METABOLISM OF SULPHUR DIOXIDE IN TWO WINE YEASTS

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SUMMARY

1. The formation of SO_2 and of three SO_2-binding compounds by two Saccharomyces yeasts (S. cerevisiae strain 350 and S. oviformis strain 723) was investigated. Taxonomic and physiological characteristics of the two yeasts have been described.

2. The production of SO_2 and of SO_2-binding compounds was influenced more by the strain of yeast used than by any other variable studied. The strain differences in sulphite formation and utilization have been studied.

3. Wines made by fermentation with S. oviformis consistently bound more SO_2 than did those made with S. cerevisiae.

(a) S. oviformis produced more acetaldehyde, pyruvic acid and α-ketoglutaric acid than did S. cerevisiae.

(b) Acetaldehyde, pyruvic acid and α-ketoglutaric acid accounted for about 97% of SO_2 bound in all wines prepared.

(c) The yeasts formed acetaldehyde, pyruvic acid and α-ketoglutaric acid during fermentation while utilizing sugar.

4. S. oviformis produced significant quantities of SO_2 from SO_4^{--}, while S. cerevisiae produced nil to trace amounts only, under similar conditions.

(a) Both yeasts utilized SO_4^{--}, methionine, cysteine, and, less efficiently, SO_3^{--} and cystine as sole sulphur sources for growth. However, SO_4^{--} was the only S-source converted to SO_2.

(b) SO_2 added before fermentation decreased SO_2 formation.

5. The production of higher amounts of SO_2-binding compounds was associated with the capacity of the yeast to produce SO_2.

(a) S. oviformis, which produced SO_2, formed more acetaldehyde, pyruvic acid and α-ketoglutaric acid than did S. cerevisiae which did not produce significant amounts of SO_2.

(b) Graded additions of SO_2 (up to 1.6 mM) to grape juice increased the production of acetaldehyde and pyruvic acid by both yeasts.

(c) Acetaldehyde (up to 20 mM) increased SO_2 formation by resting cells of the two yeasts.

6. The strain difference in SO_2 production between the yeasts was explained by the greater SO_3^{--} formation and

the lower SO_3^{--} utilization by the SO_2-forming yeast, S. oviformis.

(a) Both yeasts reduced SO_4^{--} by the following pathway :

$$SO_4^{--}\,(ext) \longleftrightarrow SO_4^{--}\,(int) \longleftrightarrow APS \longrightarrow PAPS \longrightarrow SO_3^{--} \longrightarrow S^{--}.$$

(b) Rates of SO_4^{--} uptake by the two yeasts were similar.

(c) Extracts of S. oviformis activated SO_4^{--} to APS plus PAPS four times faster than did those of S. cerevisiae.

(d) ATP-sulphurylase activity in extracts of S. oviformis was about four times greater than in extracts of S. cerevisiae.

(e) Sulphite reductase activity in extracts of S. oviformis was less than in those of S. cerevisiae.

7. The results lead to the conclusion that S. oviformis reduced SO_4^{--} to SO_3^{--} in excess of its assimilatory requirements while S. cerevisiae did not. Excess SO_3^{--} combined with SO_2-binding metabolites, thus stimulating further production of these compounds. The accumulated SO_2-binding compounds and their HSO_3^- addition derivatives diffused from the cells into the extracellular solution.

DECLARATION

I hereby declare that the work presented in this thesis has been performed by myself, except where otherwise stated in the text, and has not been submitted in any other application for a degree.

(Christopher Weeks)

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PUBLICATION

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ABBREVIATIONS

ADP	Adenosine -5'- diphosphate
AMP	Adenosine -5'- monophosphate
APS	Adenosine -5'- sulphatophosphate
ATP	Adenosine-5'-triphosphate
Co A	Coenzyme A
FAD	Flavin adenine dinucleotide
FH_4	Tetrahydrofolic acid
K_1, K_2, K_d.	Dissociation constants
MVH	Reduced methyl viologen
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Na_2-EDTA	Ethylene diamino tetra-acetic acid, sodium salt.
PAPS	Adenosine-3'-phospho-5'-phosphosulphate
Pi	Inorganic orthophosphate
PP	Pyrophosphate
POPOP	1:4-Di-2-(5-phenyl-oxazolyl)-benzene
PPO	2,5-Diphenyloxazole
S.A.M.	S-adenosyl methionine
Tris-HCl	Tris (hydroxymethyl) amino ethane hydro chloride

All temperatures are in degrees centigrade.

INTRODUCTION

The use of sulphur dioxide in wine making has increased greatly during this century such that today very little wine is prepared without it. Numerous technical articles explain and recommend the use of SO_2 (e.g. Quinn, 1940; Fornachon, 1965; Rankine, 1966). Faults occurring in Australian wines are frequently caused by the incorrect use of SO_2.

Sulphur dioxide is used to control the native microflora on grapes, to sterilize winery equipment and to preserve wine from oxidative and microbiological spoilage. The efficiency of SO_2 as a preservative depends, not only on its concentration in wine, but on the SO_2- combining capacity of wine. This, in turn, is influenced by the yeast strain used for fermentation and by the manner in which SO_2 is used during wine manufacture.

The chemistry of SO_2 in wine, and its relationship with yeast activity is discussed in connection with the use of SO_2 in wine making. Because SO_2, as SO_3^{--}, is an intermediate in the sulphur metabolism of yeasts, the formation and utilization of SO_3^{--}, together with related S-amino acid metabolism, is reviewed.

1. CHEMISTRY AND ACTION OF SO_2 IN WINE

1.1 Distribution of forms of SO_2 in wine

When SO_2, as sulphurous acid, liquid SO_2, or as $K_2S_2O_5$ solution, is added to wine, the following equilibria are established:

$$SO_2 + H_2O \overset{\alpha}{\longleftrightarrow} H_2SO_3 \overset{K_1}{\longleftrightarrow} H^+ + HSO_3^- \overset{K_2}{\longleftrightarrow} 2H^+ + SO_3^{--}$$

$$HSO_3^- \overset{Kd}{\longleftrightarrow} \text{Bound} - HSO_3^-$$

Binding compound

α = 11.3 g/100g water, 1 atm, 20°. $K_1 = 1.54 \times 10^{-2}$ M, $K_2 = 1.02 \times 10^{-7}$ M in water at 18°. (ed. Hodgman, 1961).

The inorganic species SO_2, H_2SO_3, HSO_3^- and SO_3^{--} are called, collectively, 'free SO_2', and the terms 'sulphur dioxide', 'sulphite', and 'bi-sulphite' are used synonomously here. Clearly the distribution between species is pH dependent, so 'SO_2' in wine (pH 3 to 4) is chiefly HSO_3^-, while 'SO_2' in yeast cells (pH 7) is mainly SO_3^{--}.

The bisulphite ions react reversibly with binding compounds present in wine to form 'bound SO_2', which will not reduce iodine. Bound SO_2 is decomposed to release the free binding compound and free SO_2 by strongly alkaline conditions or by boiling, which properties are used in

analytical determinations for bound SO_2 (Ripper, 1892; Rankine, 1962). Free SO_2 is a more effective preservative than is bound SO_2 and Kielhöfer (1963) concludes that SO_2 (aqueous) and H_2SO_3 are the most useful preserving molecules. The preservative action of SO_2 is due to its antiseptic and antioxidant properties.

1.2 Antiseptic action of SO_2

The toxicity of SO_2 varies with different micro-organisms and is increased at low pH and high alcohol concentration. Elliptical wine yeasts tolerate higher levels of SO_2 than do apiculate and wild yeasts (Rankine, 1963a). Extraordinarily high tolerances have been reported, for example, a *Saccharomyces* yeast in Sauternes type wine containing 700 mg/l SO_2 and a *Torulopsis* yeast fermenting black currant juice at 1200 mg/l SO_2 (Rankine, 1968b). Bacteria are more susceptible than are yeasts, and 5 mg/l of free SO_2 generally prevents growth, although many bacteria, including the homofermentative *Lactobacilli* will tolerate over 100 mg/l bound SO_2. Some heterofermentative lactic acid bacteria, e.g. *Lactobacillus hilgardii* can metabolise the acetaldehyde of the acetaldehyde - HSO_3^- complex to release free SO_2 which subsequently proves fatal (Fornachon, 1963).

The toxic effects of SO_2 may be due to its combination

with carbonyl metabolites, such as acetaldehyde and pyruvate, to block fermentative pathways (Neuberg, 1918). Kielhofer (1963) suggested that SO_2 addition to the active groups of some enzymes, and the lysis of cells by SO_2 in acid solution, killed the organisms, but he rejected the direct importance of reduction by SO_2 because other reductants with similar redox potentials do not have the same toxicity. Hernandez (1967) observed that fermentation which had been retarded by 150 to 250 mg/l SO_2 could be restored to normal by the addition of thiamine, which suggests that SO_2 obstructs the role of thiamine.

1.3 Antioxidant properties of SO_2

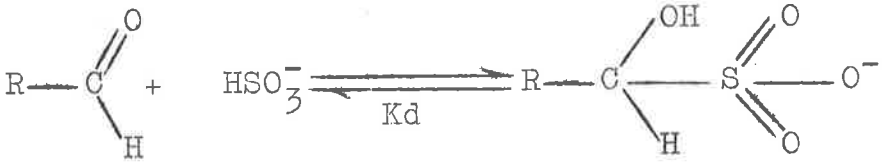
Sulphur dioxide protects wine against browning, oxidative flavour deterioration and the precipitation of oxidative casse (Fornachon, 1965).

As well as removing dissolved oxygen from wine by sulphate formation, SO_2 at 75 mg/l inactivates grape juice polyphenol oxidase (Ivanov, 1966), and so prevents enzymic oxidative browning. Higher concentrations are needed to reduce the peroxides in wines (Tagunkov, 1968). Reduction of Fe^{+++} to Fe^{++} by SO_2 decreases precipitation of ferric phosphate which is responsible for white casse or cloudiness in wines.

2. SULPHUR DIOXIDE-BINDING COMPOUNDS

Binding of SO_2 in wine has been reviewed by Joslyn (1952), Kielhöfer (1963) and Blouin (1966). The SO_2 combining capacity of wine is best given by the ratio of bound SO_2 to free SO_2, but as this ratio varies with the total concentration of SO_2, Blouin (1966) suggested that combining capacity be expressed simply as the bound SO_2, in mg/l, at 50 mg/l free SO_2.

With most binding compounds, the HSO_3^- ion adds across the double bond of a carbonyl group to form the corresponding hydroxy sulphonate (Finnar, 1957):



The reaction is reversible and the position of equilibrium is given by the dissociation constant of bound SO_2 complex:

$$Kd = \frac{[\text{Free Carbonyl}]\ \ [\text{Free } SO_2]}{[\text{Bound-}HSO_3^-\ \text{Complex}]}$$

Combination of HSO_3^- with acetaldehyde (Perkin, 1887) and with sugars (Rocques, 1898) is well known. Kielhöfer (1963) distinguished acetaldehyde-bound SO_2, glucose-bound SO_2 and 'residual SO_2'. Burroughs and Sparks (1964) used

ion exchange on Dowex 1x10 resin in the HSO_3^- form, and Blouin (1966) separated the dinitrophenylhydrazones by thin layer chromatography to extend considerably the list of known SO_2-binding compounds in cider and wine. Most of these compounds derive from the metabolism of yeasts or other micro-organisms. Phenolic compounds, notably anthocyanins and their derivatives, from grapes also bind SO_2 (Timberlake and Bridle,1967).

The most important SO_2 binding compounds in wines, with their dissociation constants are:

Compound	Kd of bound complex,M; pH 3-4
Acetaldehyde	1.5×10^{-6}
Pyruvic acid	4.0×10^{-4} to 3×10^{-4}
α-Ketoglutaric acid	8.8×10^{-4} to 5×10^{-4}
Monogalacturonic acid	3.0×10^{-2} to 1.7×10^{-2}
Glucuronic acid	5×10^{-2}
Glucose	6.4×10^{-1} to 9×10^{-1}
Arabinose	6.9×10^{-2} to 4×10^{-2}
Anthocyanins and related compounds	10^{-3} to 10^{-7}

From Blouin, 1966; Burroughs and Whiting, 1960 and Timberlake and Bridle, 1967.

Acetaldehyde, pyruvic acid and α-ketoglutaric acid account for most (average recovery, 69%) of the bound SO_2 in dry white table wines (Rankine and Pocock, 1969). The formation of these compounds during fermentation is strongly influenced by yeast strain but not by the variety of grape juice (Rankine, 1967; 1968a; and Rankine and Pocock, 1969). Higher pH of juice favours pyruvic acid and α-ketoglutaric acid formation (Rankine, 1967; 1968a). Additions of SO_2 before fermentation increase the production of acetaldehyde (Neuberg, 1918), pyruvic acid (Whiting and Coggins, 1960) and of α-ketoglutaric acid (Whiting and Coggins, 1960; Rankine, 1968a), depending on the yeast strain used. Higher concentrations of ammonia or amino nitrogen increase pyruvic acid (Whiting and Coggins, 1960) and α-ketoglutaric acid production (Rankine, 1968a) although Rankine found no correlation between the levels of these two acids. It appears that while both keto acids arise from deamination or transamination of their corresponding amino acids, pyruvic acid, but not α-ketoglutaric acid, may be formed from pathways of sugar metabolism.

Fruit damage and consequent rotting increase the SO_2 combining power of ciders (Burroughs and Sparks, 1962) and wines (Blouin, 1966), apparently because of increased galacturonic and glucuronic acids.

3. SULPHUR PHYSIOLOGY OF YEAST

Some yeasts accumulate SO_2 in their growth media, as well as producing metabolites which bind SO_2, so a consideration of yeast sulphur metabolism is relevant.

3.1 Sulphur nutrition

Suitable sulphur sources for yeast growth include SO_4^{--}, SO_3^{--}, $S_2O_3^{--}$, methionine, reduced glutathione and cysteine but not S^{--} nor ethionine (Maw, 1960). Sulphate is probably the preferred S source (Williams and Dawson, 1952). Cysteine prolongs the lag phase of yeast and inhibits growth when at higher concentrations (Maw, 1961a). Sulphate utilization is mainly, if not entirely, assimilatory.

3.2 Sulphite production

Sulphur dioxide production during fermentation of grape juice by yeast has been reported by Schanderl (1959), Dittrich and Staudenmeyer (1968), Würdig and Schlotter (1967) Mayer and Pause (1968) and Rankine and Pocock (1969). Schanderl (1959) speculated that SO_4^{--} could be reduced to SO_3^{--} during alcoholic fermentation. Added SO_4^{--} increases SO_2 production during fermentation of grape juice (Würdig and Schlotter, 1967) and SO_4^{--} is the best single source for

SO_2 formation by pure cultures of yeasts in synthetic media (Rankine and Pocock, 1969). Sulphur dioxide formation is favoured by higher pH (Rankine, 1968b), and lower temperature (Würdig and Schlotter, 1967) and is dependent on the minimum redox potential reached during fermentation (Würdig and Schlotter, 1968). There is no correlation between SO_2 and H_2S production (Mayer and Pause, 1968).

3.3 Sulphide production

Hydrogen sulphide, another product of yeast sulphur metabolism, may be produced by some yeasts. Formation of H_2S has been reviewed for wine by Rankine (1963b) and for brewery fermentations by Lawrence and Cole (1968). Yeast strain is most important, with strains of *Pichia* and *Hansenula* giving H_2S rapidly (Hernandez, 1964), *Schizosaccharomyces malidevorans* producing much H_2S (Rankine, 1964), while selected wine yeasts form little H_2S (Mayer and Pause, 1968). Several S-compounds may be converted to H_2S, but elemental sulphur, SO_2 and S-amino acids are the most common sources. Although SO_4^{--} may give rise to H_2S in some yeasts (Hernandez, 1964), addition of sulphate failed to increase H_2S production with yeasts studied by Rankine (1963b). Rankine (1964) recorded five times as much H_2S at 30° as at 15° and found more H_2S at lower pH values and at minimal Eh readings.

4. SULPHITE FORMATION FROM SULPHATE:

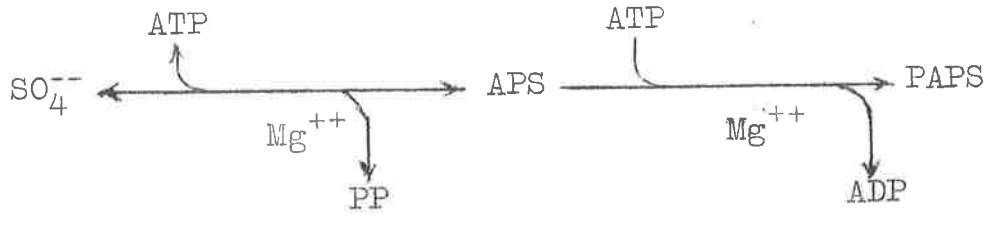
4.1 Sulphate Uptake

Sulphate uptake requires glucose and a nitrogen source, is greater in S-deficient cells (Klienzella et.al., 1959), is inhibited by azide, 2-4 dinitrophenol, iodo-acetate and (competitively) by selenate and is stimulated by ammonium ions and citrate (Maw, 1963). Klienzella et.al. (1959) suggested that uptake involved both simple diffusion and active uptake. Balogh et.al. (1969) reported both such processes contributing to methionine uptake by brewers' yeast, and Tweedie and Segal(1970) described an energy-dependent permease for sulphate uptake in Penicillium and Aspergillus. Such evidence, together with observations of extra-cellular ATP formation by yeasts (Agren and Ronquist, 1968) and localization of nucleoside phosphates around yeast cell walls (Kulaev et. al., 1967), establishes the role of ATP in SO_4^{--} uptake. However it is not certain whether SO_4^{--} is activated to APS prior to uptake or whether yeast cells accumulate an intracellular pool of inorganic SO_4^{--} which is subsequently activated.

4.2 Sulphate activation and reduction

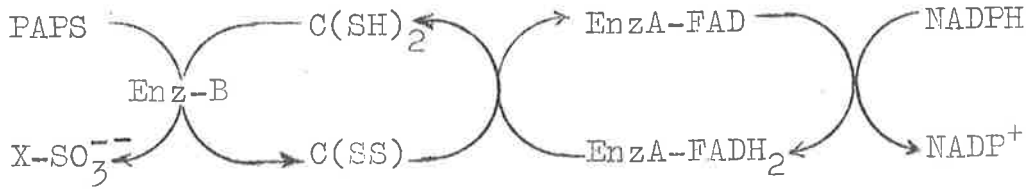
Biological reduction of sulphate has been reviewed in the last decade by Gregory and Robbins (1960), Wilson (1962), Black (1963), Postgate (1965), Nicholas (1967) and Thompson (1967).

Following studies on the ATP-dependent formation of phenyl sulphates by rat liver (Bernstein and McGilvery, 1952a; De Meio et. al, 1953) and the discovery that the first step requires SO_4^{--} activation (Bernstein and McGilvery, 1952b), Hilz and Lipmann (1955) showed that 'active sulphate' is an adenine derivative. Sulphate activation in yeast requires two steps (Bandurski et. al., 1956; Robbins and Lipmann, 1956a,b), involving ATP sulphurylase and APS kinase to form APS (Baddiley et. al. 1957) and PAPS (Robbins and Lipmann, 1957) respectively:



Activation is favoured by the rapid hydrolysis of PP (ΔF^o = -5000 cal.), and the high affinity of APS for APS kinase.

