either strain of Sacch. cerevisiae. However, the mean fatty-acyl chain lengths of cellular phospholipids also varies among organisms and must be taken into consideration when describing membrane fluidity. It appears that Δmol^{-1} values alone inadequately describe membrane fluidity as they assume a uniform membrane thickness.

Plasma-membrane composition of anaerobically grown yeasts

In an attempt to separate and assess the contribution of the two variables of fatty-acyl chain length and degree of saturation of phospholipid fatty-acyl residues to plasma-membrane fluidity, Sacch. cerevisiae NCYC 431 was grown anaerobically in media supplemented with ergosterol and specific fatty acids. The aim was to bring about changes in plasma-membrane composition and therefore fluidity, and to see if these changes could affect the permeability to SO_2. It is apparent that the two variables are closely linked as one could not be changed without affecting the other. It can be inferred from these findings that there is stringent control of plasma membrane synthesis in Sacch. cerevisiae NCYC 431 even when fatty acids are supplied exogenously.

When Sacch. cerevisiae NCYC 431 was grown anaerobically with different fatty-acid supplements there was no significant change in

the dimensions of the organisms compared with those grown aerobically. Moreover, although there was a slight decrease in the number of organisms (mg dry wt)$^{-1}$ during the mid-exponential phase of growth when grown anaerobically compared with those grown aerobically, this was not affected by the nature of the fatty-acid supplement.

It appears that membrane stability of anaerobically-grown Sacch. cerevisiae NCYC 431 is maintained by an increased synthesis of shorter chain fatty-acyl residues, which was observed in organisms grown in the presence of longer chain unsaturated fatty acids. The highly significant correlation seen between mean fatty-acyl chain lengths and values for Δmol^{-1} for cellular phospholipids indicates that there is very rigid control of membrane fluidity in organisms. There seems to be a compromise between the requirement for a fluid membrane and the requirement for a stable bilayer. When only short-chain unsaturated fatty acids are available, organisms incorporating these fatty acids also synthesize a higher proportion of longer chain saturated phospholipids to compensate and to maintain a normal functional plasma membrane. Similarly, when organisms are grown anaerobically in medium supplemented with long-chain fatty acids ($C_{20:1}$), it appears that, with incorporation of long fatty-acyl residues, shorter residues, possibly originating from cleavage of long-chain fatty acids, are also incorporated.

The relative extent to which exogenously supplied fatty acids were incorporated into anaerobically-grown Sacch. cerevisiae NCYC 431 is in general agreement with the results reported by Nes et al. (1984). The very limited incorporation of $C_{20:1}$ residues could be

attributable to the inability of these relatively lengthy residues to be accommodated into cellular membranes.

Esfahani et al. (1981a) also observed a stringent requirement for an optimal concentration of saturated fatty-acyl chains with chain length of $C_{14:0}$ and $C_{16:0}$ in phospholipids for optimal growth of a double-mutant strain of Sacch. cerevisiae. However, no conclusions were drawn from the relative saturation of cellular phospholipids in this work.

The strict conservation of membrane fluidity was noted by Watson and Rose (1980) who proposed that, when Sacch. cerevisiae NCYC 366 was grown anaerobically, multiply unsaturated fatty acids are preferentially incorporated into triacylglycerols which are not membrane components. These workers also suggest that membrane fluidity could be balanced through synthesis of phosphatidylserine and phosphatidylinositol which, having a higher proportion of saturated residues, serve to maintain a degree of rigidity in the membrane. However, my data do not support this theory as there was no significant change in the proportions of each phospholipid class under different anaerobic conditions.

Notably, under anaerobic conditions, exogenously supplied unsaturated fatty-acyl residues were incorporated preferentially into phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine and reflected by the relatively high Δmol^{-1} values calculated for these phospholipid classes. With the exception of those organisms grown anaerobically in medium supplemented with myristoleic acid, phosphatidylinositol extracted from anaerobically-grown Sacch. cerevisiae NCYC 431 always

contained fatty-acyl residues that were more saturated than those from the other phospholipid classes. The strong conservation of the highly saturated form of fatty-acyl residues in phosphatidyl-inositol, which are synthesized even when fatty acids are supplied exogenously, gives support to the theory that it is involved in second messenger systems in these yeasts.

Diffusion of sulphur dioxide and plasma-membrane composition

The permeability coefficient of SO_2 accumulation by anaerobically grown Sacch. cerevisiae NCYC 431 was affected by both the degree of saturation and mean chain length of phospholipid fatty-acyl residues but from the initial data it is not possible to ascertain how each variable has its effect. There is no direct correlation between mean fatty-acyl chain lengths in cellular phospholipids and permeability coefficient of SO_2 accumulation. Nevertheless there are two linear relationships seen between values for Δmol^{-1} calculated for cellular phospholipid fatty-acyl residues and permeability coefficient of SO_2 accumulation. However, a direct correlation between permeability coefficient of SO_2 accumulation and the ratio of mean fatty-acyl chain lengths and values for Δmol^{-1} indicates that the most important factor in controlling the rate of diffusion of SO_2 into organisms is membrane thickness, that is the distance over which diffusing molecules have to travel to enter the organism. If the mean fatty-acyl chain length is increased then, assuming a typical fluid mosaic model, the thickness of the plasma-membrane will also increase and fluidity will decrease. Membrane thickness will also be dependent on the

presence of perturbing molecules affecting the configuration of the hydrocarbon regions and on the transition temperature.

Data derived from experiments with both aerobically and anaerobically-grown yeasts show a good correlation between permeability coefficient of SO_2 accumulation and the ratio of mean phospholipid fatty-acyl chain length and value for Δmol^{-1}. Generally it is useful to consider aerobically and anaerobically-grown organisms separately because under anaerobic conditions lipid composition was artificially altered. However, for analytical purposes, there is no reason to separate the data. Data derived from experiments with Sacch. cerevisiae TC8 are consistently different to those derived from those with Sacch. cerevisiae NCYC 431 where one might expect to see better agreement, although they are well within confidence limits. These discrepancies may arise from differences in plasma-membrane composition not measured in this study or from errors most likely derived from estimation in plasma-membrane surface area. Differences in the physiological structures of the two strains of Sacch. cerevisiae are supported by data relating to the number of organisms mg^{-1} during the mid-exponential phase of growth which indicate that individual organisms of Sacch. cerevisiae TC8 are less dense than those of Sacch. cerevisiae NCYC 431.

