

# Sulphite metabolism and toxicity in Saccharomyces cerevisiae and Saccharomycodes ludwigii.

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# SULPHITE METABOLISM AND TOXICITY IN *SACCHAROMYCES CEREVISIAE* AND *SACCHAROMYCODES LUDWIGII*

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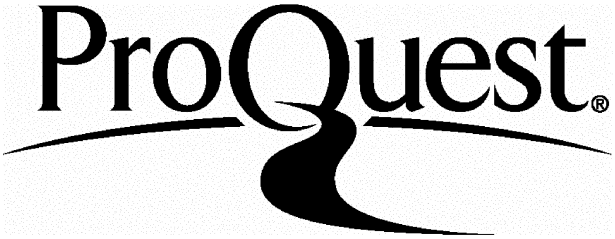

# Summary

## SUMMARY

Hydrogen sulphide production was examined in four strains of Saccharomyces cerevisiae. Hydrogen sulphide was formed in peaks of activity, coincident with the cessation of exponential yeast growth, and with depletion of an available nitrogen source in the medium. Hydrogen sulphide formation could be terminated by further additions of a nitrogen source. Addition of cycloheximide or iodoacetamide to exponentially growing cultures also caused hydrogen sulphide production and cessation of exponential growth. By terminating yeast growth at different stages, a peak of hydrogen sulphide producing activity was obtained which was similar to NADPH-sulphite reductase activity measured throughout exponential growth.

Hydrogen sulphide was formed by reduction of bound sulphite present in the medium. Sulphite was transported, separately from the carbonyl components of bound sulphite, probably by diffusion.

The toxicity of sulphite was studied in Saccharomyces cerevisiae and Saccharomycodes ludwigii. Sulphite transport into both yeasts was by diffusion although aspects of the transport superficially imitated active transport. The effects of sulphite upon amino-acid and glucose transport were examined in Saccharomyces cerevisiae. The action of sulphite upon respiration and fermentation of cells of Saccharomyces cerevisiae, differed between anaerobic and aerobic cells. This was discussed in terms of an uncoupling of glycolysis from high energy phosphorylation and the acidification of the interior of cells brought about by sulphite transport. Saccharomycodes

ludwigii was resistant to ten fold greater concentrations of sulphite than Saccharomyces cerevisiae. This resistance can be explained by the low rate of transport of sulphite into Saccharomycodes ludwigii.

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# Introduction

## HISTORICAL ASPECTS OF SULPHUR DIOXIDE AS AN ANTIMICROBIAL AGENT

Sulphur dioxide has been used as an antimicrobial agent and preservative since antiquity. In the days before modern chemical industry, sulphur dioxide gas was obtained by means of burning elemental sulphur, then known as fire and brimstone. Elemental sulphur is not, geologically speaking, a common mineral but it is found in regions of volcanic activity in Italy, notably Sicily, Japan and more recently in Texas (Green, 1976).

It is easy to understand the association, from the earliest times, between sulphur and the supernatural. Fiery volcanic vents, frequently reeking with sulphureous fumes, were deemed to be the gates of Hell. Bronze-age man would have been aware of the sulphur dioxide emanating from furnaces used in the extraction of copper or lead from their sulphide ores. The early explorer, St. Brendan, noticed a great sulphureous stench 'in the confines of Hell', whilst close to a volcanic island, probably near the modern Vestmannaeyjar group of islands (Severin, 1978). Organised religions of the early Egyptians, Hebrews and Greeks used ignited sulphur in their ceremonies, endowing its blue flames and suffocating fumes with magical purifying properties (Brent, 1965). These same properties were probably used to assist with prophesies issuing from the Delphic oracle. Nearer to home, the hot, sulphur-smelling spring water of Aquae Sulis (Bath) is still maintained to possess medicinal properties.

Long before the dawn of microbiology, fumes from burning sulphur were used as a preservative and disinfectant. In classical Greece, Homer referred to its use (Kidney, 1973) and later, in the Middle Ages, burning sulphur and burning wood were widely used to control disease (Rose, 1981). Being a gas, sulphur dioxide was invaluable in fumigating the inaccessible areas of ramshackle mediaeval dwellings. Its use soon spread to ships and their cargoes and this was probably responsible for the legendary habit of the notorious pirate Blackbeard of burning sulphur in the holds of his ship while unfortunate members of his crew were trapped below deck. Until modern times, burning sulphur, often in the form of candles, has been used to disinfect areas where disease has occurred, and it would be surprising if its use has altogether ceased today.

Sulphur dioxide has also been used, intermittently, in the production of fermented beverages for centuries. Its use probably started with grape fermentations in areas proximate to the Sicilean sulphur mines, whence its use spread to other areas and other beverages. The method of use was essentially simple; sulphur was burned inside empty casks and vessels to sterilise them and the casks were then filled whilst still containing the highly soluble sulphur dioxide gas. In this way, the must or beverage would also be exposed to the anti-microbial action of sulphur dioxide. This method was still employed recently in parts of the Australian wine industry (P. Henschke, personal communication). In cider making, this method was first recorded as being used in 1664 by John Evelyn

and it was also employed to slow and restrain cider fermentations which were proceeding at too great a rate (Beech and Carr, 1977).

The use of sulphur dioxide in the production of beer has been much less extensive than that in wine and cider. Sulphite found in beer fermentations is most likely to have originated through yeast activity (Narziß et al., 1981) or through the sulphiting of raw materials of the brewing process, such as hops (Garza-Ulloa, 1980).

## CIDER MAKING

Traditionally, cider was made by pulping apples and extracting the juice, usually by pressing the pulped fruit. The expressed juice was then fermented by the natural yeast flora of the fruit and yeast derived from contact with the processing and pressing equipment (Beech and Davenport, 1970). After fermentation of the juice to dryness, the yeast was allowed to settle and the cleared cider was racked off the lees. Considerable variation in taste and quality must inevitably have followed in the wake of the juice's undefined nutrient status and fermentation by whatever yeast (or bacteria) became dominant.