Assimilatory sulphate reducing micro-organisms, including yeasts, catalyze the reduction of PAPS to sulphite by NADPH (Wilson and Bandurski, 1958a). Dissimulatory sulphate reducers, which reduce APS, have been discussed by Peck (1962) and Nicholas (1967). The NADPH-linked sulphate reductase from yeast contains one heat stable and two heat labile fractions which have been separated (Wilson et. al., 1961) and the following reaction scheme presented (Asahi et.al., 1961):

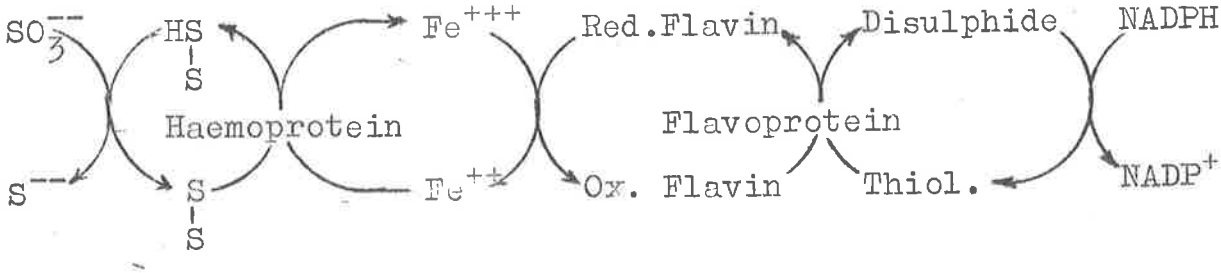


Hilz et. al. (1959, 1960) suggested that lipoic acid is the prosthetic group although Bandurski et. al. found no evidence for the involvement of lipoic acid. The product of this reduction is not free SO_3^{--} but a non-dialysable, acid-labile, SO_3^{--} bound to protein, perhaps fraction C (Torii and Bandurski, 1964, 1967). Such a model necessitates subsequent reduction, or removal, of SO_3^{--} for regeneration of fraction $C(SH)_2$ and does not explain the production of excess SO_3^{--} from SO_4^{--} discussed previously.

5. FORMATION OF SULPHIDE

5.1 Sulphite reduction

Reduction of sulphite to sulphide has been demonstrated in green plants, where activity is associated with chloroplasts (Mayer, 1961), in bacteria (Ishimoto and Nagi, 1961), in fungi (Yoshimoto et. al., 1967) and in yeast (Lezius, 1959; Wainwright, 1961). In yeast the six electron transfer occurs in one enzymic step with no detectable intermediates and is coupled to NADPH. The enzyme contains a flavin (Wainwright, 1962b), labile S and iron (Wainwright, 1967) and consists of two electrophoretic bands (Wainwright, 1967). Sulphite reductase from a mutant yeast reduces SO_3^{--} when coupled with MVH but not when coupled to NADPH, and lacks **FAD** (Yoshimoto and Sato, 1968b) which is similar to the spinach sulphite reductase of Asada et. al.(1966). The dual-protein nature of sulphite reductase is explained by Prabhakararao and Nicholas (1969) who demonstrated the presence of a sulphite reducing haemoprotein coupled to an NADPH-linked flavo protein:



Free sulphite, which is probably attached to a sulphydryl group, is reduced.

5.2 Other sources of sulphide

Although not normal intermediates in yeast metabolism, elemental S and $S_2O_3^{--}$ are reduced readily to H_2S (Rankine, 1963b; Hernandez, 1964). The reduction of S by reduced glutathione is probably non-enzymatic (Maw, 1965):

$$2\ GSH + S \longrightarrow H_2S + GSSG$$

Under oxidative conditions in *Pichia* the reverse reaction occurs (Skerman *et. al.* 1957):

$$H_2S + (O) \longrightarrow S + H_2O$$

Thiosulphate is both enzymically and non-enzymically reduced to H_2S (Lawrence and Cole, 1968; Kaji and McElroy, 1959).

The metabolism of cysteine by cysteine lyase yields H_2S, especially in the presence of thiols (Tolosa *et. al.*, 1968).

6. SULPHUR AMINO ACIDS

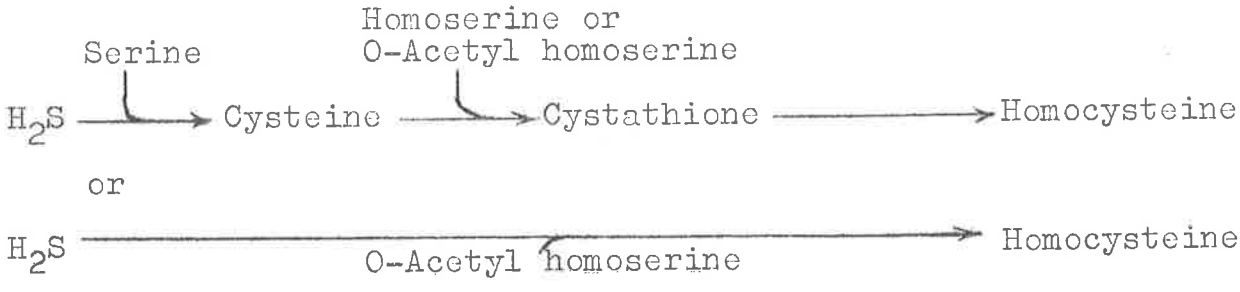
6.1 Cysteine

Sulphide condenses with l-serine in the presence of serine sulphydrase and pyridoxal phosphate (Schlossman et. al., 1962). Wolff and Black (1956) described another enzyme which catalysed the reaction of methane thiol with serine to form S methyl-L-cysteine; although this compound is a suitable S source for yeast (Maw, 1961b), the significance of the reaction is obscure.

Cysteine may be utilized for the synthesis of cystathione, glutathione, Coenzyme A and protein. Excess cysteine is metabolised by cysteine desulphydrase via β-mercapto pyruvate to pyruvate, H_2S and NH_3 (Maw, 1965).

6.2 Methionine

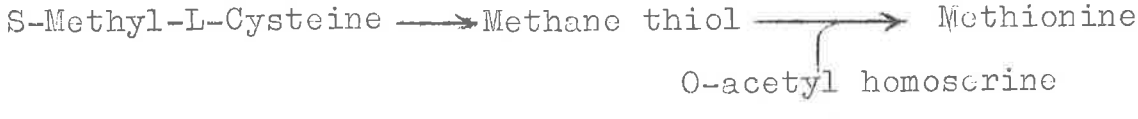
Methionine is synthesised by the methylation of homocysteine, the synthesis of which proceeds via cystathione or directly from H_2S:



In either case, homoserine derives from aspartic acid via aspartyl phosphate and aspartic semi-aldehyde (Black and Wright, 1955a,b,c). Asparto kinase is inhibited and repressed by homoserine and inhibited by methionine (Robichon-Szulmajster and Corrivaux, 1964). Homoserine is acetylated by acetyl CoA in yeasts (Nagi and Flavin, 1967), the enzyme homoserine-O-transacetylase being inhibited and repressed by methionine (Robichon-Szulmajster and Cherest, 1967).

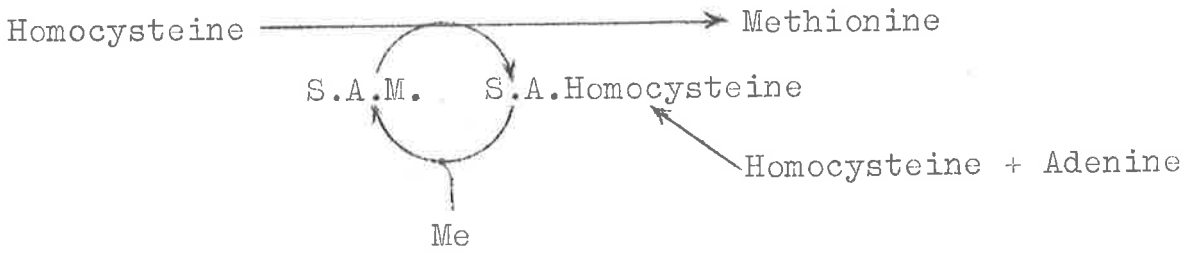
Wiebers and Garner (1967) observed that yeast could synthesise homocysteine directly from H_2S and O-acetyl homoserine by the enzyme homocysteine synthetase, which is subject to feed back inhibition by methionine.

Thiomethyl transfer from methane thiol to O-acetyl-homoserine gives methionine directly in Neurospora (Moore and Thompson, 1967); in view of the ability of yeasts to synthesise methionine from S-methyl-L-Cysteine (Maw and Coyne, 1968), perhaps this pathway also operates in yeast:



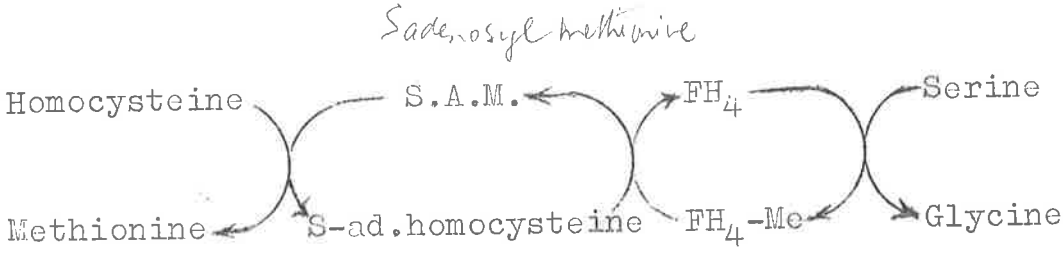
Two pathways for the methylation of homocysteine to methionine have been suggested, the role of S-adenosyl methionine, S.A.M. (Cantoni, 1953) differing:

or



Botsford and Parks (1967) favoured direct methylation of homocysteine by serine, with methionine subsequently reacting with ATP to form S.A.M. (Mudd and Cantoni, 1958; Greene, 1969). Excess methionine fed to yeasts is converted to S.A.M. (Schlenk and Zydek, 1968), the activating enzyme being induced by methionine (Pigg et. al., 1964), which suggests a storage role for S.A.M.

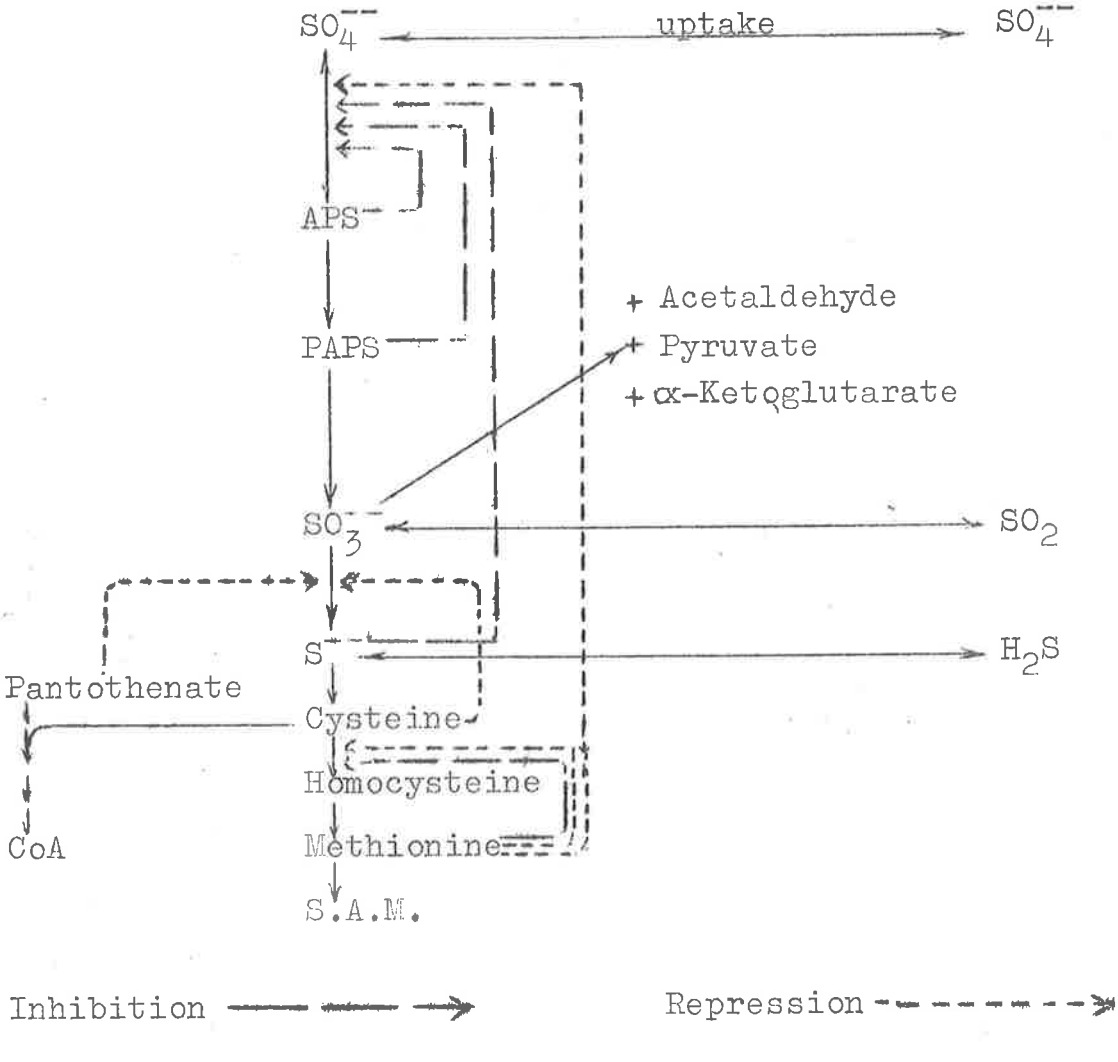
Both Saccharomyces and Candida yeasts synthesise S-adenosyl homocysteine, a precursor of S.A.M. (Duerre and Schlenk, 1962). Shapiro et. al. (1964) partially purified S.A.M.: homocysteine methyl transferase, and Pigg et. al. (1962) proposed a mechanism for the methylation of S-adenosyl homocysteine to S.A.M.:



In this scheme S.A.M. assumes the role of methyl carrier for methionine synthesis, although Duerre (1968) suggests that there is no direct methylation of S-adenosyl homocysteine to S.A.M.

7. CONTROL MECHANISMS OF SULPHUR METABOLISM

The main control mechanisms of sulphate utilization in yeast are:



As added SO_2 increases the production of SO_2-binding compounds (notably acetaldehyde, pyruvic acid and α-keto-glutaric acid), one would expect that yeasts producing SO_2 in excess of their needs would accumulate more of these SO_2-binding compounds. Accepting SO_2 production as the cause, rather than the result, of higher formation of SO_2-binding compounds, one must explain the cause of excess SO_2 production.

The release of SO_2 (and/or H_2S) from yeast cells demands either a leakage in the SO_4^{--} reduction pathway or the blockage of some subsequent synthetic pathway. As S^{--} is the first S-intermediate used for synthesis and there is no correlation between H_2S and SO_2 production, it is unlikely that blockage alone explains excess SO_3^{--} production and SO_2 release.

Sulphate activation and reduction is controlled at the ATP-sulphurylase step, by APS, PAPS and S^{--} inhibition and by methionine repression (De Vito and Dreyfuss, 1964). If sulphite reductase control (repression by pantothenate, Wainwright, 1962a; and by cysteine, De Vito and Dreyfuss, 1964) is more sensitive than ATP-sulphurylase control in a particular yeast strain, then this yeast would accumulate SO_3^{--}. There is little evidence available on the control exercised by NADPH, although Wainwright (1962a) suggested that pantothenate deficiency leads to increased NADPH and

Okuda and Takahashi (1967) reported that similar deficiency causes a lack of cytochrome oxidase in yeasts. While SO_4^{--} reduction in yeasts is primarily assimilatory, it is possible that SO_4^{--} acts as an electron acceptor to oxidize excess NADPH under strongly reducing conditions.

8. AIM OF EXPERIMENTAL WORK

Because of the importance of SO_2 in Australian wine making, an investigation of the interaction of SO_2 and yeast activity at the biochemical level, was undertaken.

The effects of added SO_2 and yeast growth on the production of SO_2 and three SO_2-binding compounds during the fermentation of grape juice by two strains of Saccharomyces yeasts were measured.

Sulphite production by reproducing cells and by resting cells, together with the effects of a number of environmental parameters, were examined.

An attempt was made to explain SO_2 formation, in terms of the activities of enzymes of the relevant biochemical pathways, in cell-free extracts of the yeasts.

MATERIALS AND METHODS

1. MICROBIOLOGICAL TECHNIQUES

1.1 Description of Yeasts

Two yeasts, Saccharomyces cerevisiae strain 350 and Saccharomyces oviformis strain 723 from the culture collection of the Australian Wine Research Institute, Adelaide, were used throughout. Strain 350, probably of European origin, was acquired from a local wine-making firm, Thomas Hardy and Son, Mile End, Adelaide, and is a well-flocculating yeast used extensively in the Australian wine industry. Strain 723 was isolated from the sediment of a fortified (28° proof spirit) wine from the Murray Valley (Angoves Pty Ltd., Renmark) in South Australia.

Standard descriptions of each yeast are as follows (following the criteria of Lodder and Kreger-Van Rij, 1952):

Saccharomyces cerevisiae HANSEN - strain 350

(i) Growth in malt extract (3 days at 25°): sediment, slight turbidity, no ring. Cells short oval to oval, singly, in pairs or short chains. 6-12 x 3-8 μ.

(ii) Growth on malt agar (3 days at 25°): buff coloured slightly raised satinny colonies. Cells as above generally slightly smaller.

(iii)Slide cultures: well developed pseudomycelium.

(iv) Sporulation (5 days on carrot wedge: oval to sausage shaped asci, 1-3 round spores per ascus.

(v) Fermentation:

Glucose	+	Maltose	+
Galactose	+	Lactose	-
Saccharose	+	Raffinose	+⅓

(vi) Sugar assimilation:

Glucose	+	Maltose	+
Galactose	+	Lactose	-
Saccharose	+	Raffinose	weak

(vii) Assimilation of nitrate: negative.

(viii) Ethanol as sole source of carbon: weak positive.

Saccharomyces oviformis OSTERWALDER - strain 723

(i) Growth in malt extract (3 days at 25°): sediment, turbidity, slight ring. Cells round, singly, in pairs or very short chains. 4-10μ.

(ii) Growth on malt agar (3days at 25°): cream to buff coloured, slightly raised satinny colonies. Cells as above, slightly smaller.

(iii)Slide culture: elongated cells but no well developed pseudomycelium.

(iv) Sporulation (7 days on Gorodkowa agar): asci round containing 1, or seldom 2, round spores per ascus.

(v) Fermentation:

Glucose	+	Maltose	+
Galactose	-	Lactose	-
Saccharose	+	Raffinose	+⅓

(vi) Sugar assimilation:

Glucose	+	Maltose	+
Galactose	-	Lactose	-
Saccharose	+	Raffinose	weak

(vii) Assimilation of nitrate: negative

(viii) Ethanol as sole source of carbon: no growth.

The optimum temperatures for growth were determined by growing the yeasts in a liquid medium of 'Yeast nitrogen base' (Difco) plus 5% w/v glucose at pH 4.5. During the logarithmic phase of growth total cell numbers were counted with a haemocytometer slide and the mean generation times were calculated. The vitamin requirements of the yeasts were determined by the methods of Lodder and Kreger-Van Rij, (1952). The findings are tabulated in Results, 1.

1.2 Media

Grape juice: 'White hermitage' (syn. Ugni blanc, Trebbiano) grapes from Seppeltsfield, S.A. were crushed, pressed and the skins removed. The juice was treated with a pectin splitting enzyme (Pectinol, Rohm and Haas) and filtered through No.7 filter pads (Ekwip, Sydney) into autoclaved 2.2 l. clear glass bottles. The juice was deemed sterile when no turbidity was observed after 5 days. Some details of the composition of the juice samples are given in Table 1.