The chemical features controlling membrane lipid fluidity are, primarily, the cholesterol/phospholipid ratio, degree of unsaturation of the phospholipid fatty-acyl chains and the concentration of membrane proteins (Shinitzky and Yuli, 1982). However, the value for Δmol^{-1} is generally regarded as an

acceptable, albeit a simplistic, measure of membrane fluidity. It assumes that the inclusion of double bonds in the hydrocarbon region of a membrane lipid results in larger gaps in the membrane because the fatty-acyl chains pack less tightly and allow greater freedom of motion. Given that diffusing molecules pass through the plasma membrane via free volumes within the bilayer, as described in the polymer matrix model in the Introduction, then the higher density of gaps in the membrane should, theoretically, allow diffusion to occur more quickly.

If the molecular packing of the fatty-acyl residues of membrane phospholipids is considered, a different conclusion may be drawn. Figure 17 shows a schematic representation of fatty-acyl chains and how they may be aligned in a membrane. Saturated chains should pack tightly in a homogeneous bilayer depending upon physical conditions, e.g. temperature, pressure and pH value. With the inclusion of one double bond in the chain, the permanent kink not only inhibits tight packing but also results in shortening the width of the membrane. The addition of a second double bond causes the chain to kink again but, because the chain effectively coils around, it should be able to pack more tightly than the singularly unsaturated chain. Notably, the second double bond causes a further decrease in membrane thickness. A third double bond has a similar effect. Fluidity is not necessarily increased by inclusion of multiply unsaturated fatty-acyl residues; indeed it may be possible for these residues to pack more tightly than mono-unsaturated chains. However, membrane thickness is decreased.

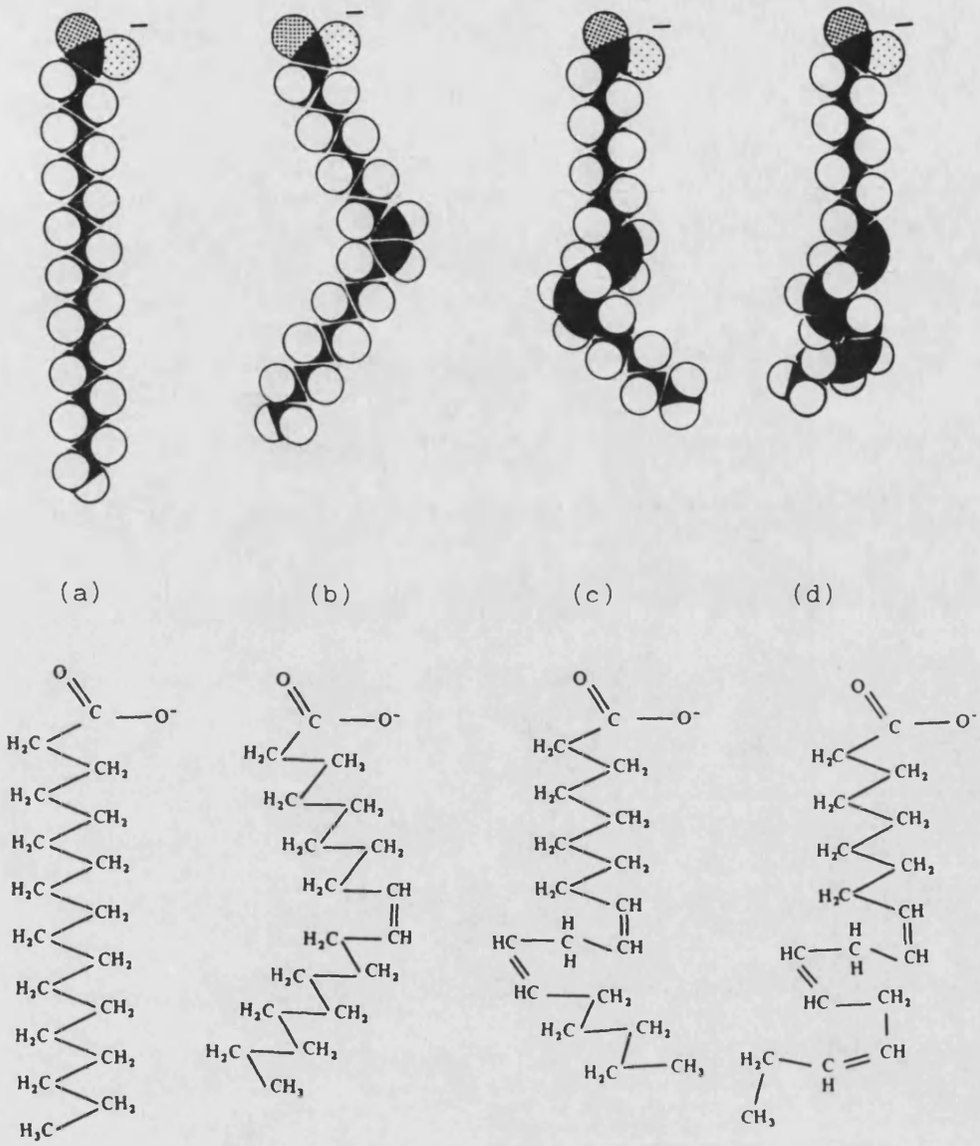


Figure 17. Space filling models and chemical structures of fatty acid anions with different numbers of double bonds: (a) stearic acid; (b) oleic acid; (c) linoleic acid and (d) linolenic acid. Adapted from Robertson (1983).

In a membrane under dynamic conditions, free rotation about single C-C bonds will result in numerous transient gauche and trans configurations. For example, oleic acid has one permanent kink but, because of stearic hindrance imposed by adjacent molecules, it is unlikely it will maintain this configuration and more likely to rotate to adopt a conformation similar to that given for linoleic acid (Figure 17). However, the transient existence of the bulky biphasic molecule does help to explain the effect on permeability to SO_2, and the excellent correlation between permeability coefficient of SO_2 accumulation and the ratio of mean chain length and Δmol^{-1} value supports the existence of these isomers.