The modern process of cider making is initially very similar. Apples, ideally bittersweet cider apples, are stored in large silos from which they are taken, washed, and the most badly damaged fruit removed by floatation.The washed apples are then milled and the juice is extracted by centrifugal force or by

pressure. The parchment-like consistency of the cider apple makes it easier and safer to press than the slippery flesh of the dessert apple. The residual apple tissue, or pomace, is sold for pectin extraction or cattle feed. The juice may then be acidified by addition of malic acid and it is then sulphited to a total concentration of the order of 1.5 mM sulphur dioxide (100 p.p.m.) of which, ideally, 0.4 mM remains as free sulphite in the juice. The pH value of the juice is of paramount importance in determining the degree of antimicrobial activity by sulphite (King et al., 1981). Little antimicrobial action is evident with a pH value greatly in excess of four. Sulphite in solution will react with available carbonyl groups to form α-hydroxysulphonates. These compounds are commonly known as 'bound sulphite' and show little antimicrobial activity (Neuberg, 1929; Rehm, 1964). Apple juices can vary considerably in their sulphite-binding ability. It has been shown that the major binding compounds in apple juices are: sugars (aldoses), acetaldehyde, xylosone, galacturonic acid, pyruvic acid, and 2-oxoglutaric acid (Burroughs and Sparks, 1964 b). These carbonyl-containing compounds react with sulphite to form a series of reversible chemical equilibria depending upon pH value, temperature and reagent concentrations.

These compounds are derived from sound fruit whereas very large amounts of these and other sulphite-binding compounds are found in damaged and rotting fruit. These include 5-oxofructose, 2-oxogluconic acid and 2,5-dioxogluconic acid (Burroughs and Sparks, 1964b). Thus, if a juice is derived from unsound and damaged fruit, more sulphite would be required to obtain the

desired antimicrobial effect. Using an average apple juice, after the addition of 1.5 mM sulphite (100 p.p.m.) about a quarter of this would be expected to remain as free sulphite after equilibrium has been attained (T. Cowland, personal communication). The effects of pH value and sulphite binding on sulphite toxicity are referred to in a later section. The free sulphite kills and inhibits growth of bacteria and wild yeasts. Generally, it is effective against most bacteria and yeasts with notable exceptions such as the cider sickness bacterium Zymomonas anaerobia which is resistant to concentrations in excess of the E.E.C. statutory limit of 3.1 mM total sulphite (Beech and Carr, 1977), and the cider spoilage yeast, Saccharomycodes ludwigii, which can resist sulphite up to concentrations of 7.5 mM (Reed and Peppler, 1973).

The sulphited juice may then be supplemented with thiamin (1 mg $l^{-1}$), an ammonium salt, at about 8 mM to increase the low nutrient levels of nitrogenous compounds in apple juice, and sugar to make the apple juice of a consistent quality.

Yeast is then added to the juice at about 0.5 g wet weight yeast $l^{-1}$. Such a massive inoculation of a pure strain of fermentative yeast easily binds the residual free sulphite in the juice and outgrows the small numbers of surviving contaminants. The fermentation is thus dominated by the inoculated fermentative yeast strain which will impart the desired flavour characteristics to the beverage. This primary

fermentation can take from two weeks to a month to complete, depending on the temperature and nutrient status of the juice. After clearing, the cidermay be blended and refermented before storage and maturation. It is usual for the malic acid in the cider to be converted into lactic acid by a Lactobacillus species. This malo-lactic fermentation will occur during maturation of the cider if it has not already happened during fermentation. After fining and clearing, the cider is racked from the lees, blended and may be sweetened. Finally it is filtered and flash-pasteurized before being dispensed into kegs or bottled. Sulphite is added to ensure the microbiological stability of the product, the amount needed depending on the acidity of the cider. A statutory limit of 3.125 mM total sulphur dioxide (200 p.p.m.) content in ciders is in force within the E.E.C. (Anon, 1973), as quoted by Hammond and Carr (1976), and sulphite additions have to be kept within this limit. However, the production of sulphite by certain strains of yeast can complicate the situation and necessitate direct chemical analysis of the sulphur dioxide content of ciders.

The yeast Saccharomycodes ludwigii is one of the major causes of spoilage of finished cider. It is discussed in detail here as a consequence of its phenomenal resistance to sulphite. This yeast has been found at most stages of the cider-making process (Beech and Davenport, 1970) but is a major problem when present in bottled cider. Here, an infection of one or two cells $l^{-1}$ can form flaky deposits in the infected bottles (Beech and Carr, 1977) and may lead to excessive gas production. Sulphite

will only slightly decrease infections and delay growth (Beech and Davenport, 1970). Such infections may be controlled by rigorous hygiene in the bottling equipment and efficient filter-sterilizing or flash-pasteurization of the cider.

Saccharomycodes ludwigii is the only species in its genus and is, perhaps, most closely related to Hanseniaspora sp. (Phaff, 1970). The cells are large, apiculate or elongate, sometimes resembling skittles, which reproduce by bipolar budding forming multiple polar bud scars (Streiblová, 1966) as quoted by Bartnicki-Garcia and McMurrough (1971). Four haploid round ascospores are formed which conjugate upon germination of the ascus (Kreger-van Rij,1969).

The nutritional requirements of Saccharomycodes ludwigii are essentially similar to those of Saccharomyces cerevisiae, being able to ferment glucose, sucrose and raffinose. It can assimilate glycerol, salicin, and lactate (latently) but is unable to utilize ethanol (Phaff, 1970).

Very little is known about this yeast and its metabolism other than the basic taxonomy. It has been reported to be an osmophobe (Beech et al., 1964), being unable to survive storage at 15°C at a specific gravity of 1,200 for more than a week, and has been reported to produce ethyl acetate (Peynaud, 1956) as quoted by Beech and Davenport (1970). Pigment formation may be induced in suitable culture conditions (Chamberlain et al., 1952). However its most noteworthy characteristic

is the ability to resist sulphite concentrations up to 7.8 mM, two and a half times the permitted maximum in ciders.

Hydrogen sulphide production can be a major problem in cidermaking. This problem has been widely reported in wine and beer fermentations but rarely in cider. However, many similarities seem to occur between the production of hydrogen sulphide in cider, and its production in wine.

Hydrogen sulphide production is usually associated with the activity of the fermenting yeast (Beech and Carr,1977) and is found to be characteristic of the yeast strain employed (Rankine, 1968). The inability of a yeast strain to form sulphide must be a factor to be considered when selecting a strain for cider production, and to date this selective criterion has probably been the most successful way of limiting hydrogen sulphide production. Yeast strains can be screened for sulphide production using small-scale fermentations containing powdered sulphur (Rankine, 1964) or on solid medium using the technique described by Molzahn (1976).