Malt extract medium: Commercial malt extract broth (Oxoid) 6.7 g/l, sucrose 50 g/l, ammonium dihydrogen phosphate 6 g/l and ammonium sulphate 1.3 g/l were dissolved in distilled water, the pH adjusted to 4.5, and the solution was

TABLE 1. Characteristics of grape juices, before addition of sulphur dioxide.

Samples were analysed as described under Methods.

Characteristic of juice	Experiment	
	Sample No. 1	Sample No. 2
Variety	'White hermitage'	'White hermitage'
Location	Barossa Valley	Barossa Valley
Picking date	3rd April, 1967	1st March, 1968
pH	3.6, adjusted to 3.4	3.3, not adjusted
Titratable acid (as g. tartaric acid/ 100 ml)	0.39	0.54
Total soluble solids (^{o}Brix)	22.5	19.0
Total sugar (g/100 ml)	19	16
Acetaldehyde (mg/l)	10	12
Pyruvic acid (mg/l)	25	10
x-Ketoglutaric acid (mg/l)	14	8
Sulphur dioxide (mg/l)	t	0

sterile filtered through No. 8 pads into autoclaved bottles.

Synthetic media: The composition of synthetic media was similar to that described by Wickeram (1951) and used routinely for the classification of yeasts (Lodder and Kreger-Van Rij, 1952). Commercial 'Yeast nitrogen base' (Difco) was used for some experiments. Other synthetic media, the composition of which are given in Appendix 1, were prepared from pure compounds to give variations of Wickerman's formula. Sugar and inorganic salts were dissolved in distilled water and autoclaved, then a ten-fold concentrated solution of vitamins was sterile filtered through 0.45μ Gelman membranes and was added aseptically.

1.3 Maintenance of cultures

For long term storage, cells were grown on malt extract agar and were transferred with a platinum loop to 15 ml of sterile 10% w/v sucrose and stored at about 2°. Cultures were renewed every six months. Inocula for experiments were taken from malt extract agar slants, which were inoculated with yeasts from sucrose cultures after one passage through malt extract broth (2 days at 25°). Slants were renewed every 2 months.

1.4 Cell Counts

Total numbers of cells were counted on a haemocytometer slide, with a double Neubauer ruling (Assistent, Germany). Where necessary, samples were diluted with 10% w/v maltose solution and were shaken with three glass beads for 3 minutes to disperse flocculated cells. Results were calculated from the average of one hundred 0.05 mm x 0.05 mm squares. Total cell numbers were also determined by measuring the absorbance at 550 nm of cultures in 10-ml spectrophotometer tubes (Bausch and Lomb, Spectronic 20). A calibration curve of absorbance against cell numbers, counted on a haemocytometer slide, was prepared for each yeast.

Viable cells were counted by serially diluting a sample of culture with sterile water, placing a drop of known volume of each dilution on the surface of a malt extract agar plate and incubating at 25°. The colonies were counted after 36 hours (Miles and Misra, 1938).

2. CHEMICAL ANALYSES

2.1 Sulphur dioxide

Sulphur dioxide in wines was determined by the aspiration method (Paul) as described by Rankine (1962). The determination of sulphite in assays involving resting cells was by the colorimetric fuchsin method of Grant (1947) using saturated mercuric chloride in 95% v/v ethanol to remove thiols (Dreyfuss and Monty, 1963). Ethanolic sodium hydroxide and saturated mercuric chloride were added directly to the reaction mixture which was then filtered. The method determines total sulphite in the reaction mixture and within the yeast cells.

2.2 Hydrogen sulphide

Hydrogen sulphide produced during fermentation was indicated by blackening in cellulose-lead acetate indicator tubes (Rankine, 1963b). Sulphide production in enzyme assays was measured by the method of Siegel (1965), with test tubes sealed with Parafilm and rubber caps.

2.3 Sulphur dioxide-binding compounds

Acetaldehyde was determined by a modification of the method of Jaulmes and Espezel (1935). A wine sample was

titrated to pH 8.5 with NaOH (Ribéreau-Gayon and Peynaud, 1958) and buffered with saturated borax. The wine was boiled and the distillate containing acetaldehyde was collected in neutral (pH 7) solution of bisulphite and Na_2-EDTA (Guymon and Wright, 1967). Excess bisulphite was quantitatively oxidized with iodine. Sodium hydroxide was added to split the acetaldehyde-bisulphite complex and the released bisulphite was determined by titration with a standard solution of iodine. About 90% of the iodine titre was added before the addition of NaOH to avoid oxidation of free sulphite by air (Burroughs and Sparks, 1961).

Pyruvic and α-ketoglutaric acids were determined enzymically by following the disappearance of NADH in a UV spectrophotometer (Unicam SP 500) at 340 nm, using lactic dehydrogenase and glutamic dehydrogenase respectively (Rankine, 1965; Peynaud et. al., 1966). The reduction of pyruvic acid was complete after 5 minutes at 20° while α-ketoglutaric acid reduction required 10 minutes at 37°.

2.4 Sugar

Total sugar: Grape juice was incubated with invertase concentrate (BDH) at room temperature for 30 minutes, then was analysed for reducing sugar (A.O.A.C. 1960).

Reducing sugar in grape juice and in wine was determined by the volumetric copper reduction method of Lane and Eynon (A.O.A.C. 1960).

Reducing sugar in synthetic media was measured by the colorimetric method of Somogyi (1945) and Nelson (1944).

2.5 Protein

The protein content of enzyme preparations was determined by the colorimetric Folin method (Cowgill and Pardee, 1957), using bovine serum albumin as a standard.

Cell suspensions were stirred into 20 volumes of acetone which was subsequently decanted. The residue was air dried, mixed with 0.5 M NaOH and the supernatant fraction was used for protein determination. Cell-free extracts were suitably diluted and made 0.5 M in NaOH.

2.6 Other analyses

The pH of samples was measured with a glass electrode om a pH-meter (Jones, Melbourne).

Titratable acid: A 5-ml sample of juice was diluted with 250 ml of recently boiled distilled water and was titrated with 0.1 M NaOH to a phenolphthalein end point.

Total solids content of juice was measured with a Balling hydrometer.

Alcohol: Wine was distilled through an inland revenue condenser (Quickfit), the refractive index of the distillate was measured with an Abbé type refractometer (Atago, Japan) and the alcohol content was ascertained by reference to appropriate tables (A.O.A.C., 1960).

3. EXPERIMENTAL DESIGN AND STATISTICS

The effects of yeast strain and of a number of environmental parameters were examined. Experiments were of factorial design and were subjected to an analysis of variance (Snedecor, 1956). Actual variables and the numbers of replicates are shown in the result tables. Significance levels (Appendix 4) were calculated from variance ratios and least significant difference values in the Results were calculated from 't' distribution tables (Fisher and Yates, 1948).

4. ENZYME PREPARATIONS AND ASSAYS

4.1 Propagation of yeasts

For experiments with resting cells and cell-free extracts, yeasts were grown usually in 2.2 l bottles containing 1.5 l malt extract medium which was inoculated with 15 ml of actively growing culture in malt extract broth (Oxoid), thus giving an initial population of 10^6 cells/ml. Unless otherwise stated, cultures were aerated with compressed air which flowed via sterile cotton wool and membrane filters through a sintered glass bubbler. Microaerophilic conditions were maintained, when required, by replacing air with nitrogen. Cells were harvested in the presence of air.

Yeasts were also grown in 500-ml conical flasks containing 250 ml of synthetic media. The inoculum was an aqueous suspension of cells from the surface of a malt extract agar slant. Initial populations were approximately 10^5 cells per ml.

All cultures were incubated at 25° and were shaken manually every 24 hours.

4.2 Harvesting of cells

Propagation cultures were centrifuged (MSE High Speed 18, England) with a continuous flow head at 25,000 x G and 3°. Small lots (250 ml) were harvested in 50-ml tubes. Sedimented cells were washed three times with 10 mM phosphate buffer at pH 4.5, and used immediately or stored at -15°.

4.3 Cell free extracts

A suspension of 5 g wet weight of yeast cells in 5 ml of 0.2 M phosphate buffer at pH 7.4 was passed twice through a French pressure cell (Aminco) at 20,000 psi and at 3°. This treatment normally gives 20 to 50% breakage of cells, based on observations of cell numbers on a haemocytometer slide. The supernatant fraction left after centrifuging at 15,000 x g for 30 minutes was retained as the crude extract and was used immediately or stored at -15°. Crude extracts

were dialysed against 0.2 M phosphate buffer (Visking 8/32 dialysis tubing) to give the dialysed extracts.

4.4 Sulphate activation and reduction

Activation of SO_4^{--} to APS and/or PAPS: Reaction mixtures contained 12 mM Na_2SO_4 (including 2 µc $Na_2{}^{35}SO_4$), 5 mM $MgCl_2$, 5 mM Na_2EDTA, 10 mM ATP, 5 mM creatine phosphate, 0.1 mg/ml creatine kinase, 0.05 mM NADPH, 10 mM sodium isocitrate, 1 mg/ml isocitrate dehydrogenase, 50 mM Tris-HCl buffer at pH 7 and 25 µl enzyme preparation in a total volume of 0.1 ml. After incubation at 30° for 1½ hours, the compounds present in 10-µl or 25-µl samples were separated by high voltage electrophoresis on paper in pH 5 citrate buffer for 1 hour at 1.5 kV. The radioactivity of the separated ^{35}S-compounds was measured by liquid scintillation counting.

ATP sulphurylase: The ATP produced from APS and pyrophosphate was measured by the luciferin-luciferase enzyme system from the firefly (Photinus pyralis) using a liquid scintillation spectrometer (Packard) with the coincidence circuit switched off. The reaction mixture contained 3.0 ml buffer (3.33 mM phosphate, 16.67 mM arsenate and 1 mM $MgCl_2$ at pH 7.3), 20 µl APS 0.185 M, 20 µl pyrophosphate 1.5 mM, 0.1 ml firefly extract and 20 µl enzyme preparation. To calibrate the system and to allow a correction for ATP-ase activity, 0,1 ml of 10^{-10} M ATP was included as an internal standard in some of the assays (Stanley and Williams, 1969; Balharry and Nicholas, 1970).

4.5 Sulphite production by resting cells

Reaction mixtures of 38 mM ammonium sulphate, 280 mM D-glucose, 100 mM phosphate buffer at pH 7 and 100 mg yeast paste in 1.0 ml were incubated in 1 x 10 cm tubes at 30° for 1 hour. Sulphite was measured (Grant, 1947) at the beginning and at the end of the incubation period. Sulphite production was recorded as the increase, over controls containing boiled yeast suspensions, in total sulphite concentration during this period.

4.6 Sulphite reduction

The sulphite reductase activity of cell-free extracts was measured by the assay of Prabhakararao and Nicholas, (1969). Enzyme preparations were incubated for 40 minutes at 30° with sulphite and an NADPH regenerating system of glucose-6-phosphate and glucose-6-phosphate dehydrogenase. Sulphide produced was determined by the colorimetric method of Siegel (1965).

5. RADIO-ISOTOPE TECHNIQUES

5.1 Uptake of sulphate

A reaction mixture (6.0 ml) containing 280 mM D-glucose, 28 mM $(NH_4)_2SO_4$, 22 mM Na_2SO_4 (labelled with 0.98 µc $^{35}SO_4^{--}$), 50 mM Tris-HCl buffer at pH 7, 0.3 mM Na_2-EDTA and 0.25 g/ml yeast cells was incubated with gentle shaking at 30°. At the times shown in Figure 7, 1.0-ml samples were withdrawn and filtered through 2.5 cm glass fibre filters (Whatman GF/C). The residue was washed with the Tris-HCl buffer and the filtrate and washings were pooled. Cells on the filter were extracted in situ with 1.0 ml absolute ethanol, followed by 1.0 ml 50% aqueous ethanol, 1.0 ml water, and, finally, 1.0 ml of the Tris-HCl buffer.

The inorganic sulphate content of each sample was determined as follows: 0.1 ml of M zinc acetate was added to samples to precipitate protein and nucleosides which were removed by filtering through Whatman 42 paper. The filtrate was maintained near 100° while 25 µl of M Na_2SO_4 and 0.5 ml of 0.2 M $BaCl_2$ in M HCl were added. The precipitated sulphate was collected on 2.5 cm filter papers (Whatman 42) and dried at 50°. Radioactivity was measured by liquid scintillation counting.

5.2 Separation of Sulphur compounds by electrophoresis

^{35}S-labelled compounds were separated by electrophoresis on 30 cm x 2 cm paper (Whatman DE 81 or Whatman 3 MM) in citrate buffer at pH 5.0 for 1 hour at 1.5 kV under carbon tetra-chloride in the tank described by Tate (1968). The paper was air dried for 2 hours at room temperature, cut into 1 cm sections and the radioactivity of each section was measured. The rates of movement of sulphate, APS and PAPS were found by using ^{35}S-labelled marker compounds.

5.3 Measurement of ^{35}S-labelled compounds

Filters and electrophoretogram sections were placed directly in a flor of 7.0 g/l PPO and 0.3 g/l POPOP in toluene and counted with a Packard Tricarb liquid scintillation spectrometer. Corrections were made for quenching.

6. REAGENTS

6.1 Biochemicals

Lactic dehydrogenase, glutamic dehydrogenase, creatine kinase, isocitrate dehydrogenase, pyrophosphatase, desiccated firely tails, AMP, ATP, creatine phosphate, NADH, $NADP^+$, NADPH, sodium isocitrate, sodium pyrophosphate and bovine serum albumin were purchased from Sigma Chemical Co., U.S.A.. 'Pectinol' enzyme was obtained from Rohm and Haas, U.S.A., and invertase concentrate was bought from British Drug Houses, Poole, England.

APS was synthesised from pyridine-sulphur trioxide and AMP by the method of Baddiley et. al. (1957) and was purified by ion exchange and high voltage elextrophoresis.

6.2 Media

Malt extract broth and malt extract agar were prepared from dehydrated media bought from 'Oxoid', Oxo Ltd, London. The dehydrated synthetic media 'Yeast nitrogen base' and 'Yeast vitamin base' were purchased from Difco Laboratories Inc., Michigan, U.S.A.. Commercial quality sucrose was bought from Commonwealth Sugar Refinery, Australia.

The composition of Synthetic Media 'A' and 'S' (details in Appendix 1) is based on the formula of Wickerham (1951). Media were prepared from A.R. grade chemicals and the following vitamins: p-amino benzoic acid and thiamine (British Drug Houses, England), folic acid and pyridoxin hydrochloride (Knoll Laboratories, Sydney), inositol (Difco Laboratories, U.S.A.), niacin (Fluka, Switzerland), riboflavin (L.Light and Co., Colnbrook, U.K.) and calcium pantothenate and biotin (Sigma, U.S.A.).

6.3 Radiochemicals

Labelled sodium sulphate, $(^{35}S)Na_2SO_4$, was bought from the Radiochemical Centre, Amersham, U.K.. $Na_2{}^{35}SO_4$ was used

to prepare (^{35}S)pyridine-sulphur trioxide from which (^{35}S)APS was prepared by the method cited (Baddiley, et. al., 1957).

6.4 Other reagents

'Analar' or equivalent grade inorganic chemicals were purchased from British Drug Houses, Ajax Chemicals and Hopkins and Williams. Toluene, POPOP, dimethyl-p-phenylene diamine sulphate and basic fuchsin were obtained from BDH, PPO from Ajax and Tris-HCl from Sigma.

Distilled and deionized (by an Elga, U.K., deionizer) water was used in the preparation of all solutions and media.

Medical grade compressed air was purchased from Australian Liquid Air. Nitrogen (industrial grade) was supplied by Commonwealth Industrial gases. Because this grade of nitrogen contains 0.2% oxygen v/v, media aspirated with it are described as 'microaerophilic', which term, as used throughout this thesis, is synonymous with 'nearly anaerobic'.

RESULTS

1. CHARACTERISTICS OF YEASTS

The origins and standard descriptions of the yeasts are given in Materials and Methods 1.1. The identity of each yeast was confirmed by the taxonomic methods of Lodder and Kreger-Van Rij. Vitamin requirements of the yeasts, determined by the method of Lodder and Kreger-Van Rij, are given in Table 2.

The optimum temperatures for growth were determined by growing the yeasts in 'Yeast nitrogen base' (Difco) plus 15% w/v glucose at pH 4.5. The optimum temperature was 30° for S. cerevisiae and 28° for S. oviformis as shown in Figure 1.

Sulphate was the preferred sulphur source for both yeasts, although methionine was almost as effective (Table 3). Sulphite also supported rapid yeast growth, especially at the higher concentration (Table 3), but only after a prolonged lag phase (Figure 2). S. oviformis grew as well on 1 mM cysteine as it did on sulphate, sulphite or methionine, but S. cerevisiae showed an extended lag phase on this substrate. However, growth of both yeasts was retarded at the higher concentration (5 mM) of cysteine. Yeasts utilized sulphide and cystine less readily than the other sulphur sources.

TABLE 2. Vitamin requirements of yeasts

Yeasts grown on malt extract agar slopes were used to inoculate 5.0 ml of 'Yeast vitamin free base' (Difco) plus all the vitamins listed except one named as deficient. After incubation at 25° for 7 days, one loopful of the culture was transferred to fresh tubes of similar media and incubated for a further 7 days.

Vitamin deficient, (all others in the table present)	Growth in the second subculture after 7 days.	
	S. cerevisiae	S. oviformis
Nil (all vitamins present)	+++	+++
Biotin	+	+
Pantothenate	+	-
Folic acid	++	++
Inositol	++	++
Niacin	++	++
p-Amino benzoate	++	++
Pyridoxin	++	++
Riboflavin	++	+
Thiamine	++	++

FIGURE 1. Effect of temperature on the growth rate of yeasts

Bottles containing 10 ml of 'Yeast nitrogen base' (Difco) plus 15% w/v glucose at pH 4.5 were inoculated with 0,1 ml of an actively growing yeast culture in malt extract broth. At 12-hourly intervals cells were counted with a haemocytometer slide (Methods 1.4) and the mean generation time during the logarithmic phase was calculated. (Means of duplicates)

○ S. cerevisiae

■ S. oviformis

FIGURE 1

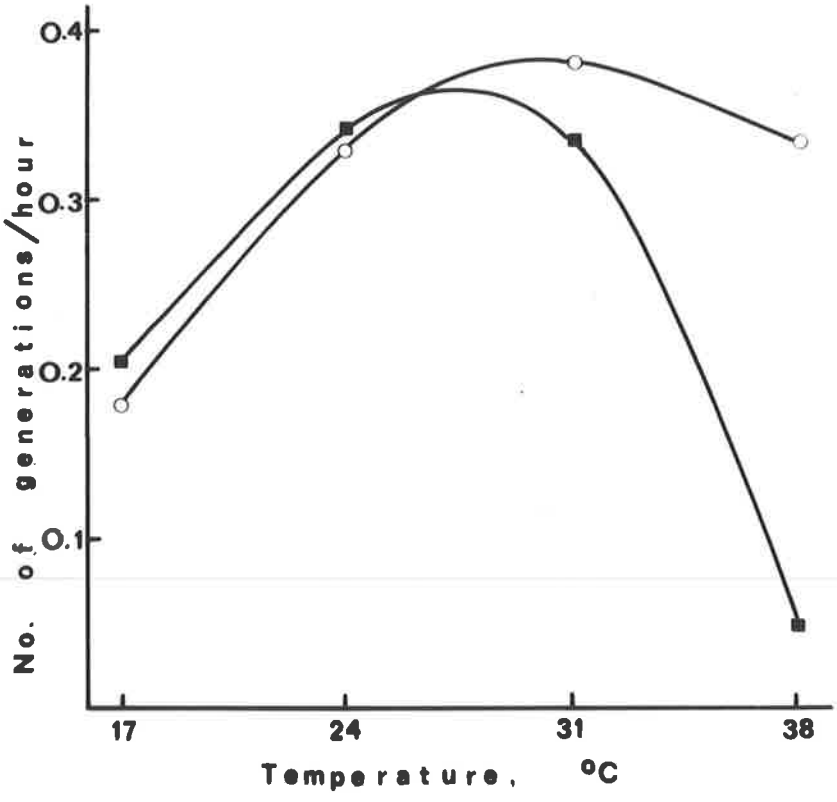


TABLE 3. Effect of sulphur source on the growth rate of yeasts

Tubes containing 8.0 ml of sulphur-free base medium (Appendix 1-2) plus an added sulphur compound were inoculated with 0.1 ml of an aqueous suspension of yeast and were then incubated at 25°. At 12-hourly intervals up to 2 days the absorbance at 550 nm of each culture was measured. Mean generation times were calculated from the time taken to double the absorbance at 550 nm during the logarithmic phase of growth. (Means of duplicates).