Theories relating to the molecular packing of plasma-membrane phospholipids raise the question of the validity of the values for Δmol^{-1} as a measure of membrane fluidity. It seems unlikely that di- or tri-unsaturated fatty-acyl residues have a two and three fold effect on increasing membrane fluidity, respectively, compared with mono-unsaturated residues. In this study, Δmol^{-1} values are useful to distinguish between the three degrees of unsaturation because of the different effects on membrane thickness rather than fluidity. The mean chain-lengths of fatty-acyl residues isolated from phospholipids in Sacch. cerevisiae NCYC 431 grown anaerobically in media supplemented with linoleic or linolenic acids were not significantly different. However, both were longer than that calculated when this organism was grown under the same conditions in media supplemented with oleic acid. This suggests that the former fatty acids have a similar fluidizing or thinning effect on the plasma membrane which is greater than that imposed by

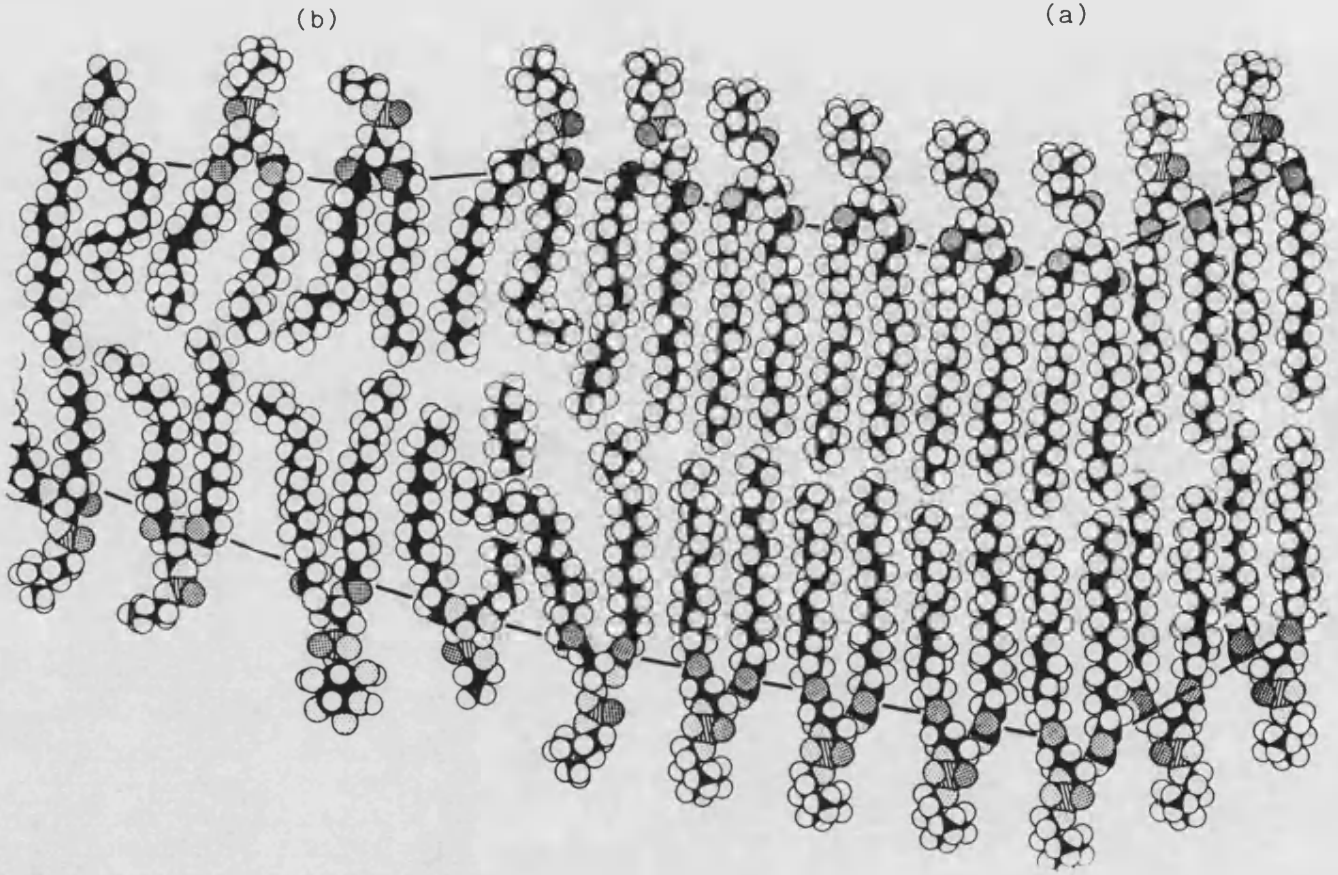
the incorporation of oleic acid. If fluidity is the primary criterion controlling incorporation of different fatty-acyl residues then, implicitly, Δmol^{-1} values are valid parameters of fluidity for singularly and doubly unsaturated residues but do not adequately describe fluidity of those membranes containing $C_{18:3}$ residues.

The direct correlation between permeability coefficient of SO_2 accumulation in yeasts and the ratio of mean fatty-acyl chain lengths and values for Δmol^{-1} supports the theory that membrane thickness determines the rate at which a molecule will diffuse across the yeast plasma membrane. The inclusion of unsaturated residues results in a shortening of fatty-acyl chains so the ratio of mean fatty-acyl chain length and value for Δmol^{-1} is proportional to the plasma-membrane thickness. The result is clearly seen in Figure 18 where the more fluid region with kinked fatty-acyl chains results in a narrowing of the membrane. A fully saturated fatty-acyl chain will be shortened by the equivalent of one methyl group in length (1.27 $\mathring{A}$) and increased in volume from about 25 to 50 $\mathring{A}^3$ when gauche rotamers are formed about two C-C bonds (Lagaly and Weiss, 1971).

These findings are in parallel with those of de Gier et al. (1968) and McElhaney et al. (1973) who, working with liposomes, examined the permeability of glycerol. They found that both by inclusion of double bonds or by decreasing the chain length of fatty-acyl residues, permeability was increased. However, in both cases, it was concluded that the increased permeability can be simply explained in terms of increased membrane fluidity.

Figure 18. A phospholipid bilayer with a crystalline region (a) where the molecules lengthen and narrow compared with the adjacent fluid molecules (b) resulting in a change in membrane thickness. Adapted from Robertson (1983).