Hydrogen sulphide production by yeast can be suppressed by addition of methionine (Wainwright, 197Cb) or pantothenic acid (Stewart et al., 1962; Wainwright, 1962b) if this vitamin is limiting the fermentation. In apple juices, Taylor (1973) has shown that pantothenic acid was not a limiting factor but that methionine addition was partially successful in suppressing sulphide production. Later work (Ranson, 1974),

again in a cider fermentation, showed that sulphide was produced during exponential yeast growth and that the amount of sulphide produced reflected the rate of fermentation by the yeast.

Elemental sulphur, whilst a prime source of hydrogen sulphide in wine due to its use as a fungicide on grapes (Acree et al., 1972), is not found in apple juices under normal conditions and is rarely a factor to be considered in cider. However, if wooden cooperage has been sterilized by burning sulphur, a residue of unburned elemental sulphur may be reduced by yeast action to sulphide (Rankine, 1963).

Bacterial contaminants, such as Zymomonas sp., can give rise to sulphide taints in cider fermentations (Millis, 1956; Carr and Passmore, 1971) but generally such contamination is contained by the low pH value and the use of sulphur dioxide (Beech and Carr, 1977). Also, other consequences of bacterial contamination are usually far more evident than any sulphide production.

## YEAST SULPHUR METABOLISM

### A. Sulphur Content of Yeast

Saccharomyces cerevisiae contains of the order of 0.3% - 0.5% dry weight of yeast as sulphur (Maw, 1963b). Some variation in this value exists, probably depending on the strain of yeast examined and the conditions of its growth (Ekström and Sandegren, 1951). In general, brewery strains of yeast have a higher sulphur content than baker's strains (Lindan and Work, 1951;

Suomalainen and Keränen, 1955).

The greater part of the yeast's sulphur content is found in the form of the sulphur-containing amino-acid residues, cysteine, cystine and methionine. Of these residues, methionine and cystine are almost entirely located in proteins while most of the cysteine residues are located in the cytoplasmic tripeptide, glutathione (Thorne, 1957). Cysteine and methionine residues represent some 30% each of the total yeast-sulphur content. Pulse-labelling experiments on Saccharomyces cerevisiae with $^{35}S$-sulphate confirmed this division of the sulphur pool with large amounts of label found as methionine and glutathione (Hartnell and Spedding, 1979). Label was also accumulated in S-adenosylmethionine. This compound plays a prominent role in cellular metabolism (Salvatore et al., 1977). It is the methyl donor for the vast majority of biological methylations (Farooqui et al., 1980), and has a major regulatory role in methionine biosynthesis (Cherest et al., 1973a). The intracellular pool of free methionine is very low (Messenguy, 1979; Ramos-Jeunehomme et al.,1979) and methionine is rapidly combined in proteins or sequestered as S-adenosylmethionine (Schlenk and De Palma, 1957; Svihla and Schlenk, 1960).

## B. Sources of Sulphur for Growth of Saccharomyces cerevisiae

Saccharomyces cerevisiae has, like all other micro-organisms, an obligate requirement for a source of sulphur for growth. The following is a description of compounds which could be

employed as a source of sulphur for this organism.

Sulphate is found widely and in abundant amounts in water supplies and in fruit juices. This is probably the most common source of sulphur for yeasts. Sulphite, while not a common naturally-occurring ion, has been available to yeast in the brewing industry for centuries. Sulphite has been shown to enter Saccharomyces cerevisiae by an active permease (Macris and Markakis, 1974) although in toxic concentrations which prohibited further metabolism of the sulphite (Macris, 1972). Schultz and McManus (1950) obtained growth using sulphite as the sole sulphur source and Hernandez (1980) reported a decline in the sulphite content of the medium during fermentation. Sulphide, whilst found in anaerobic lake muds, is unlikely to be found in large concentrations where yeast could grow and thrive. Hydrogen sulphide has been found in the juice of fresh oranges (Shaw et al., 1980) and can be formed by bacterial or yeast action (Dadds and Martin, 1973; Rankine, 1963). However, it is a volatile and highly reactive agent and, within a short time, will have become lost to the system, or reacted with other groups to form thiols of varying complexity (Neuberg and Grauer, 1952; Leppänen et al., 1980; Nishimura et al., 1980). Uptake and utilization of such compounds have not been studied in yeast and, in any event, they are unlikely to be present in sufficient concentration to act as significant yeast sulphur sources. Elemental sulphur may seem an unlikely possible sulphur source, given that it is highly insoluble in aqueous media. However, elemental sulphur can be found in grape fermentations,

as mentioned earlier, as a consequence of its use as a grape fungicide or in sterilizing cooperage (Acree et al., 1972).

Elemental sulphur can be readily reduced by Saccharomyces cerevisiae to hydrogen sulphide (Rankine, 1963; Acree et al., 1972) although this reduction is generally considered to be extracellular and is favoured by the use of finely divided, or colloidal, sulphur. Since no uptake mechanism is known for elemental sulphur, any participation by this element in yeast nutrition would have to be via hydrogen sulphide and the complex thiols which were discussed earlier.

Methionine can be regarded as one of the most suitable sulphur sources to obtain maximal yeast growth (Schultz and McManus, 1950; Anderson and Howard, 1974). Methionine uptake by Saccharomyces cerevisiae occurs on at least two permease systems (Gits and Grensen, 1967) having, respectively, a high and a low affinity for methionine. In addition, methionine may be taken up on the general amino-acid permease (Grenson et al., 1970) and perhaps by the postulated γ-glutamyl group-translocation system, although the activity of this system in yeast remains unproven(Penninickx et al.,1980; Robins and Davies, 1980; 1981,a,b). Methionine is accumulated rapidly and completely by Saccharomyces cerevisiae (Maw, 1963a; Ramos-Jeunehomme et al., 1979) although incompletely in beer fermentations (Jones and Pierce, 1964). Cysteine and cystine are not generally used as sources of sulphur by Saccharomyces species. Cystine cannot be taken up into these yeasts (Maw, 1963a). Cysteine can be taken up by

Saccharomyces cerevisiae, albeit slowly, (Maw, 1963a, Kotyk et al, 1971) and its uptake was enhanced by the presence of reduced glutathione in the medium (Maw, 1963a).However L-cysteine was found to be inhibitory to yeast growth at above 25 mg $l^{-1}$ even if an alternative sulphur source were present (Maw, 1960; 1961; 1963b).