Sulphur source.	Concentration of S source, mM.	Mean generation time, hours.	
		S.cerevisiae	S.oviformis
Na_2SO_4	1	2.3	2.5
	5	1.9	2.4
$KHSO_3$	1	4.6	4.8
	5	2.5	3.2
Na_2S	1	12.5	13.0
	5	7.5	9.5
Cysteine-HCl	1	3.4	3.6
	5	10.4	7.3
Cystine	1	4.6	5.8
	5	5.4	6.4
Methionine	1	2.4	4.2
	5	2.3	3.6

FIGURE 2. Yeast growth in media containing single sources of sulphur.

Yeasts were grown in sulphur-free base medium (Appendix 1.2) plus 1 mM sulphur compound as described in Table 3. Cell numbers were determined by measuring absorbance at 550 nm. (Means of duplicates)

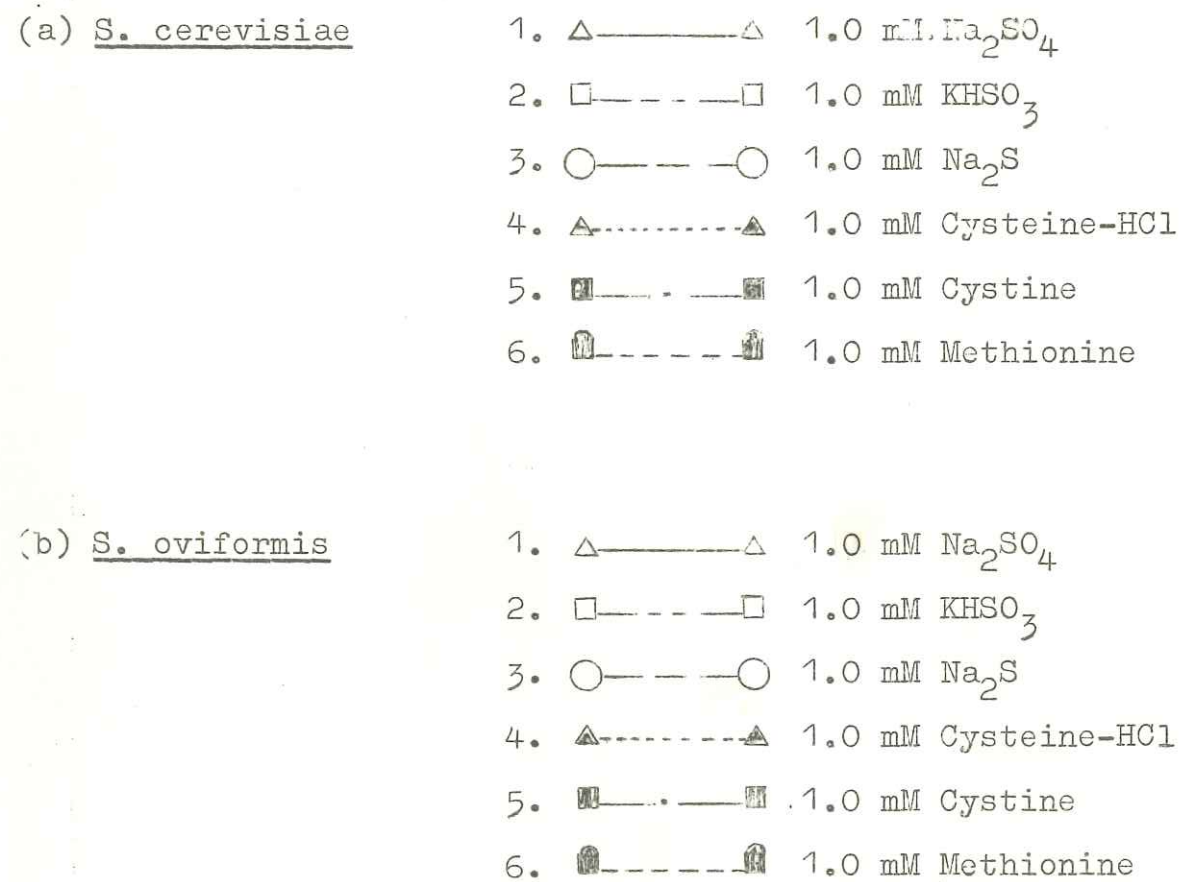
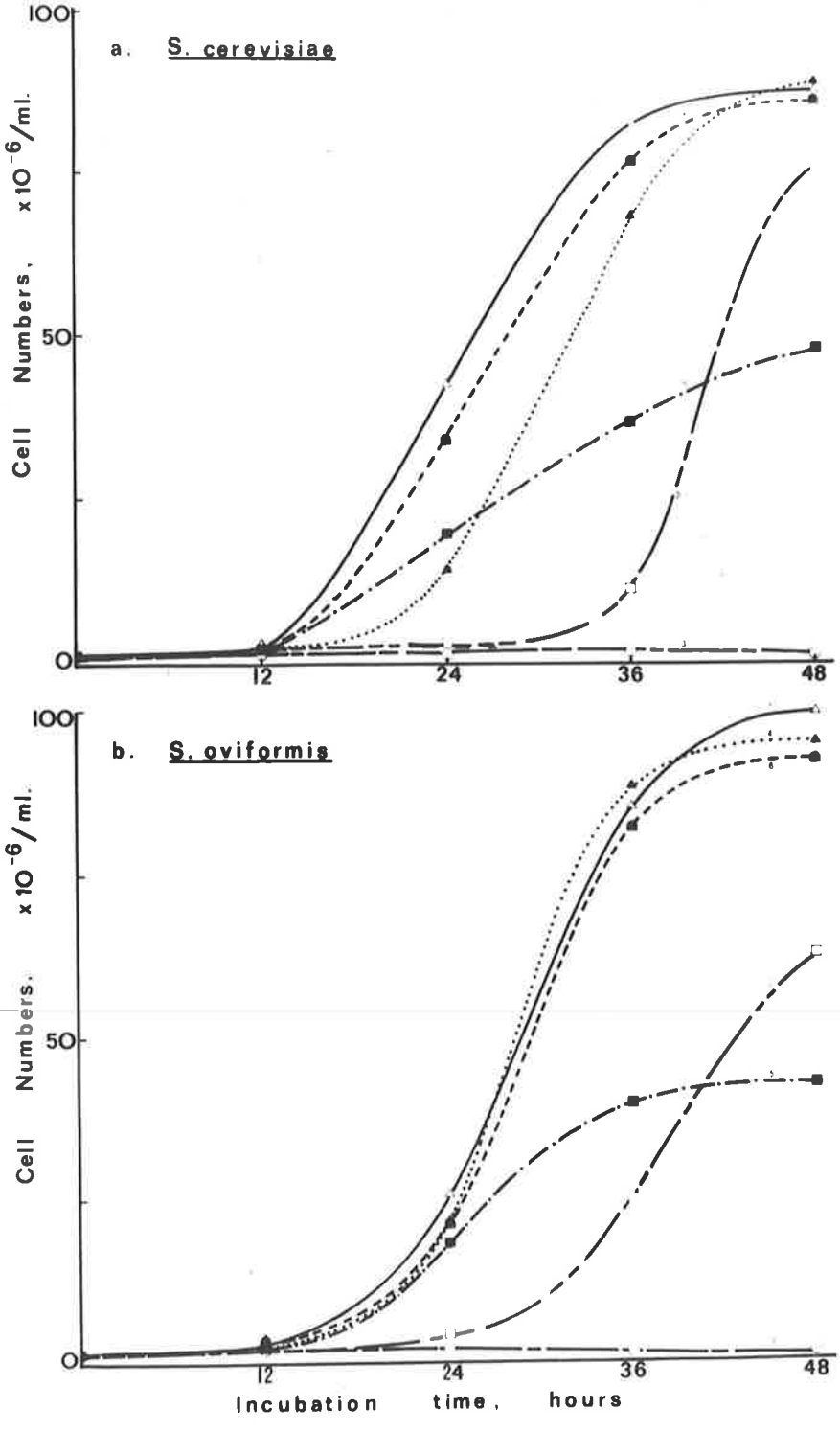


FIGURE 2


a. S. cerevisiae
b. S. oviformis
Cell Numbers, x10⁻⁶/ml.
100
50
0
12
24
36
48
Incubation time, hours

The precipitation of sulphide by metal cations probably contributed to the slow growth of yeasts in media containing Na_2S.

S. oviformis accumulated sulphite in the growth medium when grown in sulphate but not in the other sulphur sources studied. S. cerevisiae did not produce extracellular sulphite (Table 4). Neither yeast formed sulphide in any treatment, as indicated by analysis of cultures for sulphide by the method of Siegel (Methods 2.2).

TABLE 4. Sulphite concentration after yeast growth in media containing single sulphur sources

The reaction mixtures described in Table 3 were incubated at 25° for 4 days, then were centrifuged and the sulphite concentrations in the supernatant solutions were determined by Grant's method (Methods 2.1) (Means of duplicates)

Sulphur source.	Concentration of S source, mM.	Final SO_3^{--} concentration, mM	
		S.cerevisiae	S.oviformis
Na_2SO_4	1	0	0.05
	5	0	0.04
$KHSO_3$	1	0.84	0.76
	5	2.01	2.04
Na_2S	1	0	0
	5	0	0
Cysteine-HCl	1	0	0
	5	0	0
Cystine	1	0	0
	5	0	0
Methionine	1	0	0
	5	0	0

2. FORMATION AND UTILIZATION OF SULPHITE IN GROWING YEASTS

2.1 Effect of yeast strain

The production of sulphur dioxide and of SO_2-binding compounds was influenced by yeast strain more than by any other variable studied (Table 5, Figures 3 and 4). S. oviformis produced from 35 mg/l (Figure 4) to 76 mg/l (Figure 3) sulphur dioxide during the fermentation of grape juice, while S. cerevisiae produced nil to trace amounts under similar conditions.

Wines made with S. oviformis bound more sulphur dioxide than did those made with S. cerevisiae (Table 5). This was explained by the higher production of SO_2-binding compounds, namely acetaldehyde, pyruvic acid and α-ketoglutaric acid, by S. oviformis (Table 5, Figure 4). These three compounds accounted for an average of 97% of the bound sulphur dioxide in the experimental wines (Table 5).

2.2 Effect of sulphur dioxide

The effects of additions of sulphur dioxide before fermentation are shown in Figure 3.

Added sulphur dioxide reduced the formation of sulphur dioxide by S. oviformis and increased the formation of acetaldehyde by both yeasts, especially S. cerevisiae (Appendix 4.1). The addition of sulphur

TABLE 5. Influence of yeast strain and sulphur dioxide on the production of SO_2 - binding compounds

Bottles containing 1.5 l of grape juice (Sample No. 1, Table 1), prepared and sterilized as described (Methods 1.2), were inoculated with 30 ml of actively growing culture in similar juice and allowed to ferment to completion at 20°. The resultant wines were decanted, a 50-ml sample set aside for immediate analysis, and the remainder were filtered and bottled under sterile conditions. Sulphur dioxide was determined by the aspiration method (Methods 2.1) and binding compounds were determined by the methods cited previously (Methods 2.2). (Means of duplicates)

Yeast	Sulphur dioxide, mg/l						SO_2-binding compounds, mg/l			Calculated SO_2 bound to compounds, mg/l			Total calculated bound SO_2 in wines, mg/l	Bound SO_2 accounted for, %
	Added before fermentation	Total immediately after fermentation	Added at bottling	In wines after bottling										
				Free	Bound	Total	Acetaldehyde	Pyruvic acid	α-Ketoglutaric acid	Acetaldehyde	Pyruvic acid	α-Ketoglutaric acid		
S.cerevisiae	0	1	220	50	160	210	29	130	200	42	62	42	146	96
		1	320	115	175	290	21	125	185	30	78	54	162	93
	50	22	220	59	176	235	40	150	200	58	76	44	178	101
		20	320	110	220	330	44	155	185	64	96	53	213	97
	100	70	220	46	230	275	66	200	190	96	93	37	236	102
		70	320	120	265	385	63	175	180	92	109	53	254	96
S.oviformis	0	61	315	29	355	380	114	350	380	165	125	53	343	97
		76	415	46	430	475	114	380	370	166	177	73	416	97
	50	61	315	21	355	375	120	340	385	174	111	45	330	93
		72	415	41	465	505	135	350	415	196	155	76	427	92
	100	117	315	32	385	415	114	385	350	165	154	55	374	97
		120	415	60	465	525	135	400	250	196	204	57	457	98
LSD P 0.01		5	-	9	24	21	10	57	48	-	-	-	-	-

FIGURE 3. Effect of sulphur dioxide on the production of sulphur dioxide and of SO_2-binding compounds.

Grape juice (Sample No. 1, Table 1) was prepared and sterilized as described in Methods 1.2. Samples of juice were inoculated with 30 ml of actively growing yeast in similar juice and allowed to ferment to completion at 20°. The wines are decanted, filtered and bottled with added sulphur dioxide. Sulphur dioxide was determined by the aspiration method (Methods 2.1) before further SO_2 was added. Acetaldehyde, pyruvic acid and α-ketoglutaric acid were determined as described in Methods 2.2. (Means of four replicates).

Note: S.oviformis did not grow in treatments containing 200 mg/l Sulphur dioxide.

○ S.cerevisiae

▣ S.oviformis

FIGURE 3

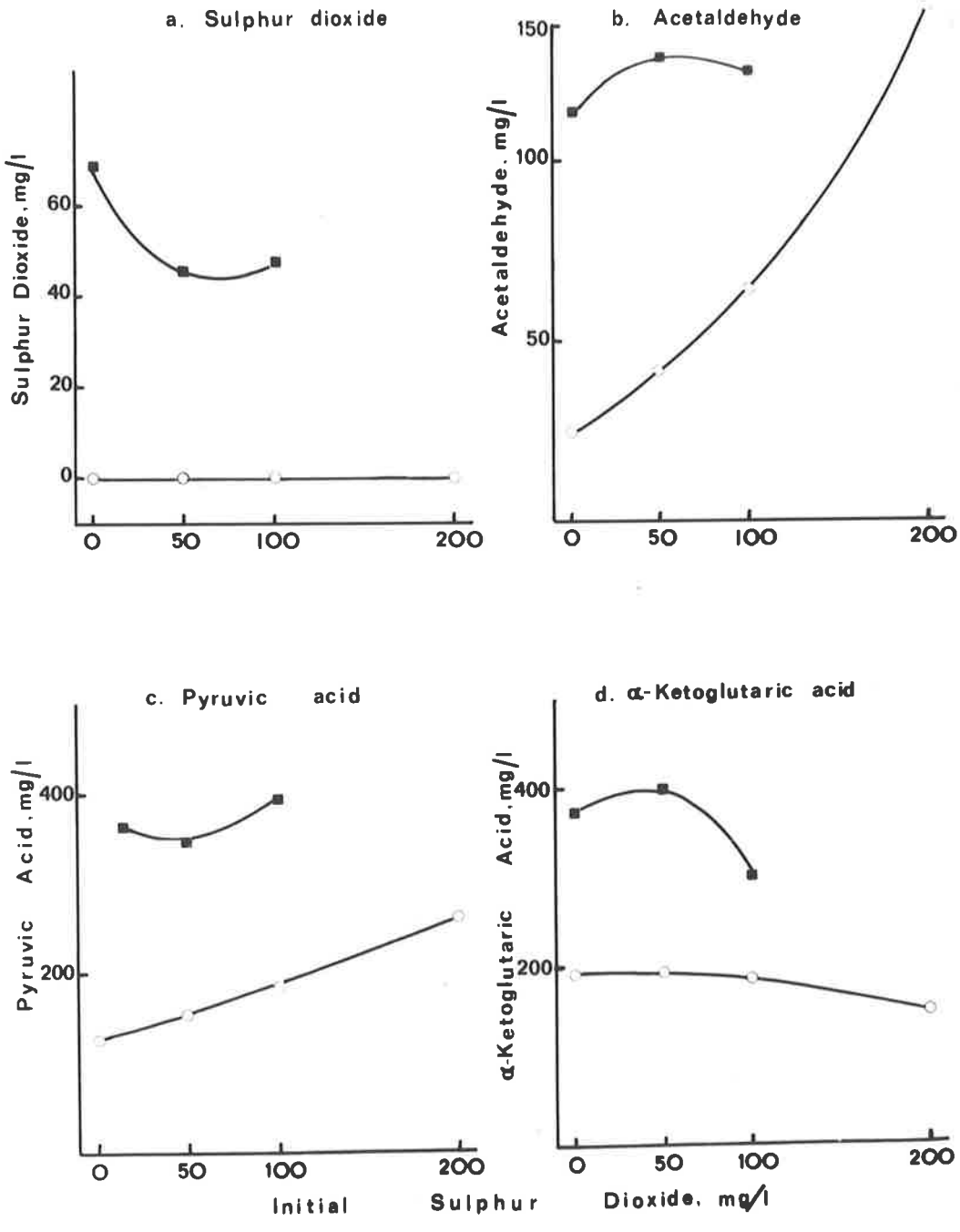

a. Sulphur dioxide
Sulphur Dioxide, mg/l
60
40
20
0
0
50
100
200
b. Acetaldehyde
Acetaldehyde, mg/l
150
100
50
0
50
100
200
c. Pyruvic acid
Pyruvic Acid, mg/l
400
200
0
50
100
200
Initial Sulphur Dioxide, mg/l
d. α-Ketoglutaric acid
α-Ketoglutaric Acid, mg/l
400
200
0
50
100
200

FIGURE 4. Production of sulphur dioxide and of sulphur dioxide-binding compounds during fermentation.