Figure 18.



Blok et al. (1975) reported enhanced permeability of liposomes at the phase-transition temperature to permeating compounds. This is a generally recognised feature attributed to a sudden increase in lipid fluidity at the transition temperature. These workers also noted a strong selectivity with respect to molecular size of the permeating molecules, and that the extent of permeability depended strongly on the length of the fatty-acyl chains in saturated lecithin liposomes (Lenaz, 1979). This finding supports the concept that, with the formation of more pores in the membrane, solutes will permeate more quickly and under these conditions fatty-acyl chain length and hence membrane thickness become the more important rate-limiting step for solute permeability.

These conclusions must not be considered in isolation. Many factors are known to influence the fluidity of a membrane and have not been considered in this Discussion. The packing arrangement of molecules in the yeast plasma membrane is altered by the proximity of proteins, sterols and different phospholipids, as well as by conditions such as temperature and osmotic pressure, all of which must be considered. This study is confined to the effects of phospholipids. The nature of the phospholipid head group is known to affect their arrangement in a bilayer but, as the relative abundance of each of the four phospholipid classes is very similar in each of the four strains studied and is not significantly influenced by inclusion of specific fatty-acyl residues, it is assumed that their influence is constant as far as these investigations are concerned. However, the importance of phospholipid head-group composition in the proper functioning of

the yeast plasma membrane must not be underestimated (Noordam et al., 1980; Trivedi et al., 1982). Further studies on the specific supplementation of phospholipids into the yeast plasma membrane are necessary.

Significantly, lower contents of phospholipid were detected in anaerobically-grown Sacch. cerevisiae NCYC 431 compared with cells grown aerobically, which may influence SO_2 uptake. Its effect in isolation is not evident but should be borne in mind. The detailed analysis of phospholipids in plasma membranes of all four yeast strains has proved valuable in improving the understanding of plasma-membrane composition in relation to SO_2 permeability but does not help to explain the toxicity of sulphite. The rate of diffusion of SO_2 into Sacch. cerevisiae NCYC 431 can be changed by selectively altering the phospholipid composition in the membrane. However it is unlikely that these changes would be great enough to affect the overall response to sulphite. If SO_2 enters a yeast at a rate of X mm $(min)^{-1}$ or at a rate five times this rate, the same intracellular equilibrium concentration will be ultimately achieved and the long-term effect will be the same. This is supported by the non-correlation between permeability to SO_2 and resistance in the four yeast strains studied. It would be interesting to extend this work to see if specific supplementation in the environment of fatty acids or sterols affects the inherent ability of a yeast to resist sulphite. Manipulation of plasma-membrane composition could, by lowering membrane stability or in some unforeseen way, affect yeast viability particularly in the presence of sulphite.

Although this work still leaves many avenues of investigation into the mode of sulphite resistance in yeasts, it is hoped that the data within will prove instrumental in furthering the present understanding of the action of sulphite on yeasts. In the context of the practical application of sulphiting agents in foods and beverages, the data confirm the importance of excluding possible sulphite-binding compounds, particularly acetaldehyde from these products.

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APPENDIX

Included in the appendix is a copy of a paper by B.J. Pilkington and A.H. Rose published in the Journal of General Microbiology. This paper contains some of the work presented in this thesis.

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Reactions of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* to Sulphite

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Sulphite inhibited growth of all four yeasts studied, *Zygosaccharomyces bailii* NCYC 563 being most sensitive and *Saccharomyces cerevisiae* NCYC 431 the least. Vertical Woolf–Eadie plots were obtained for initial velocities of ^{35}S accumulation by all four yeasts suspended in high concentrations of sulphite. Equilibrium levels of ^{35}S accumulation were reached somewhat faster with strains of *S. cerevisiae* than with those of *Z. bailii*. With all four yeasts, the greater the extent of ^{35}S accumulation, the larger was the decline in internal pH value. Growth of *S. cerevisiae* TC8 and *Z. bailii* NCYC 563, but to a lesser extent of *S. cerevisiae* NCYC 431 and *Z. bailii* NCYC 1427, was inhibited when mid exponential-phase cultures were supplemented with 1·0 or 2·0 mM-sulphite, the decrease in growth being accompanied by a decline in ethanol production. Unless growth was completely inhibited, the sulphite-induced decline in growth was accompanied by production of acetaldehyde and additional glycerol.

INTRODUCTION

Sulphite has long been recognized as a powerful antimicrobial agent (Hammond & Carr, 1976). The compound exists in solution in three forms, the proportions of which depend on pH value. At pH values below 1·8, sulphite exists predominantly as free SO_2 and at pH values above 7·2 largely as SO_3^{2-}; at intermediate pH values, it exists in various proportions as the bisulphite ion (HSO_3^-; King *et al.*, 1981). The antimicrobial action of sulphite is greatest at low pH values (Wedzicha, 1984), which explains why the compound is particularly effective against yeasts which, in general, grow best at pH values in the range 3·0–5·0 (Rose, 1987). The greater antimicrobial action of sulphite against *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii* at low pH values has been explained by the discovery that, of the three molecular forms in which sulphite exists in solution, only SO_2 enters these organisms (Stratford & Rose, 1986; Stratford *et al.*, 1987). Yeast species differ considerably in their ability to resist the antimicrobial action of sulphite. Warth (1985) found that *Kloeckera apiculata* and *Hansenula anomala* were much more sensitive to sulphite than strains of *S. cerevisiae* which is generally recognized as being a sulphite-resistant yeast. A yeast which has been reported to be even more resistant to sulphite is *Zygosaccharomyces bailii* (Thomas & Davenport, 1985; Warth, 1985).

Little is known of the physiological basis for the different degrees of sulphite resistance among yeast species. Among strains of *S. cerevisiae*, differences in resistance have been attributed to production of compounds, particularly acetaldehyde, that bind sulphite to form α-hydroxysulphonates (Burroughs & Sparks, 1964), especially when the strains are grown in the presence of sulphite (Rankine, 1968; Rankine & Pocock, 1969; Weeks, 1969). Moreover, Stratford *et al.* (1987) attributed the greater sulphite resistance of a strain of *S'codes ludwigii* as compared with one of *S. cerevisiae* to its ability to produce greater amounts of acetaldehyde. The resistance of *S'codes ludwigii* was also caused in part, it was suggested (Stratford *et al.*, 1987), by its decreased ability to accumulate sulphite. The present paper compares the physiological basis of sulphite resistance in two strains each of *S. cerevisiae* and *Z. bailii*.