S-Adenosylmethionine is actively taken up by a specific, high affinity permease in Saccharomyces cerevisiae (Murphy and Spence, 1972) and this can contribute to the sulphur requirements of this yeast. Transport of S-adenosylmethionine into sphaeroplasts was strongly inhibited by azide or dinitrophenol (Schwenke and de Robichon-Szulmajster, 1976). Inside the cell S-adenosylmethionine exerts strong repressive effects upon the sulphate reduction sequence and methionine biosynthetic enzymes (Cherest et al., 1973a; Colombani et al., 1975) although it is found to be rapidly sequestered into the yeast cell vacuole (Allen and Miller, 1980). This second transportation of S-adenosylmethionine into the vacuole occurs by an as yet unexplained mechanism (Eddy,1982) which is not inhibited by uncoupling agents or assisted by metabolic action outside the vacuole (Schwenke and de Robichon-Szulmajster, 1976). The cytoplasmic tripeptide, glutathione, can be utilized by Saccharomyces cerevisiae as a source of sulphur (Maw, 1960) although growth with this substrate is described as moderate, and not to be compared with growth using methionine or sulphate.

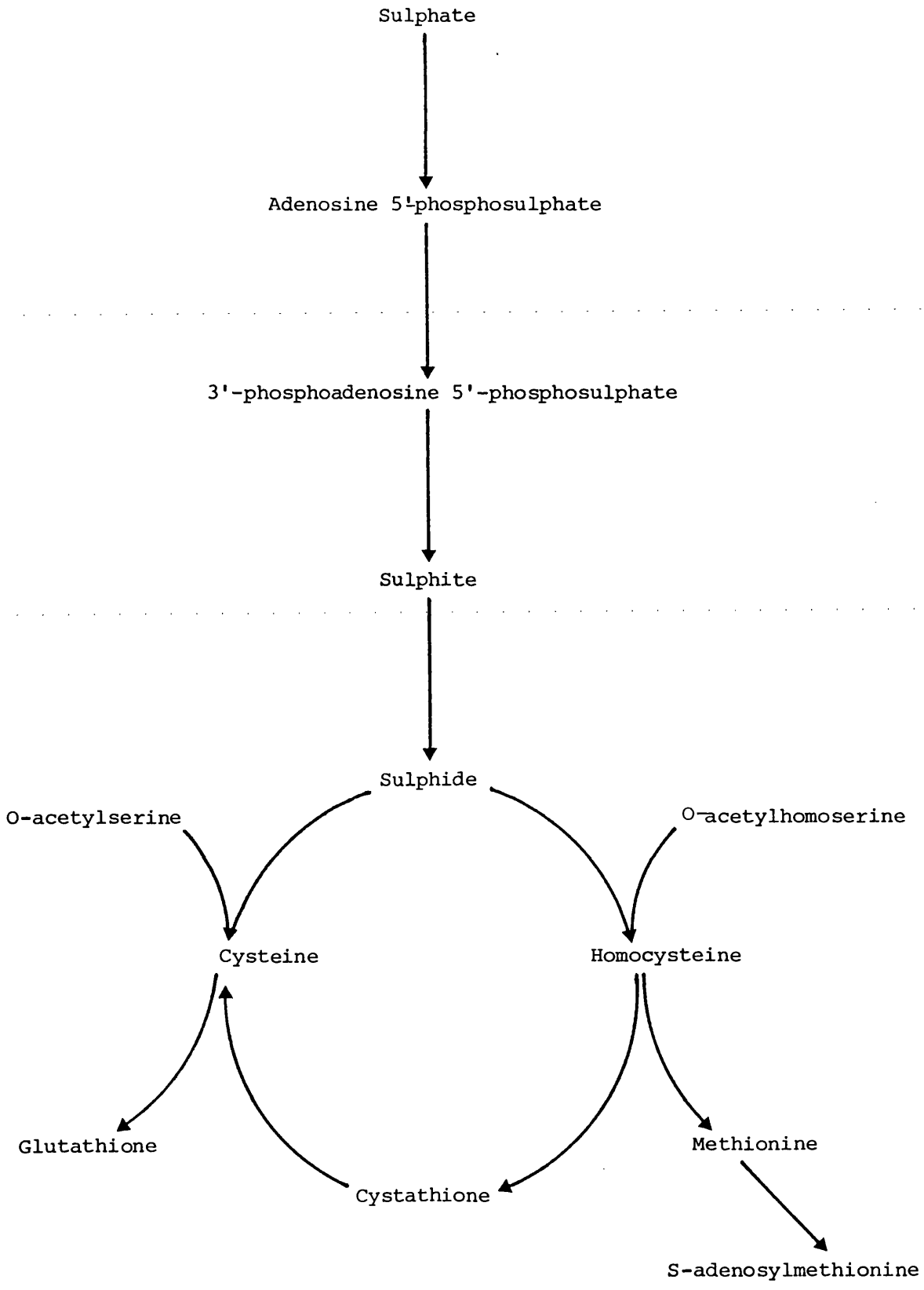



Figure 1. The position of sulphur sources within the sulphur metabolic pathway of Saccharomyces cerevisiae.

## C. Metabolism of Sulphur Sources by Saccharomyces cerevisiae

In order to be used within the cell, sulphur has to be present in a reduced form. A great part of this reduced sulphur is present as the amino-acid residues methionine and cysteine in proteins and glutathione, respectively. In most other sulphur-containing cellular constituents the sulphur is present as reduced thiol groups. If reduced sulphur sources, such as methionine, are being employed by the yeast, no further reduction is necessary but if, as is frequently the case, the oxidised sulphur compounds sulphate and sulphite are being utilized, these compounds have to be reduced to thiols before being used by yeast. This reduction process takes place in several stages and requires the expenditure of two molecules of ATP and one molecule of NADPH in order to reduce one molecule of sulphate, excluding the energy involved in uptake of sulphate. The system of enzymes involved in this process is known as the sulphate reduction sequence. As might be expected, this energy-expending sequence is repressed in the presence of methionine via its derivative S-adenosylmethionine.