Bottles containing 1.5 l of grape juice (Sample No. 2, Table 2) were prepared as described in Methods 1.2. The bottles were inoculated with 10^6 cells/ml and incubated at 22°. At daily intervals up to 5 days, then at 8 and 11 days, 50-ml samples were withdrawn, filtered and analysed. Sulphur dioxide was determined by the aspiration method described in Methods 2.1, while acetaldehyde, pyruvic acid and α-ketoglutaric acid were measured by the procedures cited in Methods 2.3. (Means of duplicates)

○ S.cerevisiae

■ S.oviformis

FIGURE 4

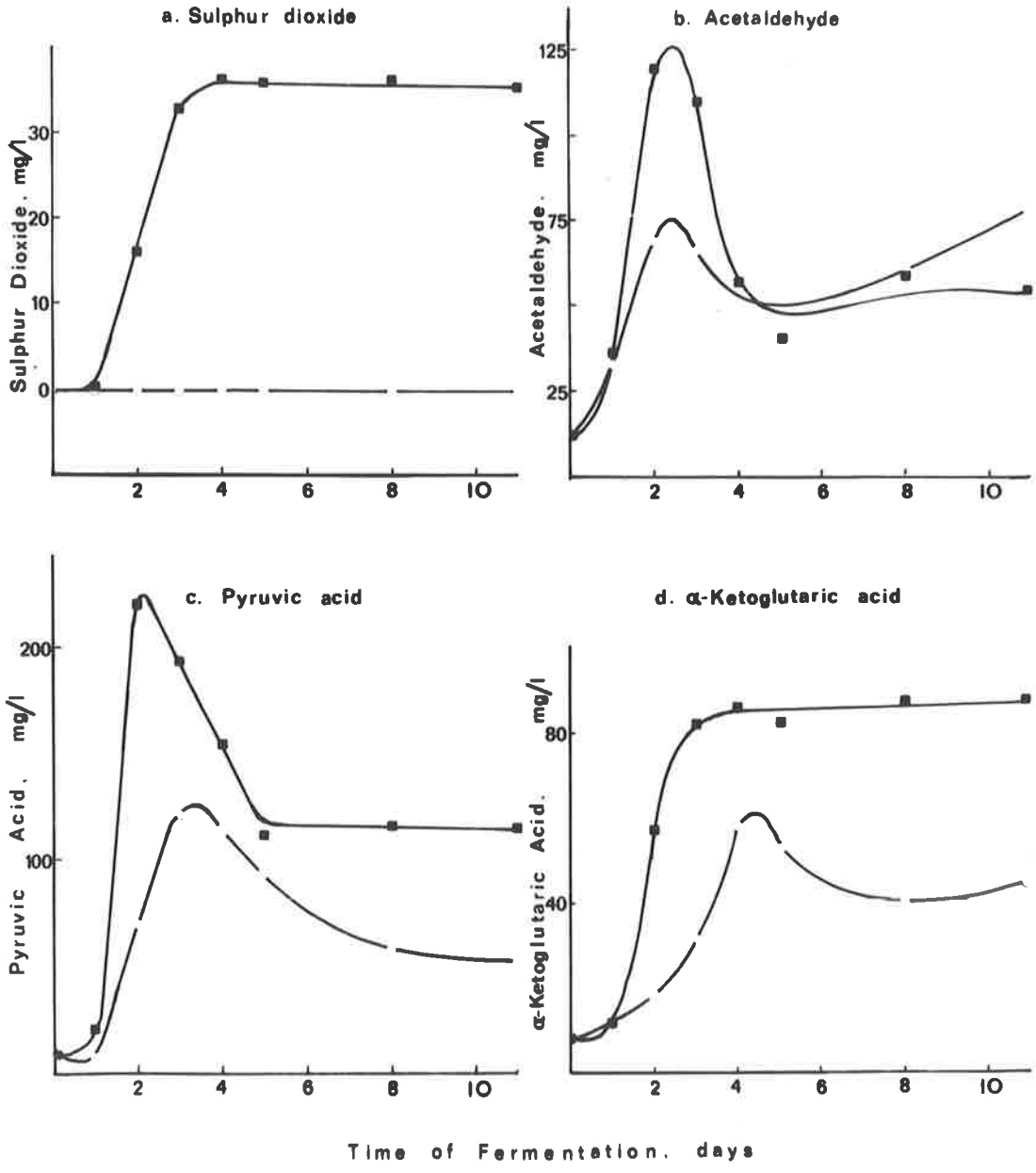

a. Sulphur dioxide
Sulphur Dioxide, mg/l
30
20
10
0
2
4
6
8
10
b. Acetaldehyde
Acetaldehyde, mg/l
125
75
25
2
4
6
8
10
c. Pyruvic acid
Pyruvic Acid, mg/l
200
100
2
4
6
8
10
d. α-Ketoglutaric acid
α-Ketoglutaric Acid, mg/l
80
40
2
4
6
8
10
Time of Fermentation, days

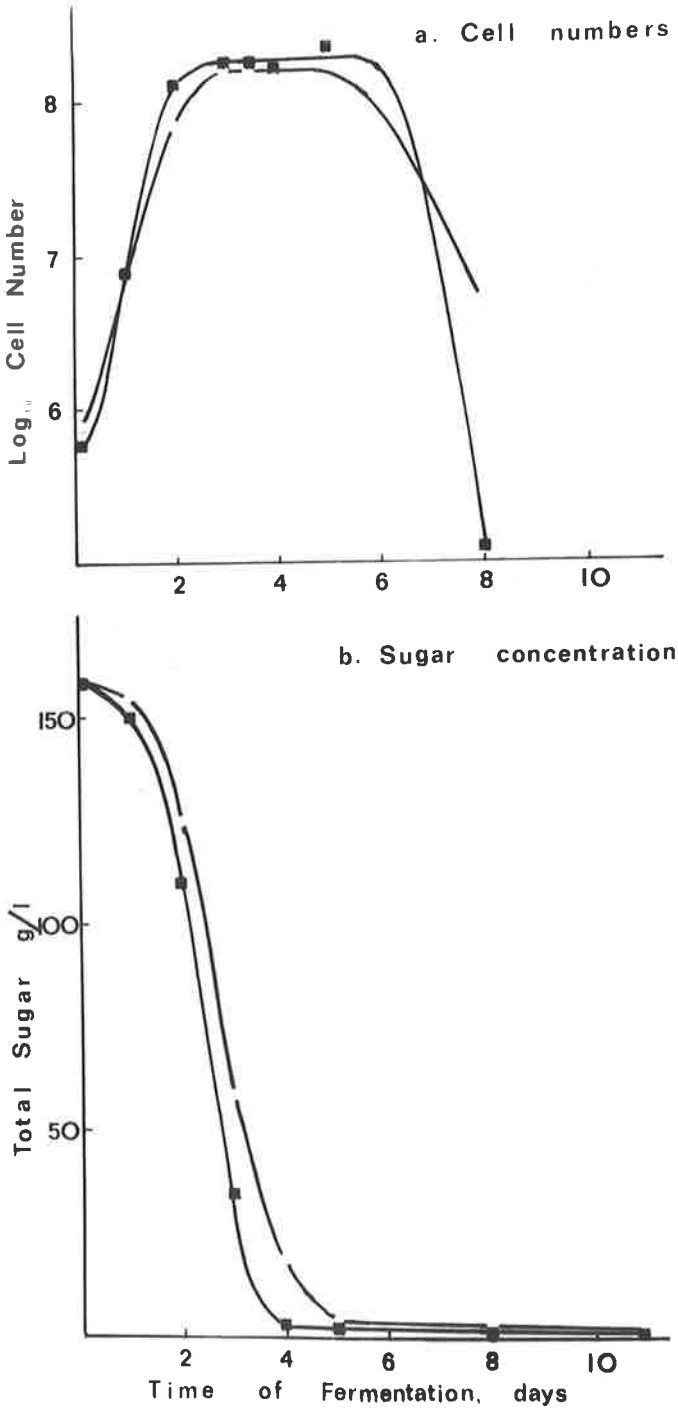
FIGURE 5. Changes in cell numbers and in sugar concentration during fermentation.

Experimental details are the same as those given in Figure 4. After gentle mechanical stirring, samples were withdrawn for cell counts (haemocytometer slide, Methods 1.4) and sugar determinations (Lane and Eynon, Methods 2.4), at the time intervals indicated in the figure. (Means of duplicates)

○ S.cerevisiae

■ S.oviformis

FIGURE 5


a. Cell numbers
Log Cell Number
8
7
6
2
4
6
8
10
b. Sugar concentration
Total Sugar g/l
150
100
50
2
4
6
8
10
Time of Fermentation, days

dioxide marginally increased production of pyruvic acid (P 0.05) and marginally reduced that of α-keto-glutaric acid (P 0.05).

Sulphur dioxide added after fermentation remained in the wines and did not affect the concentrations of binding compounds (Table 5).

The maximum concentrations of sulphur dioxide at which growth occurred were 200 mg/l for S. cerevisiae and 100 mg/l for S. oviformis. Growth and fermentation by both yeasts were visibly slower in the 100 mg/l treatments than in those with less sulphur dioxide.

Additions of sulphur dioxide had no effect on either the concentrations of alcohol, residual sugar or on the pH of the wines (Appendix 3).

2.3 Changes during fermentation

Sulphur dioxide produced by S. oviformis reached a maximum after 4 days and then remained fairly constant, while S. cerevisiae did not produce detectable amounts of sulphur dioxide (Figure 4). Comparison of sugar utilization (Figure 5) and sulphur dioxide production (Figure 4) suggests that these activities may be related. The results indicate that sulphur dioxide is produced by both resting and reproducing cells.

Figure 4 shows the progressive changes in the concentrations of acetaldehyde, pyruvic acid and α-keto-glutaric acid during fermentation. Both yeasts accumulated acetaldehyde and pyruvic acid early in fermentation (2 to 3 days) and subsequently removed about half of these compounds. S. oviformis produced higher amounts of both compounds than did S. cerevisiae. The maximum levels of acetaldehyde and of pyruvic acid coincided with the end of the logarithmic phase of growth, and decreased during the resting stage. α-Ketoglutaric acid concentrations reached maxima during the resting stage of growth and remained constant in cultures of S. oviformis, but subsequently decreased in those of S. cerevisiae.

S. oviformis reproduced more rapidly than did S. cerevisiae, reached its resting phase earlier and it removed sugar more rapidly and more completely (Figure 5).

Wine left in contact with yeast cells contained more acetaldehyde, and less free sulphur dioxide, than did similarly prepared wine which had been bottled 7 days earlier (Table 6). Total sulphur dioxide, pyruvic acid and α-ketoglutaric acid were not affected significantly (Table 6, Appendix 4.2) by autolysis of yeast cells.

TABLE 6. Effect of contact with yeast cells on the concentrations of SO_2-binding compounds in wines.

Wines were made from grape juice Sample No. 2, (Table 1) as described in Figure 4. Half the wine was bottled 11 days after inoculation with the yeasts, the other half was left in contact with yeast cells for a further 7 days when it was similarly bottled and analysed. (Means of duplicates)

Yeast	Time from inoculation to bottling (days)	SO_2 in wines after bottling, mg/l			SO_2 binding mg/l	compounds,		Calculated SO_2 bound to compounds, mg/l			Total calculated bound SO_2 in wines, mg/l	Bound accou fo: %
		Free	Bound	Total	Acetaldehyde	Pyruvic acid	α-Ketoglutaric acid	Acetaldehyde	Pyruvic acid	α-Ketoglutaric acid		
S.cerevisiae	11	110	195	305	87	51	49	125	30	14	170	87
	18	16	308	325	193	36	45	280	10	4	290	94
S.oviformis	11	155	198	355	51	123	83	74	76	26	175	89
	18	53	318	370	155	118	83	225	56	17	300	94
LSD P 0.01		83	150	90	115	33	45	-	-	-	-	-

FIGURE 6. Growth of yeasts and sulphur dioxide production in a synthetic medium.

Treatments containing 100 ml of 'Yeast nitrogen base' (Difco) plus 15% w/v glucose at pH 4.5 were sterilized by steaming at 100° for 30 minutes on 3 successive days, then were inoculated with washed cells to give 2 x 10^6 cells/ml. Cultures were incubated with gentle shaking at 15° and samples were withdrawn daily for analysis. (Means of triplicates).

(a) Reducing sugar (Somogyi and Nelson, Methods 2.4) and total cell numbers (haemocytometer slide, Methods 1.4).

○-------○ Reducing sugar, S.cerevisiae
▨--------▨ Reducing sugar, S.oviformis

×————× Cell numbers, S.cerevisiae
▲————▲ Cell numbers, S.oviformis

(b) Sulphur dioxide concentration, measured by the method of Grant (Methods 2.1).

○ S.cerevisiae

▨ S.oviformis

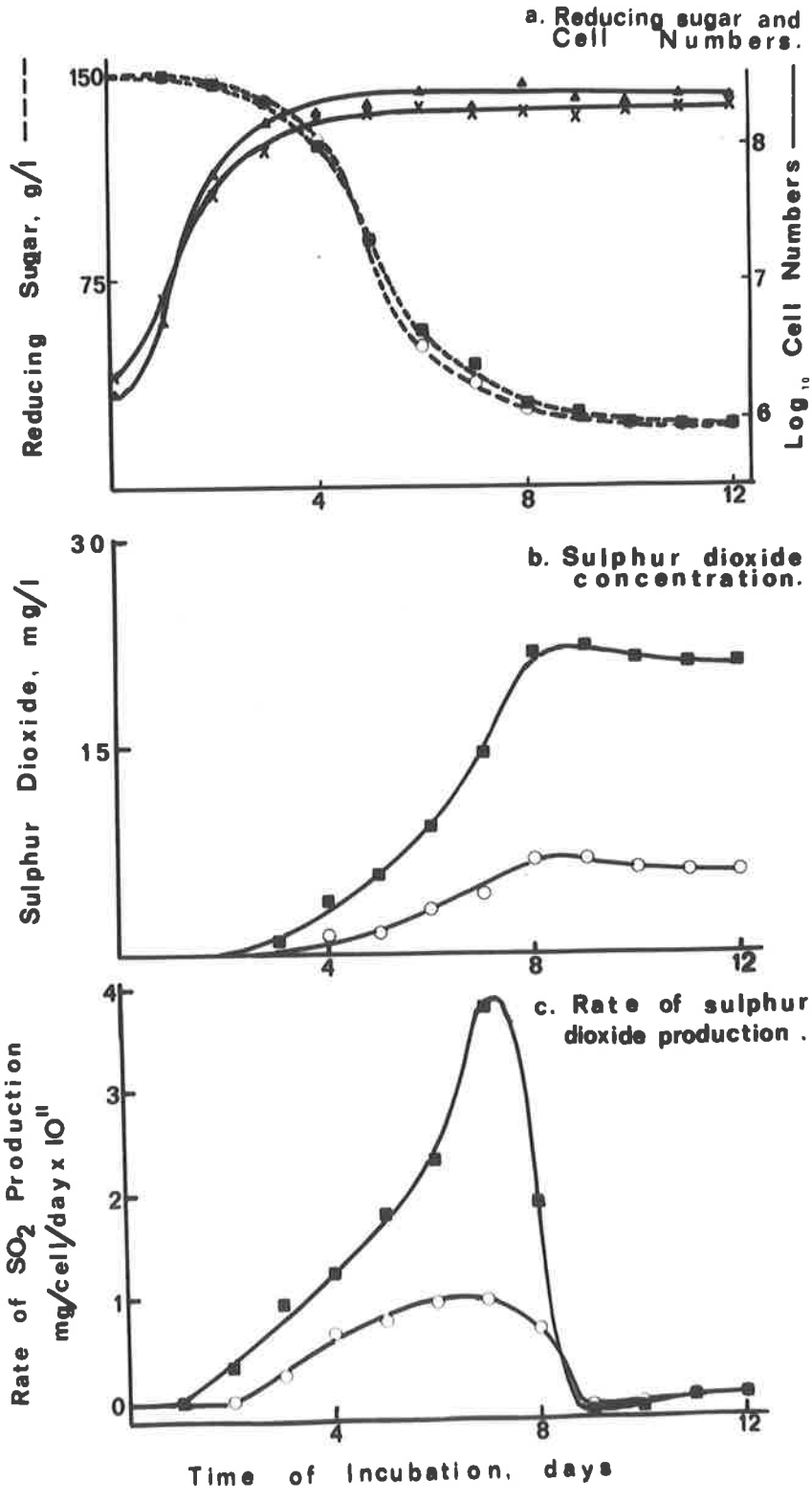
(c) Rate of sulphur dioxide production, calculated from the data given in graphs (a) and (b) above:

Rate = d/dt SO_2 production x 1/number of cells.

○ S.cerevisiae

▨ S.oviformis

FIGURE 6


a. Reducing sugar and Cell Numbers.
Reducing Sugar, g/l
Log10 Cell Numbers
150
75
8
7
6
4
8
12
b. Sulphur dioxide concentration.
Sulphur Dioxide, mg/l
30
15
4
8
12
c. Rate of sulphur dioxide production.
Rate of SO2 Production mg/cell/day x 10^11
4
3
2
1
0
4
8
12
Time of Incubation, days

2.4 Growth stage of yeast

Figure 6 shows the relationship between yeast growth, as total cell numbers, and sulphite production.

The logarithmic phase of growth of both yeasts extended from 0.5 days to approximately 3.5 days. During this period 10% of the glucose provided was utilized and some sulphur dioxide was produced.

Resting cells, which produced more sulphur dioxide than did actively growing cells, continued to form sulphur dioxide until 8.5 days when sugar utilizations had practically ceased. The rate of sulphur dioxide production per cell was highest in resting cells toward the end of fermentation; i.e. after approximately 7 days.

Sugar utilization and sulphur dioxide production in synthetic medium was lower than from grape juice (Figures 3 and 4). Table 7 shows that this was partly due to heat sterilization of the synthetic medium.

2.5 Production of hydrogen sulphide

When grape juice (sample Number 2, Table 1) was fermented at 25° in bottles fitted with lead acetate indicating tubes (Methods 2.2) no blackening was observed after 14 days. Thus neither *S. cerevisiae* nor *S. oviformis* produced free hydrogen sulphide under the conditions described.

TABLE 7. Effect of method of sterilization of the culture medium on the utilization of sugar and on the production of sulphur dioxide.

'Yeast nitrogen base' (Difco) 6.7 g/l plus 5% w/v glucose at pH 4.5 was sterilized as shown in the table, then was inoculated with 2 x 10^6 cells/ml of yeast and incubated for 8 days at 25°. Sulphur dioxide was measured by Grant"s method (Methods 2.1) and sugar was determined by the method of Somogyi and Nelson (Methods 2.4) (Means of triplicates).

Method of sterilization	Sulphur dioxide, mg/l		Residual sugar, g/l.	
	S.cerevisiae	S.oviformis	S.cerevisiae	S.oviformis
Filtered (No.8 pads, Ekwip)	9.3	51.5	6.3	5.3
Autoclaved (15 minutes at 15 p.s.i)	7.0	34.0	20.5	32.0
LSD P 0.05 =	8.7		1.6	

3. CONVERSION OF SULPHATE TO SULPHITE BY RESTING CELLS

3.1 Uptake of sulphate

When resting yeast cells were incubated with 50 mM sulphate and glucose in Tris-HCl buffer at pH 7.1, the initial rate of sulphate uptake was essentially the same for both yeast strains (Figure 7). The uptake of sulphate by *S. cerevisiae* was greater than that by *S. oviformis*. Both yeasts contained intracellular sulphate.

3.2 Effect of assay conditions on sulphite production, using a standard preparation of cells.

The reduction of sulphate to sulphite by intact cells was measured under a range of environmental conditions. For all experiments in this section, yeasts were grown in malt extract medium (Methods 1.2) and were harvested as described in Methods 4.1 and 4.2.

Both yeasts reduced sulphate to sulphite in the presence of glucose and phosphate buffer (Table 8). No added vitamins were required. Ammonium sulphate was a better substrate than was sodium sulphate.

The optimum temperature for the reduction was 30° (Figure 8). The optimum pH was approximately 7 (Figure 9). These results were used to establish the standard assay conditions described in Methods 4.5.