METHODS

Organisms. The yeasts used were *S. cerevisiae* NCYC 431, *S. cerevisiae* TC8 (Stratford & Rose, 1985), *Z. bailii* NCYC 563 and *Z. bailii* NCYC 1427. They were maintained at 4 °C on slopes of malt extract–yeast extract–glucose–mycological peptone (MYGP) agar (Wickerham, 1951).

Experimental cultures. Organisms were grown aerobically in a medium containing (l^{-1}): glucose, 20 g; $(NH_4)_2SO_4$, 3·0 g; KH_2PO_4, 3·0 g; yeast extract (Lab M), 1·0 g; $MgSO_4.7H_2O$, 30 mg; and $CaCl_2.2H_2O$, 30 mg (adjusted to pH 4·0 with HCl). This is the medium used by Stratford & Rose (1986) and is referred to as Medium A. It is, however, poorly buffered, and in experiments in which the yeasts were grown in the presence of sulphite it was replaced by Medium B which differed from Medium A in that KH_2PO_4 was omitted and replaced by 13·4 g K_2HPO_4 and 12·9 g citric acid. Under the conditions used, the pH value of cultures grown using Medium B did not fall below 4·0. Portions of medium (1 l) were dispensed into 2 l round flat-bottomed flasks which were plugged with cotton wool and sterilized by autoclaving at $6{\cdot}89 \times 10^4$ Pa for 10 min. Starter cultures (100 ml Medium A or B in 250 ml conical flasks) were inoculated with a pinhead of yeast from a slant culture and incubated at 30 °C for 24 h on an orbital shaker (200 r.p.m.). Portions of medium (1 l) were inoculated with portions of starter culture containing 0·05 mg dry wt *S. cerevisiae* NCYC 431, 0·5 mg dry wt *S. cerevisiae* TC8 or 1·0 mg dry wt of either of the *Z. bailii* strains. Growth was followed by measuring the optical density of portions of culture, measurements being related to dry wt of organism by a standard curve constructed for each strain of yeast. Organisms were harvested from mid exponential-phase cultures, containing 0·5 mg dry wt *S. cerevisiae* ml^{-1} or 0·25 mg dry wt *Z. bailii* ml^{-1}, by filtration through a membrane filter (0·45 μm pore size; 50 mm diam.; Oxoid) and washed twice with 10 ml 30 mM-citrate buffer (pH 3·0).

Assessment of sulphur dioxide tolerance. The ability of the yeasts to grow in Medium B containing different concentrations of sulphite was measured using Dynatech Microplates. Organisms were harvested from mid exponential-phase cultures by centrifugation (12000 *g* for 2 min) and resuspended in fresh medium (pH 4·0) to give a suspension containing 0·1 mg dry wt ml^{-1}. Cell suspension (170 μl) was pipetted into each well of a microtitre plate leaving one well empty to use as a blank. Sodium metabisulphite (30 μl) diluted in fresh medium was added to each well giving final concentrations of sulphite ranging between zero and 3·3 mM across the plate. The blank well was filled with 200 μl water and the plate gently shaken for a few seconds to mix the suspensions. Replicate plates were prepared, covered, sealed in an airtight container with some moist tissue paper to minimize evaporation and incubated at 30 °C on an orbital shaker (200 r.p.m.). Using a Dynatech Microplate Reader (MR600), set at 600 nm, optical densities were measured at intervals up to 6 h after adjusting to zero against the blank well. Cells tended to settle to the bottom of the wells so the plates were gently agitated before optical densities were measured.

Measurement of sulphite accumulation. To measure initial velocities of sulphite accumulation, organisms grown in Medium A were washed twice with 30 mM-citrate buffer (pH 3·0) containing 100 mM-glucose, suspended in the same buffer at 10 mg dry wt ml^{-1} and the suspension allowed to equilibrate for 3 min at 30 °C. A reaction mixture consisting of 30 mM-citrate buffer (pH 3·0) containing 100 mM-glucose and 10–200 μM-[^{35}S]sulphite (0·20 μCi ml^{-1}; 1 μCi = 37 kBq) was prepared in a universal bottle and warmed to 30 °C in a water-bath. Labelled sulphite was stored at −20 °C in 5 mM-EDTA under nitrogen gas in 0·5 ml portions (0·1 mCi ml^{-1}) to prevent oxidation. Portions (300 μl) of the suspension of organisms were dispensed into microcentrifuge tubes (Eppendorf). Using a 1·5 ml multi-dispense syringe pipette, 1·25 ml of labelled sulphite reaction mixture was added to the organisms and the suspension quickly mixed by refilling and emptying the syringe. After exactly 4 s, 1·5 ml of the suspension was rapidly filtered through a membrane filter (0·45 μm pore size; 25 mm diam.; Millipore) which had been washed with 5 ml 10 mM-sulphite in 30 mM-citrate buffer (pH 3·0). After filtration, three 1 ml portions of buffered sulphite solution of the same concentration as used in the experiment were used quickly to wash the organisms and filter. Filters with organisms were then placed in scintillation vials containing 7 ml Optiphase Safe (Fisons). Radioactivity in the vials was measured in an LKB Rackbeta liquid scintillation spectrometer (model 1217).

To measure the extent of sulphite accumulation, washed organisms grown in Medium A were suspended in glucose-containing citrate buffer as already described. Labelled sulphite was added to a suspension containing 2 mg dry wt ml^{-1} giving a final concentration of 0·1–5·0 mM-sulphite (0·2 μCi ml^{-1}) and the suspension incubated at 30 °C. At appropriate time intervals, three 1 ml portions of suspension were filtered through prewashed filters as already described. The organisms were washed with three 1 ml portions of 30 mM-citrate buffer containing sulphite at the concentration used in the experiment. Radioactivity was measured as already described. Background activity was estimated by repeating the procedure without organisms to check washing efficiency and to make sure that sulphite was not binding to filters.