The sulphate reduction sequence is the means by which sulphate is transported into the cell, reduced to sulphite and thence to sulphide. This may then be combined with a carbon/nitrogen skeleton to form, eventually, the sulphur-containing amino acids, methionine and cysteine. An understanding of this pathway and its regulation and control is of fundamental importance in elucidating yeast sulphur metabolism and its abnormalities and

imbalances which can lead to the over-production of sulphite or sulphide and their secretion into the environment.

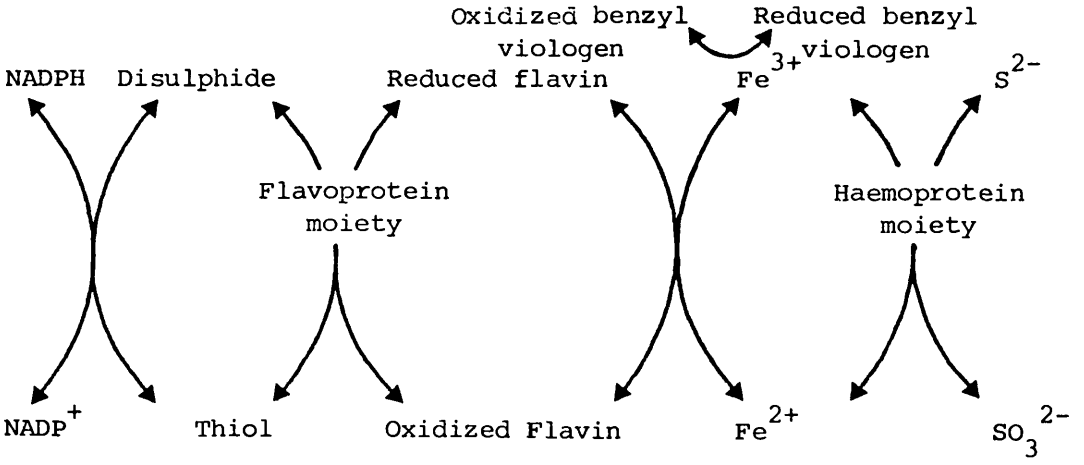
In Saccharomyces cerevisiae, sulphate is actively transported by an energy-dependent process (Kleinzeller et al., 1959; Kotyk, 1959; McCready and Din, 1974) which probably involves the activity of two permeases (Breton and Surdin-Kerjan, 1977). The sulphate is 'activated' by reaction with ATP to form APS, (adenosine 5'-phosphosulphate; Robbins and Lipmann, 1958). This reaction is catalysed by the enzyme ATP-sulphurylase, a key enzyme and probably the rate-limiting step in the sequence (Bonish and Eschenbruch, 1976; Eschenbruch and Bonish, 1976). Thereafter occurs the second activation step involving the enzyme APS-kinase to combine APS with ATP to form PAPS (3'-phosphoadenosine 5'-phosphosulphate; Robbins and Lipmann, 1958), and the reduction by the enzyme PAPS-reductase to form sulphite. It has been postulated (Hammond and Carr, 1976) that sulphite is not released free into the cytoplasm but remains attached to an enzyme complex until its further reduction by sulphite reductase. Sulphite permeation has been studied in yeast only in large and toxic concentrations (Macris, 1972; Macris and Markakis, 1974). Under these conditions, the uptake appears to be an active carrier-mediated process. However, the large amount of sulphite taken up remained in the small-molecule fraction and was not further metabolized (Macris, 1972). The vastly lower level of uptake required for nutrition by sulphite, such as is described by Schultz and McManns (1950), has not been studied. Sulphite is reduced to sulphide by the enzyme sulphite reductase. Since

this enzyme controls a major method of hydrogen sulphide production by yeast, its activity and regulation will be discussed here in detail.

Sulphite reductase is a multisubunit enzyme (Wainwright, 1962a, 1967) requiring reduced nicotinamide adenine dinucleotide phosphate (NADPH) to catalyse the reduction of sulphite to sulphide (Prabnakarao and Nicolas, 1969):

$$SO_3^{2-} + 3NADPH \longrightarrow H_2S + 3NADP^+$$

This enzyme has been partially purified and characterized (Prabnakarao and Nicholas, 1969; 1970; Yoshimoto and Sato, 1968a, b; 1970). In Saccharomyces cerevisiae sulphite reductase, an electron transfer occurs from NADPH via flavin and a chromophore to sulphite. Prabnakarao and Nicholas (1969; 1970) discovered that flavin and haem protein participate in the electron flow and termed this enzyme a haemoflavoprotein.




Model of Sulphite Reductase Action (Prabnakarao and Nicolas, 1969).

While the *in vivo* reducing power is supplied by NADPH, sulphite reductase has also been found capable of reducing sulphite using reduced cytochrome *C* or benzyl viologen. It has a Km value for reduced cytochrome *C* of $6 \times 10^{-5}$ M (Yoshimoto and Sato, 1968a, b; 1970), the Km value for sulphite being $1.4 \times 10^{-5}$ M.

Sulphite reductase is inhibited by sulphide and also by high concentrations of sulphite or nitrite. This inhibition is non-competitive (Yoshimoto and Sato, 1968a, b). It has been demonstrated that there is no effect on sulphite reductase activity by sulphate, methionine, cysteine, thiosulphate or serine. The activity of this enzyme is found to rise during exponential yeast growth and to fall with the onset of the stationary phase (Dott and Trüper, 1978). Synthesis of sulphite reductase is repressed by methionine (Cherest *et al.*, 1971; Dott and Trüper, 1978) although de Vito and Dreyfuss (1964) found that this inhibition was only partial in their strain of yeast. Cysteine was also found to repress sulphite reductase synthesis (de Vito and Dreyfuss, 1964; Dott and Trüper, 1978), while sulphite and djenkolic acid appear to have little or no effect upon repression of this enzyme.