FIGURE 7. Uptake of ^{35}S sulphate by resting cells

A yeast suspension (0.25 g yeast paste/ml) was incubated with 280 mM glucose plus 50 mM Na_2SO_4 (labelled with 160 µcurie/l of $^{35}SO_4^{--}$) in 50 mM Tris-HCl buffer at pH 7 at 30° (Methods 5.1). Sulphate in the substrate and that present in cells was determined by liquid scintillation counting. (Means of triplicates)

(a) Sulphate removed from the substrate, calculated by subtracting final from original sulphate concentration. (µmoles SO_4^{--}/g protein)

○ S.cerevisiae

■ S.oviformis

(b) Sulphate within cells, determined directly in extracts of cells, (µmoles SO_4^{--}/g protein)

○ S.cerevisiae

▩ S.oviformis

FIGURE 7

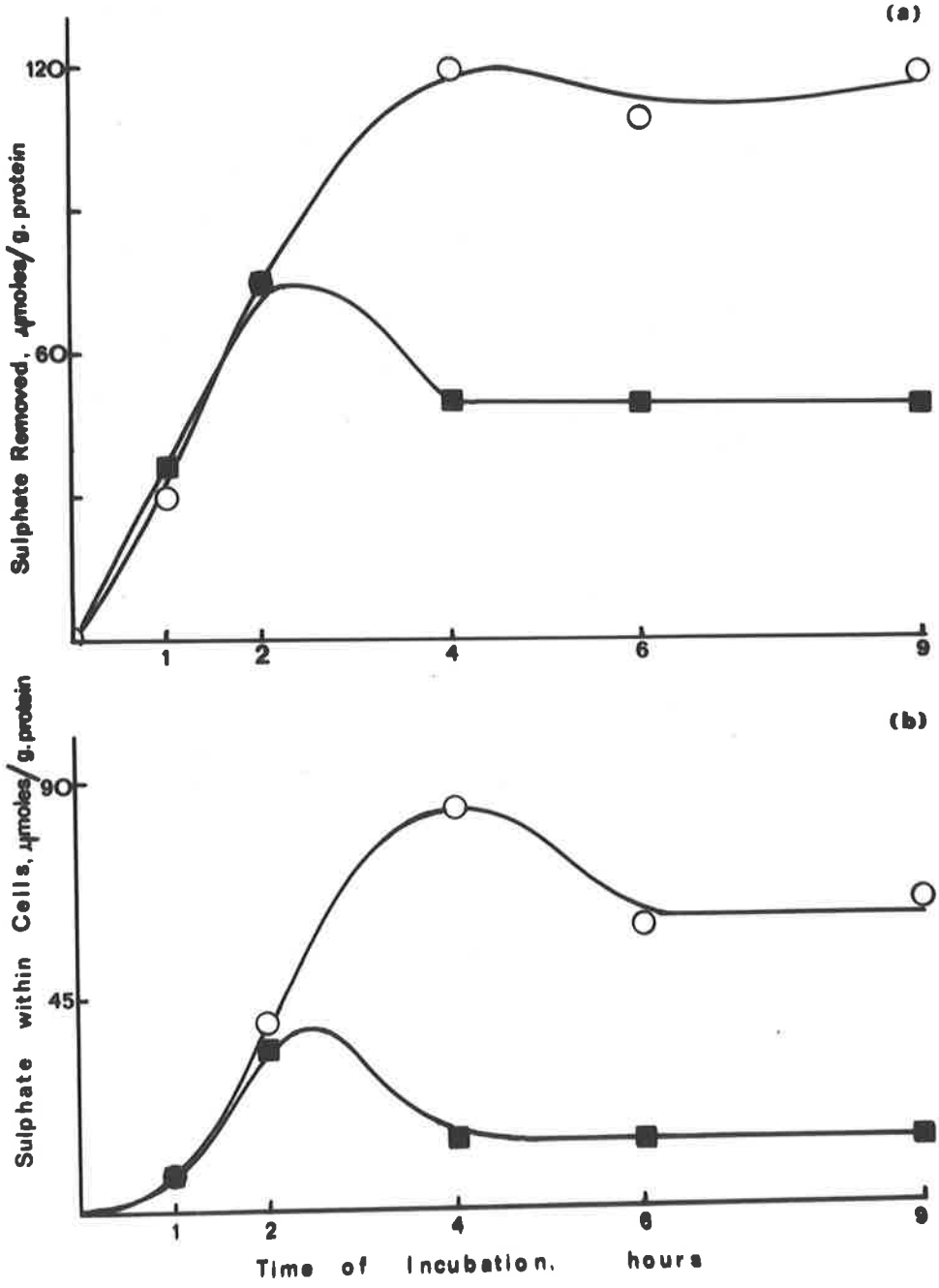

(a)
120
60
Sulphate Removed, μmoles/g. protein
1
2
4
6
9
(b)
90
45
Sulphate within Cells, μmoles/g. protein
1
2
4
6
9
Time of Incubation, hours

TABLE 8. Substrate requirements for the production of sulphite by resting cells.

Reaction mixtures contained 0.5 g wet weight yeast (grown in malt extract medium and harvested as described in Methods 4.1 and 4.2), the substrates listed in the table and 10 mM phosphate buffer in a final volume of 5.0 ml. Mixtures were incubated at 25° for 6 hours when sulphite production was measured by Grant's method (Methods 2.1). (Means of triplicates).

Substrate	Mean sulphite production, µmoles SO_3^{--}/hr/g wet wt yeast	
	S.cerevisiae	S.oviformis
Buffer + 6.7g/l 'Yeast nitrogen base' (Difco) + 280 mM glucose + 38 mM $(NH_4)_2SO_4$.	0.4	2.4
Buffer + 38 mM $(NH_4)_2SO_4$ + 280 mM glucose	0.4	2.2
Buffer + 38 mM Na_2SO_4 + 280 mM glucose	0.1	1.5
Buffer + 280 mM glucose	0	0
Buffer + 38 mM $(NH_4)_2SO_4$	0	0

LSD Body of table P 0.05 = 0.3

FIGURE 8. Effect of temperature on sulphite production in whole cells.

Yeast cells, grown in malt extract medium under the standard conditions described in Methods 4.1, were incubated with 280 mM glucose and 38 mM sulphate at pH 4 at a range of temperatures shown in the figure. Sulphite produced after 6 hours was measured by Grant's method (Methods 2.1). Specific activity was calculated in μmoles SO_3^{--}/hour/g yeast protein. (Means of triplicates)

○ S.cerevisiae

■ S.oviformis

FIGURE 9. Effect of pH on sulphite production in whole cells.

Yeast cells, grown in malt extract medium under the standard conditions described in Methods 4.1, were incubated with 280 mM glucose and 38 mM sulphate at 30°. Reaction mixtures were buffered with 100 mM phosphate buffer at the pH values shown in the figure. Sulphite produced after 1 hour was measured by Grant's method (Methods 2.1). Specific activity was calculated in μmoles SO_3^{--}/hour/g yeast protein. (Means of triplicates).

○ S.cerevisiae

■ S.oviformis

FIGURE 8

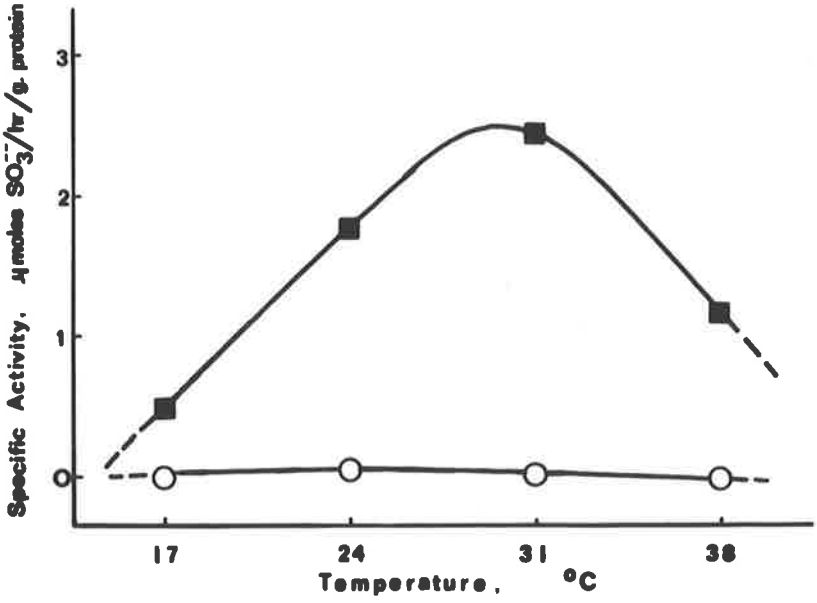
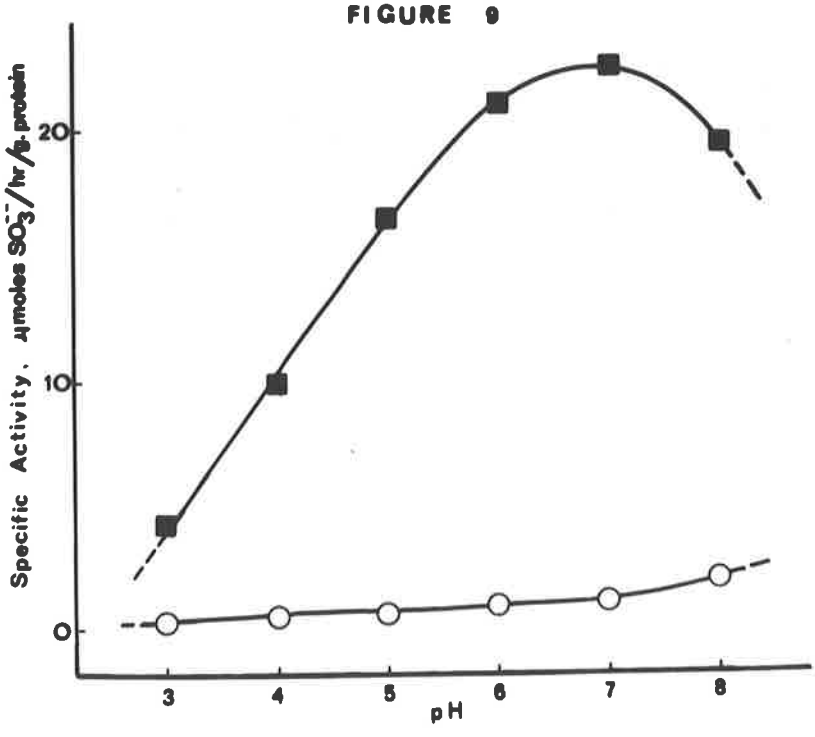


FIGURE 9



The supply of oxygen during assay did not affect the activity of sulphite production (Table 9).

The presence of sulphur dioxide-binding compounds in the assay mixture had little effect on sulphite production. Sulphite production by S. oviformis was stimulated by acetaldehyde up to 20 mM, but at 50 mM it was markedly depressed. At 50 mM concentrations, pyruvic acid and α-ketoglutaric acid slightly reduced the formation of sulphite. S. cerevisiae produced very little sulphite in all treatments (Figure 10).

3.3 Effect of growth conditions on sulphite production

Yeasts were grown under a variety of environmental conditions. The reduction of sulphate to sulphite by resting cells was measured by the standard assay described in Methods 4.5

The specific activity of sulphite production, in μmoles/hour/g. yeast protein, increased with higher sulphate concentration (up to 50 mM) in the growth medium (Figure 11). Sulphite formation was higher in yeasts grown anaerobically than in those grown in air (Table 10).

The effects of adding methionine, calcium pantothenate and cysteine to a synthetic medium are shown in Table 11. Addition of pantothenate stimulated

TABLE 9. Activity of sulphite production in whole cells under aerobic and microaerophilic conditions.

Yeasts were grown in malt extract medium (Methods 1.2), harvested (Methods 4.2) and assayed for sulphite producing activity (Methods 4.5) in Thunberg tubes. The tubes were evacuated and refilled three times with either oxygen or nitrogen and equilibrated for 10 minutes. Then a suspension of yeast cells and the substrate were mixed and incubated for 1 hour at 30°. (Means of triplicates).

Assay conditions	Specific activity, μmoles SO_3^{--}/hr/g yeast protein	
	S.cerevisiae	S.oviformis
Aerobic (under oxygen)	1.8	20.2
Microaerophilic (under nitrogen)	2.0	21.0

LSD body of table P 0.05 = 1.1

FIGURE 10. Effect of SO_2-binding compounds on sulphite producing activity

Yeast cells, grown in malt extract medium under the standard conditions described in Methods 4.1, were assayed for sulphite production (Methods 4.5) in the presence of graded concentrations of three SO_2-binding compounds, acetaldehyde, pyruvic acid and α-ketoglutaric. Specific activity is given in μmoles SO_3^{--}/hour/g yeast protein. (Means of triplicates).

(a) Acetaldehyde. ○ S.cerevisiae
■ S.oviformis

(b) Pyruvic acid. ○ S.cerevisiae
■ S.oviformis

(c) α-Ketoglutaric acid. ○ S.cerevisiae
■ S.oviformis

FIGURE 10

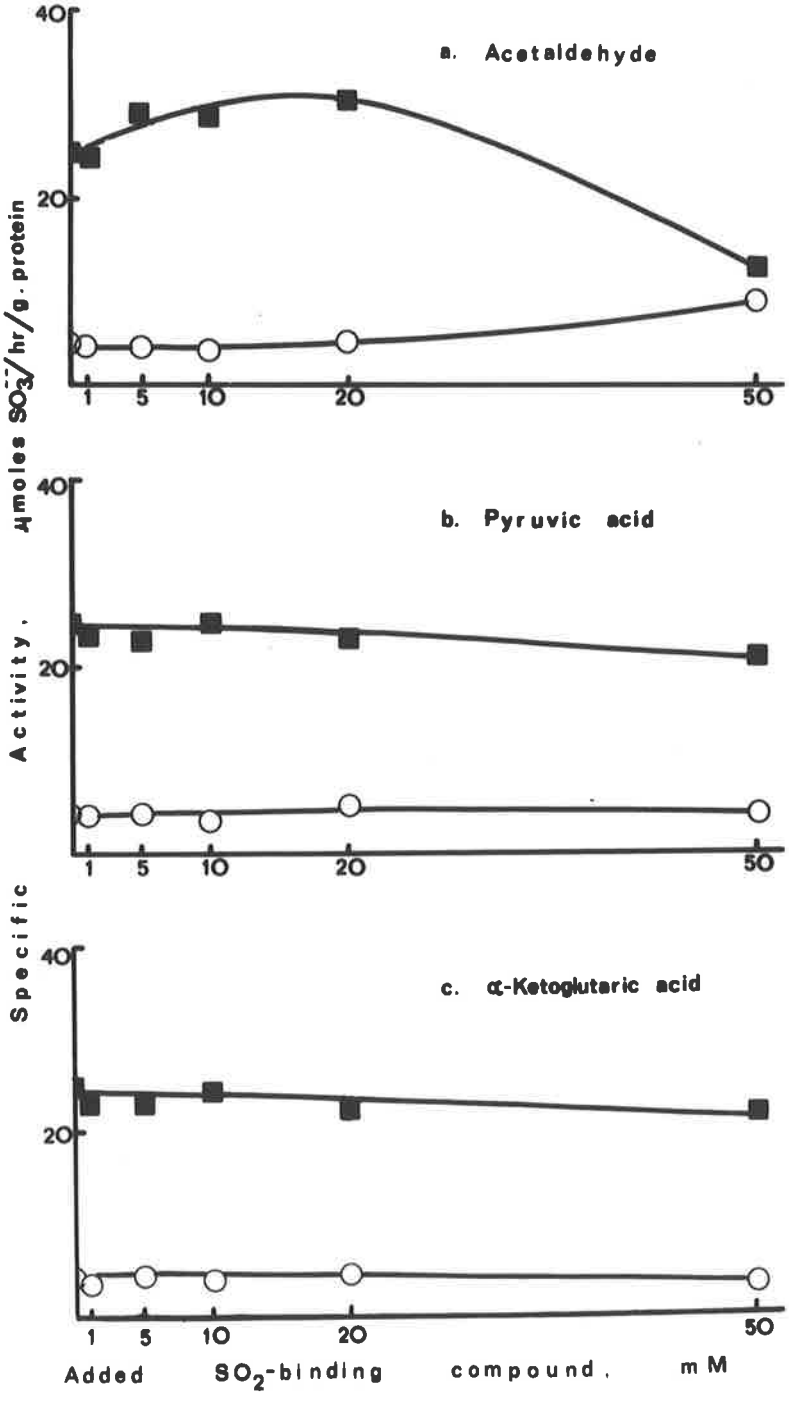

a. Acetaldehyde
b. Pyruvic acid
c. α-Ketoglutaric acid
Specific Activity, μmoles SO_3^{--}/hr/g. protein
40
20
1
5
10
20
50
Added SO_2-binding compound, m M

FIGURE 11. Effect of sulphate concentration during yeast growth on sulphite producing activity.

Yeasts were grown in the basic medium 'S' (Appendix 1.3) supplemented with 2, 10 or 50 mM concentrations of sulphate. Cells were harvested, washed and the specific activity of sulphite production was determined (Methods 4.5). (Means of triplicates)

○ S.cerevisiae

■ S.oviformis

FIGURE 11

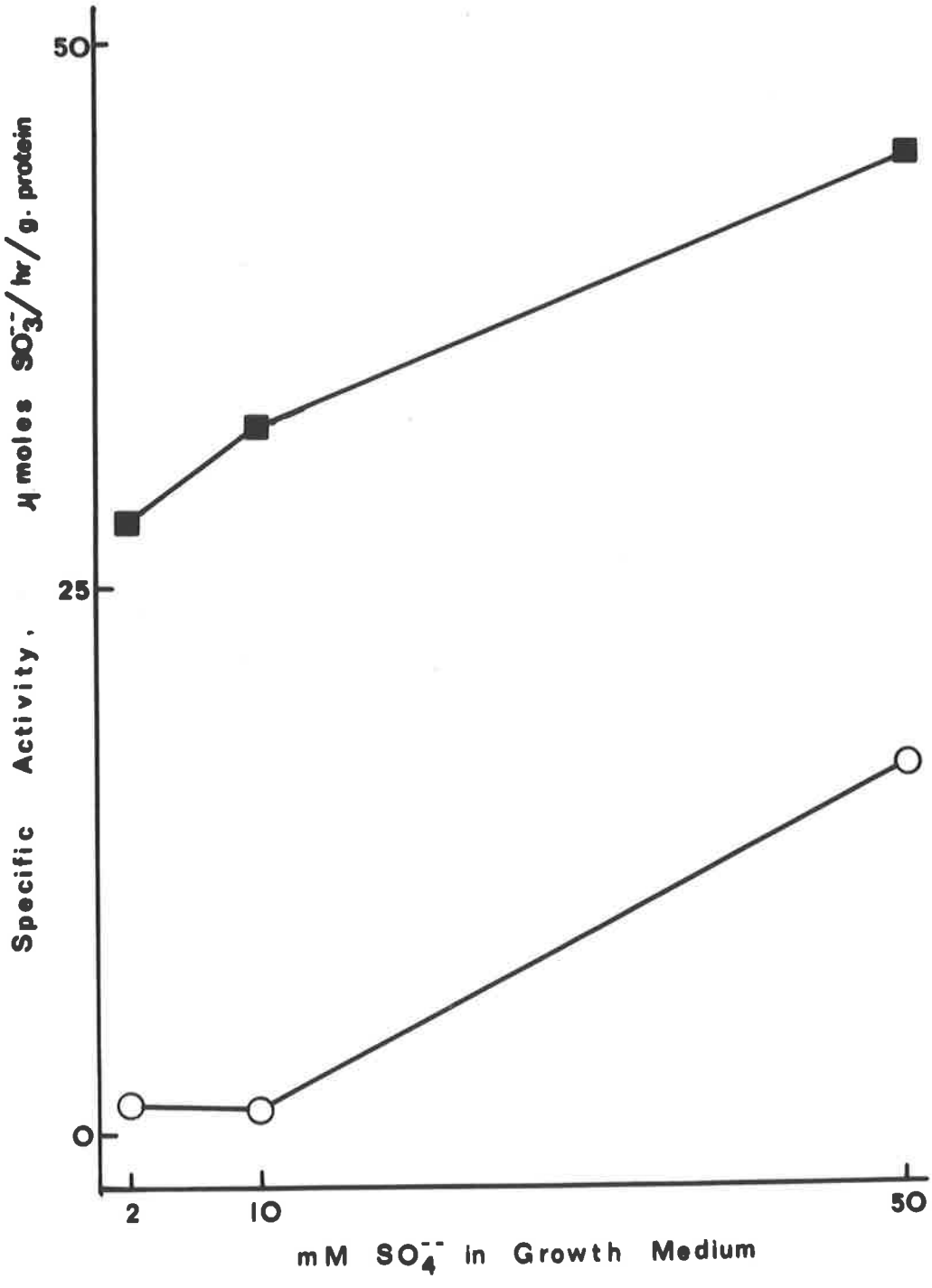

50
25
0
Specific Activity, μ moles SO_3^{--}/hr/g. protein
2
10
50
mM SO_4^{--} in Growth Medium

TABLE 10. Sulphite producing activity of yeasts grown under aerobic and under microaerophilic conditions.