Measurement of plasma-membrane area of organisms. Dimensions of organisms were measured by observation in a light microscope fitted with an eyepiece graticule. In calculating membrane areas, it was assumed that organisms of *S. cerevisiae* were spheres and those of *Z. bailii* cylinders with rounded ends.

Measurement of intracellular water volume. Volumes of intracellular water in organisms in suspension were

calculated by measuring the differential distribution of ^3H_2O, which equilibrates with both extracellular and intracellular water, and D-[1-^{14}C]mannitol which is excluded by the plasma membrane. Preliminary experiments established that mannitol was not accumulated by any of the yeasts examined. To do this, washed organisms were suspended at 10 mg dry wt ml^{-1} in 30 mM-citrate buffer (pH 3·0) containing 100 mM-glucose and [^{14}C]mannitol at 0·01, 1·0 or 100 mM. The suspensions were incubated for 60 min at 30 °C and filtered through washed membrane filters (0·45 μm pore size; 50 mm diam.; Oxoid). The membranes were then washed with non-radioactive mannitol at the concentration used in the experiment, placed in scintillation vials containing 7 ml Optiphase Safe and radioactivity was measured as already described. To measure the volume of intracellular water, a suspension of washed organisms (10 mg dry wt ml^{-1}) grown in Medium A was prepared as already described. To 15 ml of suspension was added 10 mM-[^{14}C]mannitol (0·02 μCi ml^{-1}) and 0·2 μCi ^3H_2O ml^{-1}. Suspensions were incubated with continuous stirring at 4 °C for 10 min. Six 1 ml portions of suspension were then centrifuged in microcentrifuge tubes (Eppendorf) for 3 min at 12000 *g*. Duplicate 200 μl portions of supernatant from each tube were added to scintillation vials containing 7 ml Optiphase Safe and radioactivity was measured as previously described. Radioactivity in the suspension of organisms was measured by placing 12 200 μl portions of suspension in scintillation vials containing 7 ml Optiphase Safe.

Measurements of intracellular pH values. Intracellular pH values of organisms grown in Medium A were calculated by determining the equilibrium distribution of propionic acid across the plasma membrane (Conway & Downey, 1950). Washed organisms, suspended (5 mg dry wt ml^{-1}) in 30 mM-citrate buffer (9 ml) containing 100 mM-glucose, were allowed to equilibrate after adding 1 ml 0·1 mM-[2-^{14}C]propionic acid (0·25 μCi ml^{-1}) at 30 °C. After 1, 2, 4, 6, 8 and 10 min, duplicate 300 μl portions were taken from the suspension, rapidly filtered through washed membrane filters (0·45 μm pore size; 25 mm diam.; Millipore) and washed with 4 × 1 ml 0·01 mM-propionic acid at 4 °C. The filters with organisms were transferred to scintillation vials as already described. Once the time for equilibration had been ascertained, replicate measurements were obtained by sampling after 5 min incubation. Intracellular pH values were calculated from the expression derived by Waddell & Butler (1959):

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

where $R = TA_i \cdot V_e / TA_e \cdot V_i$, pH_i and pH_e are the internal and external pH values, TA_i and TA_e the intracellular and extracellular total amounts of propionic acid, V_i and V_e the intracellular and extracellular volumes and pK_i and pK_e the dissociation constants for propionic acid in the internal and external environments. The internal and external dissociation constants for propionic acid were calculated from the Davies (1962) simplified version of the Debye–Hückel equations. Values for pK_i and pK_e were calculated to be 4·75 and 4·86, respectively.

Analytical methods. Free SO_2 was assayed by the method of Burroughs & Sparks (1964), which assumes that dissociation of bound SO_2 is minimized by lowering the pH value to 1·5. Acetaldehyde, glycerol and pyruvate were determined by using assay kits (Boehringer). Ethanol was determined by GLC as described by Beavan *et al.* (1982).

Chemicals. All reagents used were AnalaR or of the highest grade available commercially. Amersham supplied radioactively labelled chemicals

RESULTS

Effects of sulphite on growth

Sulphite inhibited growth of all four yeasts at concentrations up to and including 3·3 mM as assessed by the microplate method (Fig. 1). *Z. bailii* NCYC 563 was the most sensitive and *S. cerevisiae* NCYC 431 the least.

Accumulation of sulphite

Vertical Woolf–Eadie plots (Hofstee, 1959) were obtained with initial velocities of accumulation by all yeasts suspended in high concentrations of SO_2 (Fig. 2). However, at low concentrations of SO_2 and especially with *S. cerevisiae* NCYC 431, there was considerable deviation from the vertical. Equilibrium levels for accumulation of sulphite equivalents were reached somewhat faster with the strains of *S. cerevisiae* than with those of *Z. bailii* although all four strains had reached these levels after 10 min irrespective of the concentration of sulphite. As suspensions of organisms accumulated equilibrium levels of sulphite equivalents measured after 10 min incubation, intracellular pH values declined (Fig. 3). The greater the extent of accumulation of sulphite equivalents, the larger was the decline in internal pH value. Equilibrium accumulation values, and therefore decline in internal pH values, were smallest for *Z. bailii* NCYC 1427 (Fig. 3).

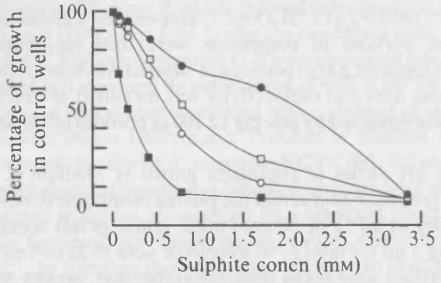

Fig. 1

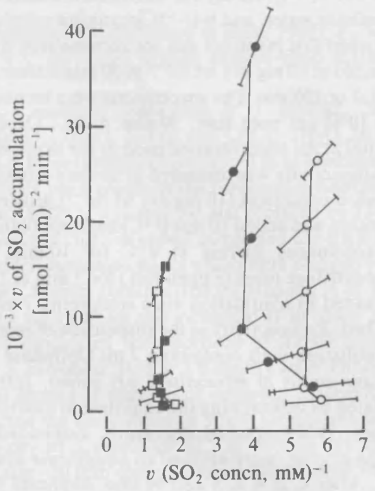

Fig. 2

Fig. 1. Effect of sulphite concentration on growth of *S. cerevisiae* TC8 (○), *S. cerevisiae* NCYC 431 (●), *Z. bailii* NCYC 1427 (□) and *Z. bailii* NCYC 563 (■) in Medium B in microtitre wells. Values quoted are the means of measurements on eight separate plates. The maximum variation was ±10%.