## D. Regulation and Control of Methionine Biosynthesis and the Sulphate Reduction Sequence

### i. Repression by Methionine, Cysteine and Sulphite

Hydrogen sulphide and sulphite production by yeasts has been demonstrated repeatedly to be repressed by methionine (Okuda and Uemura, 1965; Anderson, 1970; Eschenbruch, 1972a,b; Eschenbruch et al., 1973). Further work by Wainwright (1970b) on methionine-requiring mutants of Saccharomyces cerevisiae showed that hydrogen sulphide was generated from sulphate, sulphite or thiosulphate, when these mutants were cultured in low concentrations of methionine. An examination of the enzymes involved showed that the following were repressible by methionine; the sulphate permease (Breton and Surdin-Kerjan, 1977), ATP-sulphurylase (de Vito and Dreyfuss, 1964; Cherest et al., 1969; Colombani et al., 1975), sulphite reductase (Cherest et al., 1971; Dott and Trüper, 1978), homocysteine synthetase (Cherest et al., 1971), homoserine O-transacetylase (de Robichon-Szulmajster and Cherest, 1967), aspartokinase and homoserine dehydrogenase (Cherest et al., 1971). It has been found that, of these methionine-repressible enzymes, some may be grouped together by their apparently co-ordinated mode of repression and by their response in all cases to mutations of methionyl-tRNA synthetase and the regulatory mutation eth $2^r$. Such enzymes have been termed methionine group 1 enzymes (Cherest et al., 1971), and include the sulphate permease, ATP-sulphurylase, sulphite reductase, homocysteine synthetase and homoserine O-transacetylase. It has been speculated that the two remaining enzymes of sulphate assimilation, APS-kinase

and PAPS-reductase, are repressible by methionine (Eschenbruch and Bonish, 1976). Little work has been done on these enzymes, greater effect having been exerted on the rate limiting ATP-sulphurylase (Bonish and Eschenbruch, 1976). However, in terms of economy to the yeast, their repression by methionine is logical since both the preceeding and succeeding steps are also methionine repressed.

It has been shown that methionine has little or no repressive activity but that repression is imposed on group 1 enzymes *via* the activated forms of methionine, methionyl t-RNA and S-adenosylmethionine (Cherest *et al.*, 1971). Mutations of Met t-RNA synthetase abolish repression except that by exogenous S-adenosylmethionine which appears to show a separate repressive effect (Cherest *et al.*, 1973a,b; Colombani *et al.*, 1975). It has been demonstrated that methionyl t-RNA synthetase is not directly involved but that repression is enforced by the total methionyl t-RNA content (Surdin-Kerjan *et al.*, 1973). Thus added methionine is probably activated to both methionyl t-RNA and S-adenosylmethionine to exert a dual repression.

The other methionine-repressed enzymes, aspartokinase and homoserine dehydrogenase, are not co-ordinated with the Group 1 repression and have a different methionine mediated repression (Cherest *et al.*, 1971). Cysteine has been implicated in the repression of some stages of sulphate reduction but the interaction of this repression with that of methionine has not been elucidated. Cysteine has been reported to repress

ATP-sulphurylase (Heinzel and Trüper, 1978) and sulphite reductase (de Vito and Dreyfuss, 1964; Dott and Trüper, 1978). Sulphite has also been demonstrated to repress ATP-sulphurylase (Heinzel and Trüper, 1978).