Yeasts were grown in a malt extract medium (Methods 1.2), aspirated with air or nitrogen, as described in Methods 4.1. Cells were harvested, washed and the specific activity of sulphite production was determined (Methods 4.5). (Means of triplicates).

Growth conditions	Specific activity, µmoles SO_3^{--}/hr/g yeast protein	
	S.cerevisiae	S.oviformis
Aerobic (aspirated with air)	1.3	29
Microaerophilic (aspirated with nitrogen)	2.0	34

LSD body of table P 0.05 = 3.5

Growth conditions	Yield of cells, g wet weight/l	
	S. cerevisiae	S. oviformis
Aerobic	11.3	9.6
Microaerophilic	3.3	2.5

TABLE 11. Effect of methionine, pantothenate and of cysteine in the growth medium on the activity of sulphite production.

Yeasts were grown in the basal medium 'A' with added compounds as shown in the table (Appendix 1.) for 5 days at 25°. Cells were harvested, washed and the specific activity of sulphite production was determined (Methods 4.5). (Means of duplicates).

Growth medium	Specific activity, µmoles SO_3^{--}/hr/g yeast protein	
	S.cerevisiae	S.oviformis
Basal medium	1.5	11.5
Basal medium plus methionine, 20 mg/l.	2.5	13.0
Basal medium plus calcium pantothenate, 400 µg/l.	2.0	24.5
Basal medium plus methionine 20 mg/l plus calcium pantothenate 400 µg/l.	1.0	21.0
Basal medium plus methionine 20 mg/l plus calcium pantothenate 400 µg/l plus cysteine 20 mg/l.	2.0	32.5

LSD body of the table P 0.05 = 3.0

sulphite production by S. oviformis, but this effect was repressed by the presence of methionine. The addition of methionine alone did not affect activity. The addition of cysteine apparently removed the methionine repression and further stimulated sulphite production by this yeast. The activity of S. cerevisiae was low in all cases and no significant differences were observed with the added compounds.

4. METABOLISM OF SULPHATE BY CELL-FREE EXTRACTS

4.1 Activation and reduction of sulphate

Sulphate activation and reduction by the two yeasts was investigated in a reaction mixture containing yeast enzymes, 12 mM Na_2SO_4 (labelled with $^{35}SO_4^{--}$) and ATP and NADPH regenerating systems (Methods 5.1). Figure 12 shows that 'active sulphate' (i.e. APS and PAPS) was the main product, with small amounts of sulphite being formed. An unidentified ^{35}S fraction was detected near the origin of electrophoretograms. When a reaction mixture was subjected to electrophoresis on Whatman 3 MM paper, APS and PAPS separated; most of the 'active sulphate' was PAPS.

S.oviformis activated more sulphate than did S. cerevisiae (Table 12). Dialysis decreased activity. Addition of isocitric dehydrogenase did not increase sulphate reduction by either yeast, while added pyrophosphatase stimulated sulphate activation by one yeast, S. oviformis. The initial rate of sulphate activation by S. oviformis was three to four times greater than that by S. cerevisiae (Table 13). This is explained by the higher ATP sulphurylase activity of S. oviformis over S. cerevisiae shown in Table 14.

The concentrations of sulphite in treatments containing

FIGURE 12. Electrophoretograms of reaction products of (^{35}S)-sulphate activation and reduction.

Dialysed extracts were prepared from yeasts grown aerobically in malt extract medium (Methods 4.1, 4.3). A 0.1-ml reaction mixture containing 25 µl of dialysed yeast extract, 12 mM Na_2SO_4 (labelled with ^{35}S) and sources of ATP and NADPH was incubated for 1 hour at 30°. The products were separated by high voltage electrophoresis of a 10 µl sample on Whatman DE81 paper as described in Methods 5.2. The electrophoretograms were cut into 1 cm x 2 cm sections and the radioactivity of each was determined by liquid scintillation counting (Methods 5.3).

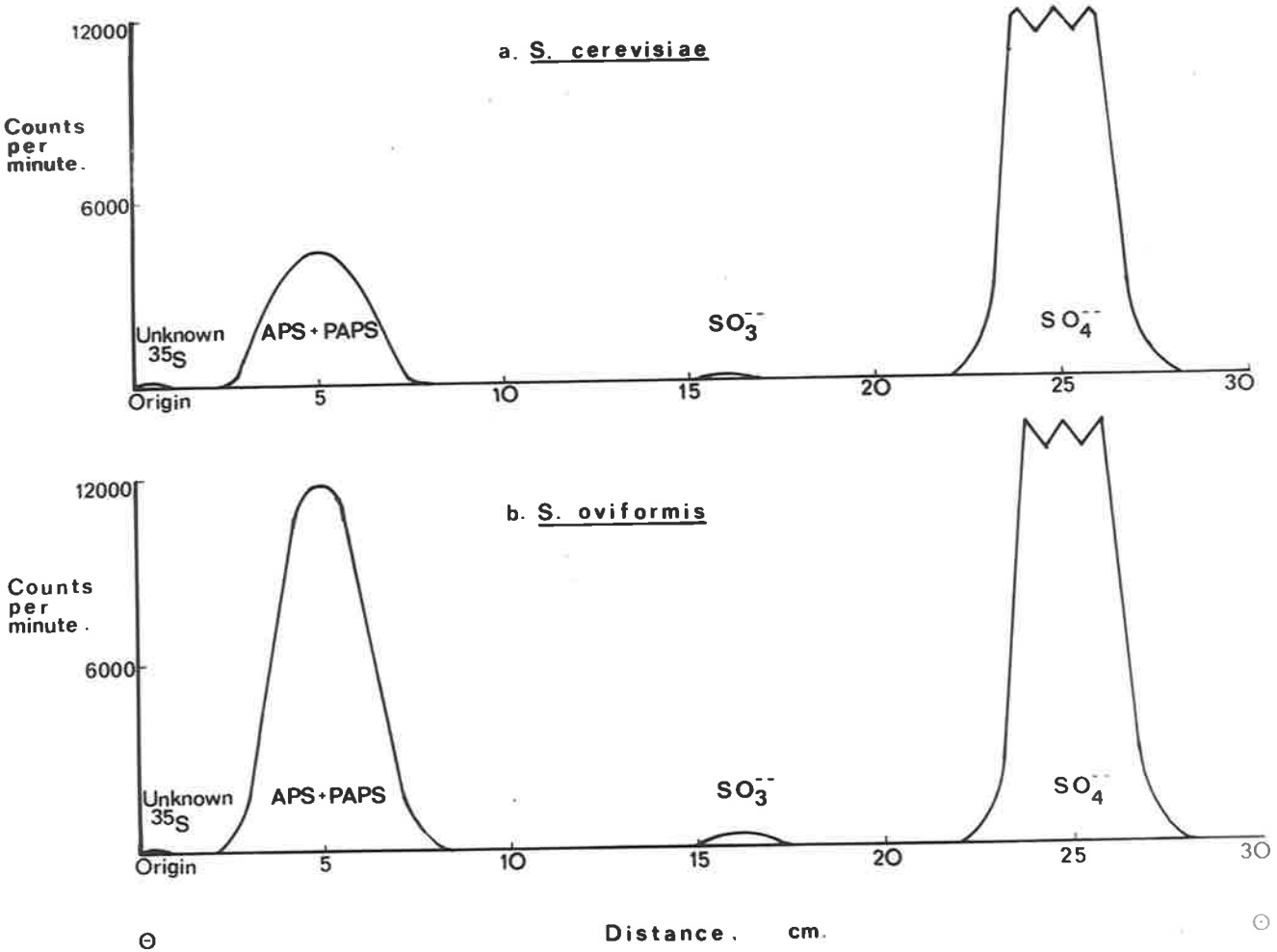

a. S. cerevisiae
12000
Counts per minute.
6000
Unknown 35S
APS + PAPS
SO3--
SO4--
Origin
5
10
15
20
25
30
b. S. oviformis
12000
Counts per minute.
6000
Unknown 35S
APS+PAPS
SO3--
SO4--
Origin
5
10
15
20
25
30
Distance. cm.

FIGURE 12

TABLE 12. Activation of sulphate by cells and cell-free extracts of the yeasts.

Yeasts were grown aerobically in malt extract medium (Methods 1.2, 4.2) and extracts were prepared as described in Methods 4.3. The 0.1-ml reaction mixture containing 25 µl of enzyme source, 12 mM $Na_2{}^{35}SO_4$ and sources of ATP and NADPH is described in Methods 4.4. Samples were incubated for 1 hour at 30°, then ^{35}S-labelled compounds were separated by high voltage electrophoresis on Whatman DE81 paper (Methods 5.2) and determined by liquid scintillation counting.

Source of enzyme	Concentration of ^{35}S compounds after incubation, mM.			
	S.cerevisiae		S.oviformis	
	SO_4^{--}	APS + PAPS	SO_4^{--}	APS + PAPS
Intact cells	11.78	0.20	11.80	0.18
Crude extract	11.52	0.47	10.85	1.10
Dialysed extract	11.73	0.25	11.50	0.49
Dialysed extract + 5µg pyrophosphatase	11.78	0.24	11.20	0.80
Dialysed extract + 5µg pyrophosphatase + 5µg isocitrate dehydrogenase.	11.76	0.25	11.20	0.72
Boiled crude extract	12.00	0	12.00	0

TABLE 13. Initial rate of sulphate activation by yeasts

Results from the experiment described in Table 12 were used to calculate the rates of sulphate activation.

Source of enzyme	Rate of formation of APS plus PAPS, µmoles S/hour/g protein	
	S.cerevisiae	S.oviformis
Crude extract	18.5	60
Dialysed extract*	12.5	48

* Means of triplicates; LSD P 0.05 = 11

TABLE 14. ATP sulphurylase activity of cell free extracts

Extracts were prepared from yeasts grown aerobically in malt extract medium (Methods 1.2, 4.3). The enzyme was assayed by measuring ATP production, from pyrophosphate and APS, with the luciferin-luciferase enzyme system as described in Methods 4.4. (Means of duplicates).

Yeast	Specific activity, µmoles ATP/hr/g protein
S.cerevisiae	50
S.oviformis	173
LSD P 0.01	26

dialysed extracts of S. oviformis averaged 8.0 µM compared with 1.5 µM in those of S. cerevisiae.

4.2 Reduction of sulphite

Sulphite reductase in cell free extracts was assayed in a reaction mixture containing 3 mM $KHSO_3$ and an NADPH regenerating system in 0.2 M phosphate buffer at pH 7.4. Both sulphite utilization (Grant, 1947) and sulphide formation (Siegel, 1965) were measured. Grant's method was insufficiently sensitive to detect differences in sulphite utilization between the two yeasts. S. cerevisiae produced sulphide under the assay conditions while S. oviformis produced nil to trace amounts (Table 15). Sulphite reductase activity was higher in yeasts grown with a lower oxygen supply.

The failure of S. oviformis extracts to form free sulphide was investigated further by incubating extracts of this yeast with a dialysed extract of bakers' yeast (Mauri Bros. and Thompson, Sydney) in the sulphite reductase assay mixture. The results (Table 16) indicate that S. oviformis contained a dialysable, heat-labile inhibitor of the bakers'yeast sulphite reductase. This inhibitor was not investigated further. Although no sulphite reductase was found in extracts of S. oviformis, it is

TABLE 15. Sulphite reductase activities of dialysed cell-free extracts

Yeasts were grown in malt extract medium (Methods 1.2) or synthetic medium 'C' (Appendix 1.2) as described in Methods 4.1 and 4.2. Sulphite reductase activity of extracts was assayed in 3 mM sulphite plus an NADPH regenerating system as described in Methods 4.6. (Means of duplicates).

Growth conditions	Specific activity, μmoles S^{--} formed/hr/g protein	
	S.cerevisiae	S.oviformis
Malt extract, aerobic.	12	0
Malt extract, microaerophilic.	21	0
Medium 'C', aerobic.	4	0
Medium 'C', microaerophilic.	6	1

LSD body of table P 0.01 = 3

TABLE 16. Inhibition of bakers' yeast sulphite reductase by S.oviformis

Fractions by S.oviformis were included in the sulphite reductase assay mixture (Methods 4.6) with a dialysed cell free extract of commercial bakers' yeast (the 'Fraction I' of Prabhakararao and Nicholas, 1969). Sulphide formation was measured. (Means of duplicates)

Fraction of S.oviformis added	Activity µmoles/hr/g.	% inhibition
Nil	20	0
Crude extract	4	80
Dialysed extract	18	10
Boiled crude extract	19	5
LSD body of table P 0.05 =	4	

possible that the enzyme was present in amounts too small to be measured by this assay method.

DISCUSSION

Two morphologically and physiologically similar strains of the genus Saccharomyces differ considerably in their capacity to produce SO_2 and compounds which combine with SO_2. The strain difference in the metabolism of SO_2 in the two yeasts has been examined by physiological and biochemical techniques. A model is presented to explain the results.

1. PRODUCTION OF SO_2-BINDING COMPOUNDS

1.1 Wines fermented by S. oviformis bound more SO_2 than did those fermented by S. cerevisiae. The former wines (S. oviformis) contained more acetaldehyde, pyruvic acid and α-ketoglutaric acid than did the latter. These results indicate that there is a strain difference between the two yeasts in their capacity to form acetaldehyde, pyruvic acid and α-ketoglutaric acid, and secondly, that these three compounds contribute significantly to the SO_2-combining capacity of wine.

Both yeast strains accumulated acetaldehyde, pyruvic acid and α-ketoglutaric acid during the fermentation of sugar. However S. oviformis produced more of these

compounds than did S. cerevisiae. The importance of yeast strain on the composition of wine has been recognised increasingly in recent years (Rankine, 1968b). Lafon-Lafourcade and Peynaud (1966) reported considerable variation in the pyruvic acid and α-ketoglutaric acid production by several yeasts and concluded that the amounts formed were related to yeast species. The data presented here and those of Rankine (1965, 1968a) indicate that the differences are due to variations between strains rather than between species.

Kielhöfer (1963) found that SO_2 bound to acetaldehyde and to glucose accounted for about 70% of the measured bound SO_2 in a number of white wines. Studies by Burroughs and Sparks (1964) with ciders and by Blouin (1966) with wine have increased greatly the number of known SO_2-binding compounds; the more important ones are listed on page 6. Several other ketones (oxaloacetic, α-ketobutyric and α-ketoisovaleric acids, acetone, acetoin and diacetyl) and sugars (fructose and sucrose) also bind SO_2 but are present at such low concentrations or bind SO_2 so weakly (i.e. the dissociation constants of the HSO_3^- addition compounds are high) that they are relatively unimportant in binding SO_2 in wines. The concentrations of sugars and of polyphenolic compounds are relatively low in white wines, so the compounds which bind SO_2 are mainly yeast metabolites

formed during fermentation. Acetaldehyde, pyruvic acid and α-ketoglutaric acid were studied here because they are key metabolic intermediates and because the data of Burroughs and Sparks (1962, 1964) and of Blouin (1966) indicate their capacity to bind SO_2 in wine.

The SO_2 calculated to be bound to acetaldehyde, pyruvic acid and α-ketoglutaric acid in the wines described here averaged 97% of the measured bound SO_2, indicating that these three bound compounds accounted for almost all the bound SO_2 in these wines. This represents a very high recovery of bound SO_2 compared with, for example, the 70% recovery of Rankine and Pocock (1969). However the wines described here had atypically high concentrations of the SO_2-binding compounds listed, which would therefore contribute relatively more to the SO_2-combining capacity of the wines.

1.2 When SO_2 was added to grape juice before fermentation the SO_2-combining capacity of the subsequent wines increased, due to increased formation of SO_2-binding compounds, notably acetaldehyde and pyruvic acid.

The pioneering work of Neuberg (1918), who found that adding SO_2 to yeasts fermenting glucose increased the formation of acetaldehyde and glycerol, indicates that acetaldehyde is derived from sugars via the Embden-Meyerhof

glycolytic pathway. When SO_2 is added, HSO_3^- combines with acetaldehyde preventing this compound from being reduced to ethanol by NADH, so that the latter is then oxidized by glyceraldehyde forming glycerol.

Pyruvic acid may be derived from two sources. Results in this thesis show that pyruvic acid, an intermediate in the alcoholic fermentation of sugar by yeasts, accumulated early in fermentation reaching a maximum when sugar utilization was most rapid. Subsequently, pyruvic acid utilization exceeded its formation, thus its concentration fell. These results, together with those of Lafon-Lafourcade and Peynaud (1966) who observed a silimar relation between pyruvic acid formation and sugar concentration, suggest that pyruvic acid is formed from glucose. Some amino acids are degraded to pyruvic acid during fermentation. Whiting and Coggins (1960), who reported higher pyruvic acid formation in the presence of SO_2 and also in media rich in amino nitrogen, especially asparagine, suggested that asparagine was metabolised via α-keto-succinamic acid and oxaloacetic acid to form pyruvic acid. Lafon-Lafourcade and Peynaud (1966) reported higher pyruvic acid production when amino acids replaced ammonium sulphate as the nitrogen source in fermentation media.

Whatever the origin of pyruvic acid, its formation apparently exceeds its utilization early in fermentation,

thus it accumulates and is subsequently metabolised. Addition of SO_2 inhibits the utilization of pyruvic acid, resulting in an accumulation of this compound. This inhibition may occur in two ways. Firstly, HSO_3^- reacts with pyruvic acid to form 2-hydroxy-2-sulphonyl propanoic acid which is an unsuitable substrate for pyruvate carboxylase and other enzymes which metabolise pyruvic acid. Secondly, SO_2 may react with thiamine pyrophosphate, the co-factor of pyruvate carboxylase. Such an inhibition is indicated by the observation (Lafon-Lafourcade and Peynaud, 1966) that the production of pyruvic acid was greatly increased in thiamine deficient media, and by the report of Hernandez (1967) that a fermentation which had been retarded by SO_2 was restored to normal by adding thiamine.