Fig. 2. Woolf–Eadie plots for accumulation of molecular SO_2 by *S. cerevisiae* TC8 (○), *S. cerevisiae* NCYC 431 (●), *Z. bailii* NCYC 1427 (□) and *Z. bailii* NCYC 563 (■) suspended in 30 mM-citrate buffer (pH 3·0) containing 100 mM-glucose at 30 °C. Concentrations of molecular SO_2 were calculated from the data of King *et al.* (1981). Bars indicate SD.

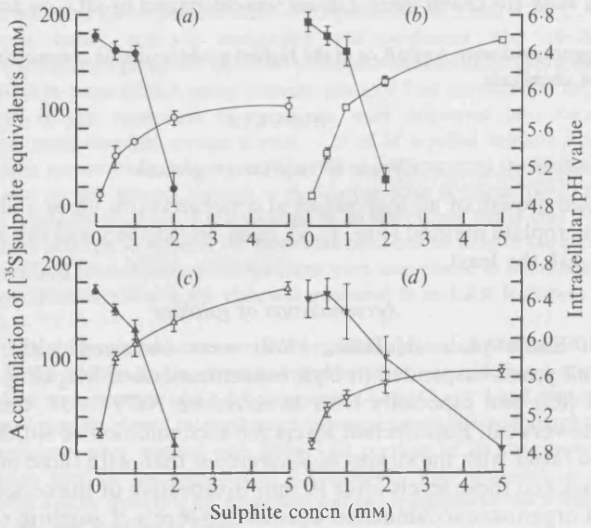

Fig. 3. Relationship between extent of accumulation of sulphite equivalents (open symbols) and intracellular pH values (closed symbols) in *S. cerevisiae* TC8 (*a*), *S. cerevisiae* NCYC 431 (*b*), *Z. bailii* NCYC 563 (*c*) and *Z. bailii* NCYC 1427 (*d*). Measurements were made after organisms had been suspended in buffer for 10 min. Values quoted are means of at least three determinations. Bars indicate SD.

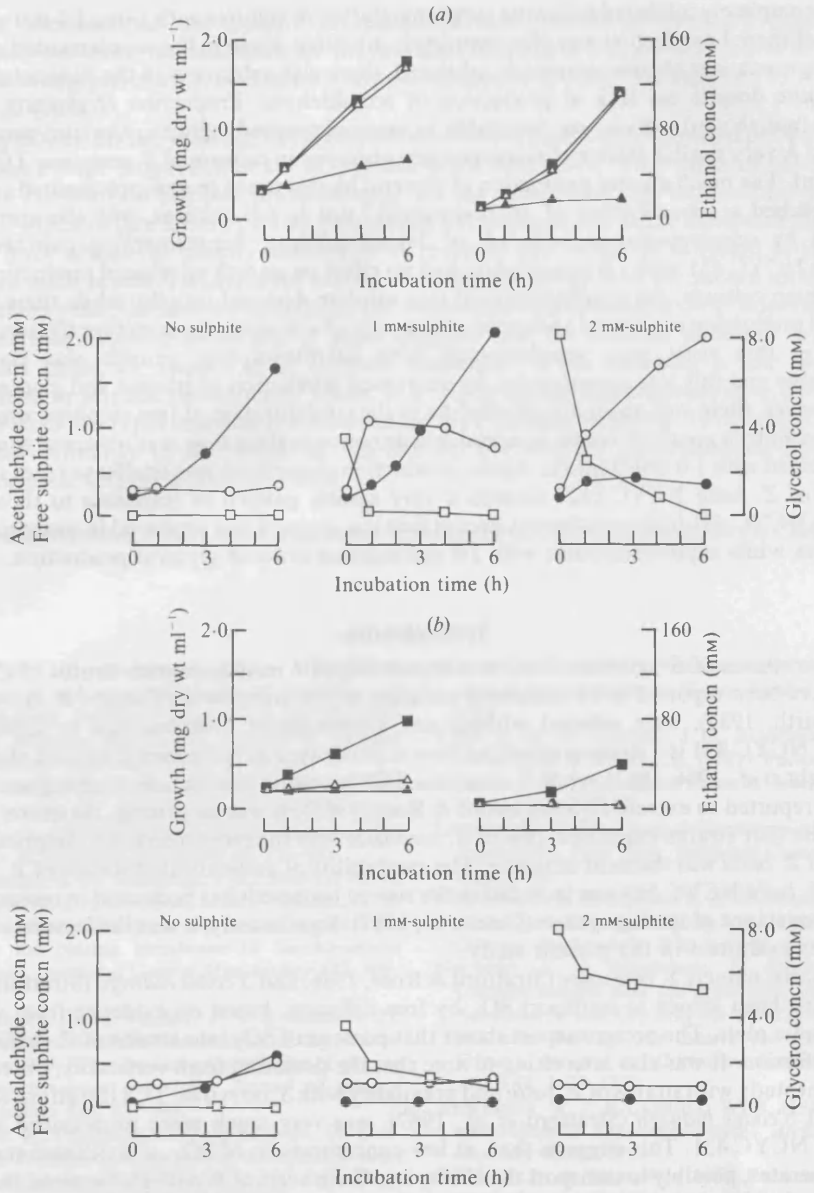

Fig. 4. Effect of supplementing cultures of *S. cerevisiae* NCYC 431 (*a*) and *Z. bailii* NCYC 563 (*b*) with sulphite (■, control, △, 1·0 mM, ▲, 2 mM) on growth and ethanol formation. Also shown are the effects of these supplementations on concentrations of acetaldehyde (○), glycerol (●) and free sulphite (□) in culture supernatants. After supplementing cultures with sulphite, they were observed for a further 6 h. Values quoted are the means of three separate determinations. The maximum variation in values for concentrations of acetaldehyde and free sulphite was $< 10\%$; for concentrations of ethanol and glycerol the variation was $\pm 15\%$.