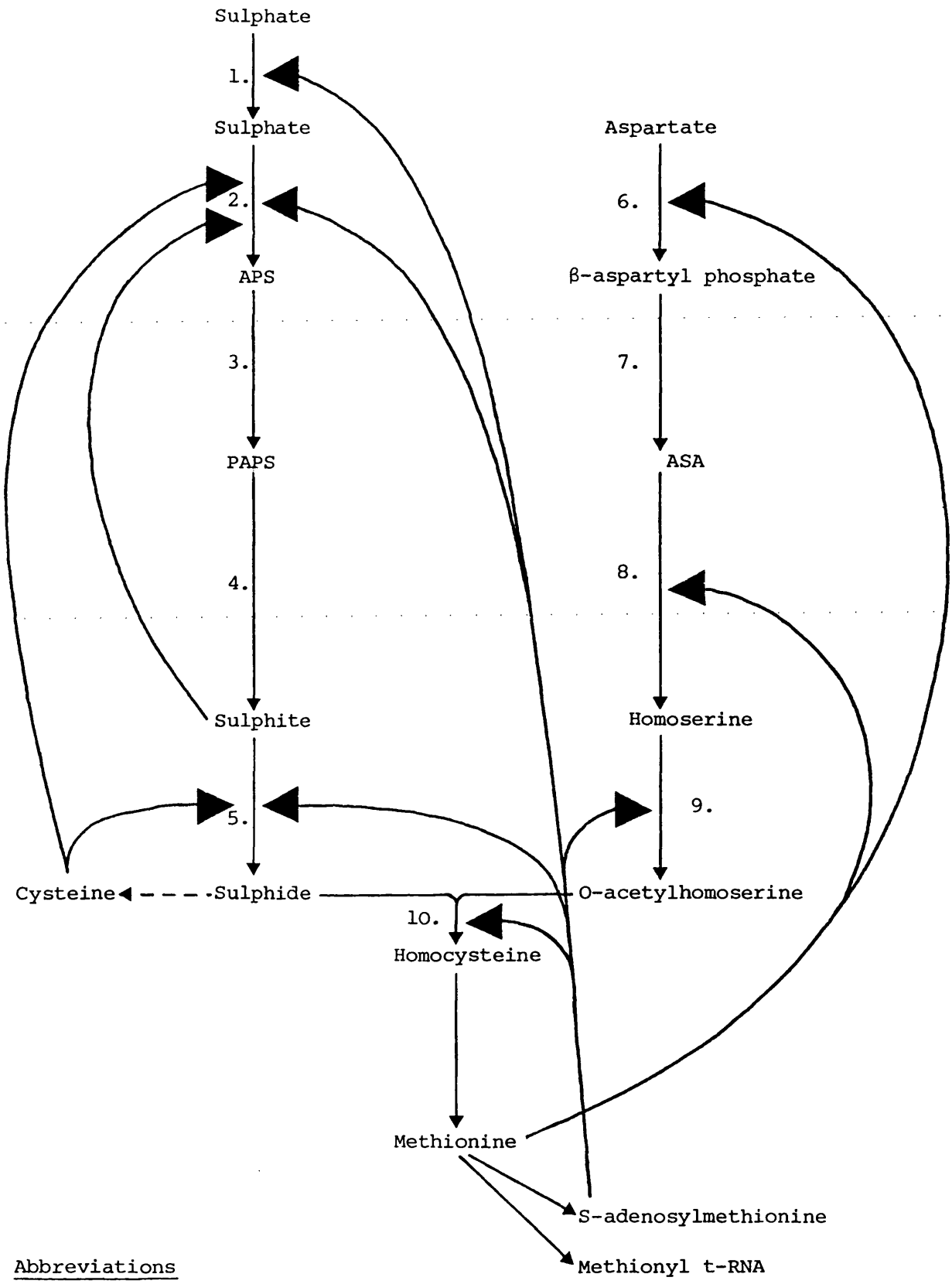
ii. Fine Control of Sulphate Reduction

Whereas methionine and cysteine dominate coarse repressive control of sulphate reduction, they have little or no role in the fine control of allosteric feedback inhibitions on this pathway. As might be expected, almost all of these inhibitions are found operating in the early stages of sulphate uptake and metabolism. The later stages seem to be under little control, but this may be a reflection of the comparative dearth of experimentation on these stages. Sulphate uptake is inhibited by endogenous sulphate and probably by adenosine 5'-phosphosulphate (Breton and Surdin-Kerjan, 1977) and a non-competitive inhibition of the sulphate permease in yeast, by sulphite, has been reported (Kleinzeller et al., 1959; McCready and Din, 1974). Adenosine triphosphate sulphurylase, probably the rate-limiting step in sulphate assimilation (Bonish and Eschenbruch, 1976; Eschenbruch and Bonish, 1976), is competitively inhibited by sulphide and non-competitively inhibited by adenosine 5'-phosphosulphate (APS) and 3'-phosphoadenosine 5'-phosphosulphate (PAPS) (de Vito and Dreyfuss, 1964; Heinzel and Trüper, 1976). Sulphite reductase activity is inhibited by sulphide and by very high concentrations of sulphite or nitrite. This inhibition is non-competitive (Yoshimoto and Sato, 1968a,b)

Figure 2. Repressive control of the sulphate reduction sequence and methionine biosynthetic pathway

1. Sulphate permease
2. ATP-Sulphurylase
3. APS-kinase
4. PAPS-reductase
5. Sulphite reductase
6. Aspartokinase
7. ASA-dehydrogenase
8. Homoserine dehydrogenase
9. Homoserine O-transacetylase
10. Homocysteine synthetase

Repressive Feedback ⟶




Abbreviations

APS - Adenosine 5'-phosphosulphate

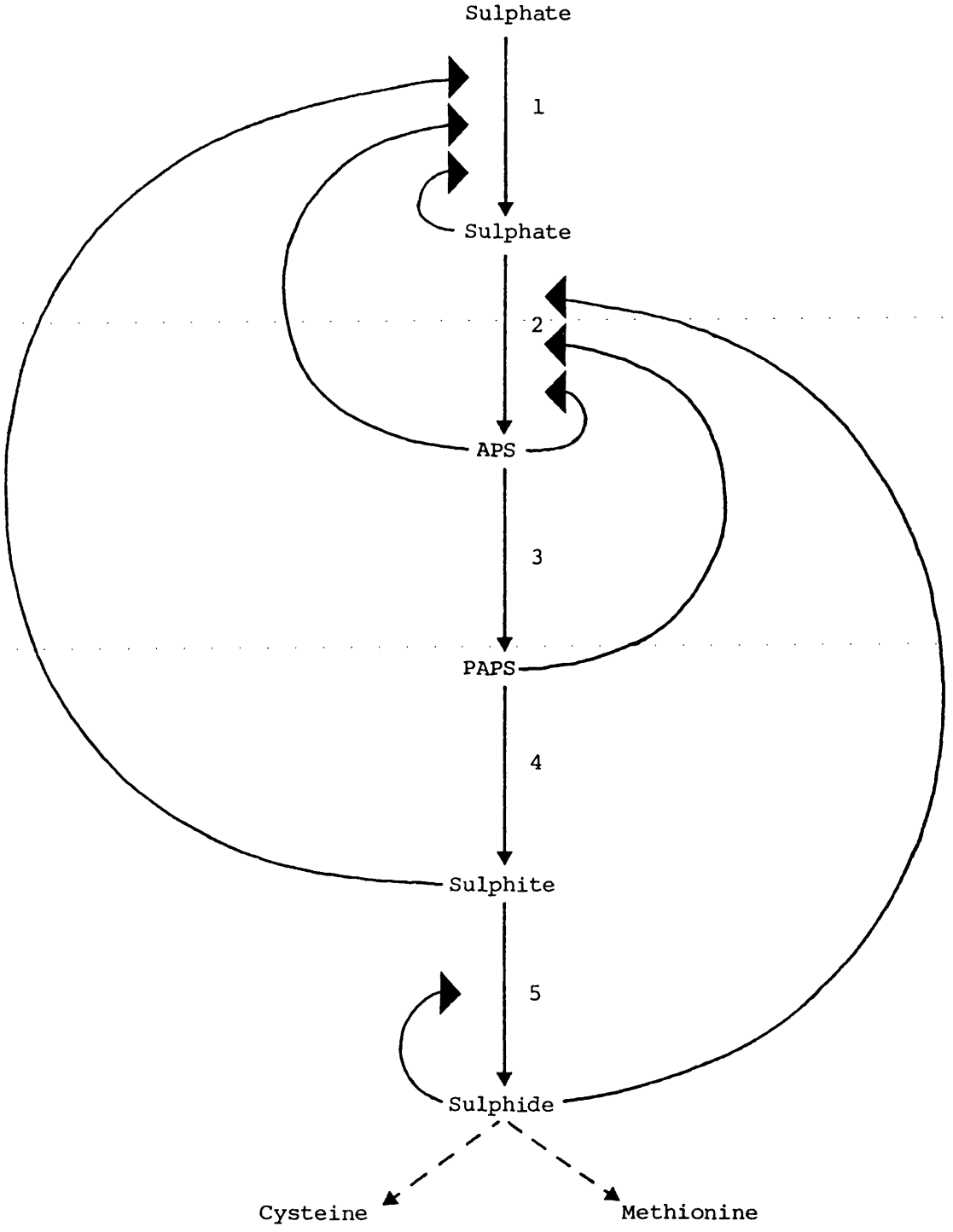
PAPS - 3'-phosphoadenosine 5'-phosphosulphate