With some yeasts, additions of SO_2 also increased the formation of α-ketoglutaric acid (Whiting and Coggins, 1960; Rankine, 1968a). Production of α-ketoglutaric acid, which is formed from glutamic acid (Lafon-Lafourcade and Peynaud, 1966), was increased by low ammonium concentration, high pH and high temperature, and was influenced by the variety of grape juice (Rankine, 1968a). In the experiment described here (Table 5), the addition of SO_2 before fermentation did not affect the concentration of α-ketoglutaric acid in the wines made with either of the two yeast strains. All wines contained high concentrations of

α-ketoglutaric acid, compared with those described by Blouin (1966) and Rankine (1968a). Although the interaction of yeast strain with any of the environmental conditions listed above may have resulted in high α-ketoglutaric acid production, it is probable that autolysis of yeast cells, while wines were left in contact with the yeast sediment until all fermentations were complete, was a major cause. This high formation of α-ketoglutaric acid, even in the absence of SO_2, probably masked any stimulatory effect of SO_2.

1.3 The results suggest that the production of SO_2-binding compounds was associated with the capacity of the yeast to produce excess SO_2. This hypothesis is supported by the following observations:

(a) S. oviformis, which formed SO_2, produced higher concentrations of the SO_2-binding compounds than did S. cerevisiae which formed very little SO_2.

(b) Addition of SO_2 increased the production of SO_2-binding compounds, namely acetaldehyde and pyruvic acid. This increase was proportional to the strength of binding (i.e. inversely proportional to the dissociation constant of the HSO_3^- addition compound), and was less in S. oviformis which produced SO_2.

(c) When acetaldehyde was added to intact cells, SO_2 formation increased.

Data published by Rankine and Pocock (1969) support this view. The SO_2-combining capacity (as mg/l bound SO_2 at 100 mg/l free SO_2) of four wines described by them was positively correlated with the amount of SO_2 formed by the four yeasts used in the preparation of the respective wines.

As SO_2 stimulates the production of SO_2-binding compounds, any accumulation of sulphite, irrespective of whether it is added externally or formed by the yeast, would be expected to increase the formation of these compounds by combining with them and preventing their further breakdown.

2. PRODUCTION OF SO_2 BY INTACT CELLS

2.1 Cells of S. oviformis produced 10 to 20 fold more SO_2 than did S. cerevisiae under similar conditions. The formation of sulphur dioxide from SO_4^{--} required glucose. Presumably the metabolism of glucose provided ATP and NADPH for sulphate activation and reduction (Bandurski, 1965).

Most yeast strains used for primary fermentation do not produce significant quantities of SO_2. Burroughs (1958) reported that the SO_2 content of cider decreased marginally during fermentation, indicating that SO_2 was not formed. Similarly, the bakers' yeast used by Bandurski et. al. (1956, 1965) did not produce free SO_2. In extracts of this yeast,

SO_3^{--} formation equalled SO_3^{--} utilization and free inorganic SO_3^{--} was not released during SO_4^{--} reduction (Torii and Bandurski, 1964).

However some yeasts do produce SO_2 from SO_4^{--} (Würdig and Schlotter, 1967; Dittrich and Staudenmeyer, 1968; Rankine and Pocock, 1969), demonstrating that inorganic SO_3^{--} is released under some circumstances.

2.2 Increased concentrations of SO_4^{--} during yeast growth stimulated the SO_2 producing capacity of harvested cells when assayed at constant SO_4^{--} concentration. Apparently SO_4^{--} additions increased the amount of at least one of the three enzymes involved in the activation and reduction of SO_4^{--}. These results partly explain the observation of Würdig and Schlotter (1967) that SO_2 formation was increased when SO_4^{--} was added to fermenting grape juice.

The capacity of cells of S. oviformis to form SO_3^{--} was increased by low oxygen supply during yeast growth, but not by similar conditions during the assay for SO_4^{--} reduction, which indicates that lack of oxygen increased the amount rather than the activity of enzymes which cause SO_2 production. The results suggest that SO_4^{--} may act as a terminal electron acceptor in a respiratory capacity to oxidize NADPH, when yeasts are grown under microaerophilic conditions.

3. SULPHATE METABOLISM IN CELL-FREE EXTRACTS

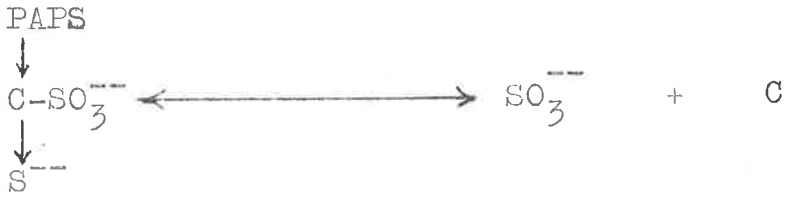
3.1 Electrophoretic data reported here indicate that both the yeasts studied reduced SO_4^{--} via APS and PAPS to SO_3^{--} as has been described for bakers' yeast (Hilz et. al., 1959). Before reduction, SO_4^{--} is activated to APS and then to PAPS by two ATP-dependent reactions catalysed by ATP sulphurylase and APS kinase respectively (Robbins and Lipmann, 1956b). Reaction rates differed markedly; in S. oviformis, the conversion of SO_4^{--} to APS plus PAPS was four times as rapid as in S. cerevisiae. This was subsequently explained by enzyme assays which showed that the ATP sulphurylase activity in cell-free extracts of S. oviformis was almost four times greater than in those of S. cerevisiae. If, as appears likely, the activation of SO_4^{--} to APS is the rate limiting step in the formation of SO_3^{--} from SO_4^{--}, then the differences in the ATP-sulphurylase activities explain the observed strain differences for SO_2 production in the two yeasts.

3.2 Sulphite reductase activity, measured as S^{--} formation from SO_3^{--}, in S. cerevisiae was similar to that in the bakers' yeast (another strain of S. cerevisiae) described by Prabhakararao and Nicholas (1969). However, the strain of S. oviformis used in the present work did not produce

detectable S^{--} under these assay conditions. Because S. oviformis utilized both SO_4^{--} and SO_3^{--} for the synthesis of S-amino acids, it is assumed that in crude extracts of this yeast sulphate reductase activity was too low to be detected.

Several workers (Wainwright, 1961, 1967; Yoshimoto and Sato, 1968a; Prabhakararao and Nicholas, 1969) have studied SO_3^{--} reduction by Saccharomyces yeasts but none has reported products other than S^{--}. Therefore it is reasonable to conclude that both yeasts studied utilized SO_3^{--} via S^{--} using a sulphite reductase and that the low S^{--} formation in S. oviformis is a true reflection of a diminished SO_3^{--} utilization in this yeast.

3.3 Thus, net SO_2 production can be explained by SO_3^{--} formation exceeding SO_3^{--} utilization, as indicated by the comparison of the reaction rates of ATP sulphurylase and sulphite reductase in S. cerevisiae and in S. oviformis. Torii and Bandurski (1964) claimed that the product of SO_4^{--} reduction in yeast was not free SO_3^{--} but protein bound SO_3^{--} (C-SO_3^{--}), which was a suitable substrate for SO_3^{--} reduction (Bandurski, 1965). The release of SO_3^{--} from yeast cells and the utilization of SO_3^{--} as a sulphur source, reported herein, require that this protein-SO_3^{--} complex dissociates reversibly to some extent:



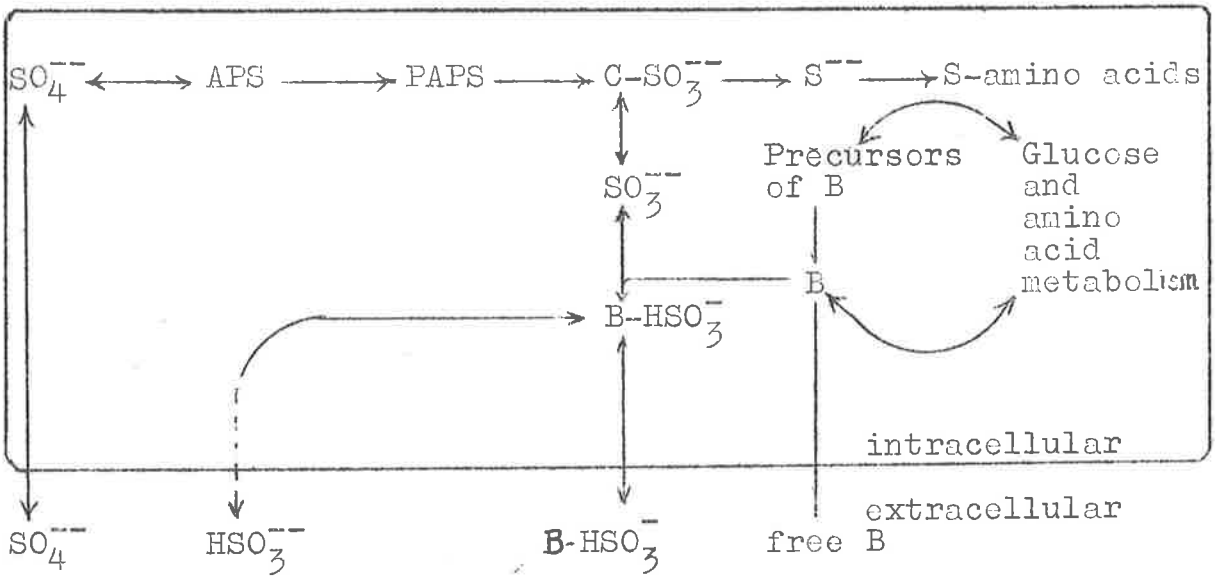
Thus, differences in enzymic activity which increase the concentration of $C\text{-}SO_3^{--}$ result in increased formation of inorganic SO_3^{--}.

3.4 Sulphite reductase in yeast is repressed by pantothenic acid and by cysteine, while methionine represses ATP sulphurylase (De Vito and Dreyfuss, 1964). Sake yeast studied by Okuda and Uemara (1965) contained active sulphite reductase when grown in media containing 10 µg/l pantothenic acid but had no sulphite reductase when grown with 200 µg/l pantothenic acid. Wainwright (1970) suggested that pantothenic acid deficiency results in less O-acetyl homoserine with consequent accumulation of S^{--}. Additions of either pantothenic acid or of cysteine would therefore decrease SO_3^{--} utilization and additions of methionine would decrease SO_3^{--} formation. The results presented here, that additions of pantothenic acid and of cysteine increased the SO_2 producing capacity of harvested cells, and that addition of methionine reduced SO_2 formation, are consistent with the suggested control mechanisms (De Vito and Dreyfuss, 1964; Okuda and Uemara, 1965). Further, the

small amounts of pantothenic acid which repress sulphite reductase may explain the low activity of this enzyme in extracts of S. oviformis described here. A detailed study of control systems was not attempted, since this is beyond the scope of this thesis.

4. TENTATIVE MODEL

The following schematic representation of a yeast cell is proposed to explain the inter-relationships of SO_2 in the metabolism of the two yeasts:



Note: C = protein, described by Torii and Bandurski, 1964.

B = SO_2-binding compounds, especially acetaldehyde, pyruvic acid and α-ketoglutaric acid.

In S. oviformis, but not in S. cerevisiae, SO_4^{--} activation and reduction proceeds more rapidly than does SO_3^{--} utilization, so C-SO_3^{--} accumulates. The reversible dissociation of C-SO_3^{--} provides intracellular inorganic SO_3^{--}.

Sulphite, either added externally or produced endogenously from SO_4^{--} reduction as in S. oviformis, combines with SO_2-binding metabolites B to displace enzyme equilibria in favour of increased formation of these compounds.

Because the concentration of B exceeds that of HSO_3^- in both yeasts, any SO_2 produced diffuses from the cell as B-HSO_3^-, together with excess B, rather than as free inorganic SO_3^{--} or HSO_3^-.

APPENDIX 1. Composition of synthetic media used for the propagation of yeasts.

1.1 Basal medium

Compound		Concentration, mg/l
Sugar	Sucrose	5×10^4
Salts	KH_2PO_4	1360
	NaCl	100
	$MgSO_4$	100
	H_3BO_3	0.5
	$CuSO_4.5H_2O$	0.04
	$CaCl_2$	500
	KI	0.1
	$FeCl_3.6H_2O$	0.2
	$Na_2MoO_4.2H_2O$	0.2
	Mn citrate	0.4
	$Zn(NO_3)_2$	0.4
Vitamins	Biotin	0.002
	Folic acid	0.002
	p-Aminobenzoic acid	0.2
	Nicotinic acid	0.4
	Pyridoxin HCl	0.4
	Riboflavin	0.2
	Thiamine HCl	0.4
	Inositol	2
Amino acids	l-Histidine HCl	10
	d-l-Tryptophan	20

1.2 Modifications to basal medium

Medium	Modications	Concentration, mg/l		
Synthetic medium S.		S2	S10	S50
Basal medium	plus $(NH_4)_2SO_4$	0	1060	5020
	plus Na_2SO_4	0	0	1420
	plus NH_4Cl	4000	3180	0
	plus Ca pantothenate	0.4	0.4	0.4
Synthetic medium A				
Basal medium	plus $(NH_4)_2SO_4$	5000		
	plus methionine	0 or 20		
	plus cysteine	0 or 20		
	plus Ca pantothenate	0 or 0.04		
Synthetic medium C				
Basal medium	plus sucrose	5×10^4 (=10^5 total)		
	plus $(NH_4)_2SO_4$	5×10^3		
	plus $(NH_4)_2HPO_4$	10^3		
	plus Ca pantothenate	0.02		
Sulphur-free medium				
Basal medium	less $MgSO_4$ plus $MgCl_2$	500		
	less sucrose plus glucose	5×10^4		
	plus $(NH_4)_2HPO_4$	5×10^3		
	plus Ca pantothenate	0.4		

APPENDIX 2. Progressive changes in yeast cell numbers and concentrations of SO_2 and of SO_2-binding compounds during the fermentation of grape juice.

Grape juice (Sample No. 2, Table 1) was prepared and inoculated as described (Methods 1.2). After stirring, 1-ml samples were withdrawn for cell counts and 50 ml samples were withdrawn for analysis, at the times shown on the table. Analyses were done by the methods described (Methods 2.1, 2.3 and 1.4). (Means of duplicates).

Yeast	Incubation time(hr)	Total Cells ($\times 10^{-6}$)	Viable cells ($\times 10^{-6}$)	Total sugar (g/l)	Sulphur dioxide mg/l	Acetaldehyde mg/l	Pyruvic acid mg/l	α-Ketoglutaric acid mg/l
S.cerevisiae	0	1.0	0.8	160	0	12	10	8
	12	3.4						
	24	10	8	155	0	33	7	12
	36	26						
	48	80	70	125	0	68	70	19
	60	155						
	72	260	160	58	0	66	123	31
	84	270						
	96	245	135	19	0	50	115	58
	108	650						
	120	360	190	3.8	0	55	94	53
	192	165	50	1.7	0	61	59	40
	264	-	-	1.6	0	78	53	45
S.oviformis	0	0.8	0.6	160	0	12	10	8
	12	4.5						
	24	16.5	8	150	0	36	20	11
	36	75						
	48	150	130	110	16	120	220	57
	60	175						
	72	210	175	35	33	115	195	82
	84	355						
	96	190	135	2.1	37	57	155	86
	108	405						
	120	270	220	1.1	36	40	110	82
	192	265	0.1	1.0	37	58	118	88
	264	-	-	1.0	36	53	118	88

APPENDIX 3. Reducing sugar and alcohol concentrations and pH of wines.

Wines listed in 'Experiment 1' were made from grape juice No. 1 (Table 1) and are described in Table 5 and Figure 3. 'Experiment 2' wines were made from juice No. 2 (Table 1) and are described in Table 6 and Figures 4 and 5.

Treatment		S.cerevisiae			S.oviformis		
Experiment (1)		Sugar	Alcohol	pH	Sugar	Alcohol	pH
SO_2 before	SO_2 after	g/l	% v/v		g/l	% v/v	
0	220	1.1	10.8	3.20	0.9	11.6	3.15
	320	1.3	10.7	3.10	0.8	11.4	3.10
50	220	1.1	11.0	3.05	0.7	11.2	3.10
	320	1.0	11.5	2.95	0.8	11.1	3.00
100	220	3.7	10.6	2.90	0.8	11.4	3.00
	320	3.5	11.1	2.95	0.9	11.5	3.00
Experiment (2)							
Bottled after 11 days		1.6	8.3	3.20	1.0	8.5	3.15
Bottled after 18 days		1.6	8.3	3.20	1.0	8.6	3.20

APPENDIX 4. Statistical data

4.1 Effect of sulphur dioxide on the production of SO_2 and of SO_2-binding compounds by yeasts fermenting grape juice.

Independent variable	Significance						
	Total SO_2 after fermentation.	SO_2 after bottling			Acetaldehyde.	α-Ketoglutaric acid.	Pyruvic acid
		Free	Bound	Total			
Yeast strain	***	***	***	***	***	***	***
SO_2 before	***	ns	***	***	***	*	*
SO_2 after	ns	***	***	***	ns	ns	ns
Yeast x SO_2 before	ns	ns	ns	ns	**	ns	ns
Yeast x SO_2 after	ns	***	*	ns	ns	ns	ns
SO_2 before x SO_2 after	ns	ns	ns	ns	ns	ns	ns

Refer in text : Table 5, Figure 3

4.2 Effect of time of bottling on the production of SO_2 and of SO_2-binding compounds by yeast fermenting grape juice.

Independent variable	Significance					
	SO_2 in wine after bottling			Acetaldehyde.	α-Ketoglutaric acid.	Pyruvic acid
	Free	Bound	Total			
Yeast strain	*	ns	*	ns	**	*
Time bottled	**	*	ns	**	ns	ns
Yeast x time	ns	ns	ns	ns	*	ns

Refer in text : Table 6

4.3 Effect of method of sterilization of media on SO_2

Independent variable	Significance	
	Sulphite produced	Residual sugar
Yeast strain	***	***
Sterilization method	**	***
Yeast x method	*	***

Refer in text : Table 7

4.4 Effect of assay conditions on sulphite production

Independent variable	Significance	Refer in text
Yeast strain	***	Table 9, Figures 8, 9 and 10
Temperature	***	Figure 8
Yeast x temperature	***	
pH	***	Figure 9
Yeast x pH	***	
Oxygen supply	ns	Table 9
Yeast x oxygen supply	ns	
SO_2-binding compounds	*	Figure 10
Yeast x compounds	**	

4.5 Effect of growth conditions on sulphite production

Independent variable	Significance	Refer in text
Yeast strain	***	Tables 10 and 11, Figure 11
Sulphate concentration	***	Figure 11
Yeast x sulphate	ns	
Aeration	*	Table 10
Yeast x aeration	ns	
Adding met., pant. and cysteine	*	Table 11
Yeast x addition	*	

4.6 Enzyme activities in dialysed cell free extracts

Independent variable	Dependent variable	Significance	Refer in text
Yeast strain	Total sulphate activation	***	Table 13
Yeast strain	ATP sulphurylase	***	Table 14
Yeast strain	SO_3^{--} reductase	***	Table 15
Media	"	***	"
Aeration	"	**	"
Yeast x medium	"	***	"
Yeast x aeration	"	*	"
Medium x aeration	"	ns	"
Inhibitor treatments	SO_3^{--} reductase	**	Table 16

KEY to tables in Appendix 4.

*** = significant at 0.001 level of probability

** = significant at 0.01 level of probability

* = significant at 0.05 level of probability

ns = not significant

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