Production of binding compounds by organisms grown in the presence of sulphite

The effect of sulphite on growth of each of the yeasts in 1 litre cultures (Medium B) was assessed by adding the compound to mid exponential-phase cultures, and measuring the effect on density of organisms and on concentrations in culture filtrates of acetaldehyde, ethanol, glycerol, pyruvate and free sulphite over the following 6 h. Growth of *Z. bailii* NCYC 563 was

virtually completely inhibited following supplementation of cultures with 1·0 or 2·0 mM-sulphite (Fig. 4*b*). Ethanol production was also completely inhibited. Even in the supplemented cultures in which growth was almost completely inhibited, there was a decrease in the concentration of free sulphite despite the lack of production of acetaldehyde. Production of glycerol and of pyruvate (not shown), which was detectable in unsupplemented cultures, was also completely inhibited. A very similar pattern of responses was observed in cultures of *S. cerevisiae* TC8 (data not shown). The much greater production of glycerol by this strain in unsupplemented cultures, which reached a concentration of approximately 7 mM in 6 h cultures, was also completely inhibited by supplementation with 1·0 or 2·0 mM-sulphite. Supplementing cultures of *S. cerevisiae* NCYC 431 with 1·0 mM-sulphite had no effect on growth or ethanol production (Fig. 4*a*). In these cultures, the concentration of free sulphite declined rapidly, while there was an increased production of glycerol and rapid appearance of acetaldehyde in culture filtrates. When cultures of this yeast were supplemented with 2·0 mM-sulphite, growth was decreased considerably and this was accompanied by decreased production of ethanol and glycerol (Fig. 4*a*). However, there was again a rapid decline in the concentration of free sulphite, which was accompanied by a greater increase in acetaldehyde concentration than was observed in cultures supplemented with 1·0 mM-sulphite. Again, production of pyruvate was unaffected (not shown). Cultures of *Z. bailii* NCYC 1427 showed a very similar pattern of responses to those of *S. cerevisiae* NCYC 431 (data not shown), except that less glycerol was produced in unsupplemented cultures while supplementation with 1·0 mM-sulphite lowered glycerol production.

DISCUSSION

The two strains of *S. cerevisiae* used to compare sulphite resistance with strains of *Z. bailii*, which have been reported to be extremely resistant to the compound (Thomas & Davenport, 1985; Warth, 1985), were selected without any knowledge of their reaction to sulphite. *S. cerevisiae* NCYC 431 is a strain originating from a distillery, and has a high tolerance of ethanol (Cartwright *et al.*, 1986, 1987), while *S. cerevisiae* TC8 is a strain used in cider-making and which has been reported to excrete H_2S (Stratford & Rose, 1985). It was surprising, therefore, to find that, of the four strains examined, one of *S. cerevisiae* was the most tolerant to sulphite while a strain of *Z. bailii* was the most sensitive. The availability of authenticated strains of *Z. bailii* is limited. *Z. bailii* NCYC 563 was included in the survey because it has been used in research into sulphite resistance of spoilage yeasts (Cole *et al.*, 1987). Significantly, it was the least resistant of the strains examined in the present study.

Two yeasts, namely *S. cerevisiae* (Stratford & Rose, 1986) and *S'codes ludwigii* (Stratford *et al.*, 1987), have been shown to transport SO_2 by free diffusion, based on evidence from vertical Woolf–Eadie plots. The present report shows that passage of SO_2 into strains of *Z. bailii* is also by free diffusion. It was also interesting to note that the deviation from verticality, observed in the present study with strains of *Z. bailii* and previously with *S. cerevisiae* TC8 (Stratford & Rose, 1986) and *S'codes ludwigii* (Stratford *et al.*, 1987), was very much more pronounced with *S. cerevisiae* NCYC 431. This suggests that, at low concentrations of SO_2, a facilitated transport system operates, possibly to transport the HSO_3^- ion. With vertical Woolf–Eadie plots, the value at the intercept on the abscissa is equivalent to the permeability coefficient for passage of SO_2 into the organism (Laidler, 1977). It is clear, therefore, that the two strains of *Z. bailii* have lower permeability coefficients than either of the *S. cerevisiae* strains.

Our discovery of a correlation between ability of yeasts to grow in the presence of sulphite and sulphite-induced production of acetaldehyde suggests that production of this sulphite-binding compound contributes significantly to the resistance. It is also noteworthy that the two most sulphite-resistant yeasts examined, namely *S. cerevisiae* NCYC 431 and *Z. bailii* NCYC 1427, are able to produce large amounts of acetaldehyde when growth was almost completely inhibited by 2·0 mM-sulphite. Excretion of acetaldehyde together with glycerol in cultures of *S. cerevisiae* supplemented with sulphite has been known for many years (Neuberg & Reinfurth, 1918, 1919), and constitutes Neuberg's second form of fermentation (Nord & Weiss, 1958). Our data are in general agreement with the finding of Neuberg & Reinfurth (1919) that, in the presence of

sulphite, acetaldehyde and glycerol are produced in equimolar amounts by strains of *S. cerevisiae*. Moreover, the data show for the first time that this is true also for strains of *Z. bailii*. Production of glycerol by *Z. acidifaciens* (now recognized as *Z. bailii*) was reported by Nickerson & Carroll (1945).

When SO_2 enters the yeast cell, it encounters an environment which is around pH 6·5 with the result that a large proportion of the SO_2 is converted into HSO_3^-. This explains the ability of yeasts to concentrate sulphite intracellularly. At the same time, the intracellular pH value declines, which in turn lowers the transmembrane pH gradient and hence dissipates the proton-motive force across the plasma membrane. A result of this would be to retard or inactivate processes, such as active transport of solutes, that require energy from the proton-motive force. The discovery that the decrease in internal pH value following accumulation of sulphite is not of the same magnitude in all strains of yeast suggests that the internal buffering capacity of organisms might be important in sulphite resistance. While invoking a role for energy metabolism in sulphite resistance of yeasts, it is worth noting that exposure of *S. cerevisiae* to sulphite leads to a rapid decrease in the content of ATP (Schimz & Holzer, 1979) which has been attributed primarily to the action of sulphite on the enzyme glyceraldehyde-3-phosphate dehydrogenase (Hinze & Holzer, 1986).

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