ASA - L-aspartyl β-semialdehyde

Figure 3. Feedback Inhibitions in the Sulphate Reduction Sequence

1. Sulphate permease
2. ATP-sulphurylase
3. APS-kinase
4. PAPS-reductase
5. Sulphite reductase

Feedback Inhibition 




Abbreviations: APS - Adenosine 5'-phosphosulphate

PAPS - 3'-phosphoadenosine 5'-phosphosulphate

ASA - L-aspartyl β-semialdehyde

## E. Sulphite Production

Most strains of Saccharomyces cerevisiae can produce sulphite during fermentation, normally at concentrations of between 0.1 mM and 0.4 mM. However, certain yeast strains, under comparable conditions, can produce in excess of 1.5 mM sulphite (Dittrich and Staudenmayer, 1968; Würdig and Schlotter, 1968, 1970, 1971; Minarik, 1972). These have been dubbed "sulphite-forming or high sulphite-forming" strains. This phenomenon has been intensively investigated, in recent years, because of its potential legal consequences (Würdig and Schlotter, 1971). Individual yeast strains have been shown to differ markedly in their sulphite-producing abilities (Rankine and Pocock, 1969) and it is probable that several different metabolic imbalances and abnormalities can cause sulphite production. This seemingly contradictory evidence may be explained by different mechanisms of sulphite formation operating in the various yeast strains examined.

The source of sulphur for production of sulphite is almost invariably sulphate (Eschenbruch, 1974a). However small amounts of sulphite may be produced from cysteine and reduced glutathione (Rankine and Pocock, 1969) and from elemental sulphur (Dittrich and Staudenmayer, 1970).

### i. Factors Influencing Sulphite Production

Sulphite has been found to be produced throughout fermentation, but with a peak of production half way through the fermentation (Feuillat, 1980), and to parallel ethanol

production by some strains of Saccharomyces cerevisiae (Dott et al, 1976). Elevated temperatures (30°C) and lower pH values have been reported to lower sulphite production (Brewer and Fenton, 1980). Dott et al. (1976) reported that sulphite formation was favoured by aerobic conditions, whilst Brewer and Fenton (1980) found the opposite effect. Oxidation of sulphite to sulphate may be involved here. A fermentable sugar is required for sulphite production as would be expected considering the expenditure of ATP in the reduction of sulphate to sulphite. (Dott et al., 1976) and it has been observed that biomass production by sulphite-forming strains is somewhat lower than that of other strains. High concentrations of sulphate have been reported to increase sulphite production in high sulphite-forming strains, up to concentrations of 7.8 mM (Würdig and Schlotter, 1971; Minarik, 1972; Feuillat et al., 1979; Feuillat, 1980).

Sulphite has also been shown to suppress further production of sulphite by yeast (Feuillat et al., 1979; Feuillat, 1980). Brewer and Fenton (1980) reported that hydrogen sulphide could decrease sulphite formation, whilst Amati et al. (1978) could not find this effect in their yeast strain. The amino acids, methionine and cysteine, have been widely reported as lowering sulphite production by up to 50% (Minarik and Navara, 1973; Heinzel and Trüper, 1978; Feuillat et al., 1979; Brewer and Fenton, 1980; Feuillat, 1980).

ii. Causes of Sulphite Formation

Possible causes of sulphite formation can be broadly

divided into two categories. One possible cause is a restriction of metabolite flow below sulphite, as suggested by Dittrich and Staudenmayer (1968), such as a lowered activity of sulphite reductase. An alternative is an enhanced or uncontrolled flow of metabolite upstream of sulphite, in the sulphate uptake and activation stages, as has been suggested by Eschenbruch (1974 a). In different strains of yeast either or both phenomena may occur. These effects could arise through a number of regulatory modifications to enzymes, all of which would appear in high sulphite-forming yeast strains.

A series of mutant strains ($SR^-$) of Saccharomyces cerevisiae which accumulate sulphite and form little free hydrogen sulphide have been found to possess very low sulphite reductase activity (Zambonnelli et al., 1975). These mutants are leaky since they are able to grow using sulphate as the sole sulphur source. Similar strains, producing sulphite but unable to produce hydrogen sulphide, have also been reported (Acree et al., 1972; Bonish and Eschenbruch, 1976; Dott and Trüper, 1978).

Some high sulphite-producing strains of yeast excrete hydrogen sulphide in moderate quantities (Eschenbruch, 1974a)and have sulphate reductase activity comparable with normal strains (Dott and Trüper, 1976). Furthermore, it has been demonstrated that, in low sulphite-producing strains, no correlation exists between sulphite reductase activity and the amount of sulphite formed (Bonish and Eschenbruch, 1976). It thus seems likely that, in low sulphite-forming strains and some high sulphite-

forming strains, the rate of sulphite production is governed by the rate of assimilation and activation of sulphate. The rate-limiting step in sulphate assimilation and activation has been identified as the ATP-sulphurylase primary activation stage (Bonish and Eschenbruch, 1976; Eschenbruch and Bonish, 1976) and high ATP-sulphurylase activity has been reported by these workers in high sulphite-producing yeast strains while similar strains have not been found to possess enhanced activity of this enzyme (Heinzel and Trüper, 1978). Sulphate uptake has been shown to be abnormally rapid in strains of high sulphite-forming yeasts (Eschenbruch, 1972b; Minarik and Navara, 1973). Other evidence showed no difference between low and high sulphite-forming yeast strains with regard to sulphate uptake (Dott et al., 1976).

Sulphite production can be controlled to a certain extent by additions of methionine or cysteine (Minarik and Navara, 1973). These compounds probably cause repression of sulphate permease and ATP-sulphurylase. However, one high sulphite-forming strain examined had a sulphate permease which was irrepressible by methionine (Dott et al., 1976). Sulphite has been demonstrated to suppress its own synthesis (Eschenbruch et al., 1973) and to repress ATP-sulphurylase in normal yeast strains. However, in some high sulphite-forming strains, no repressive activity by sulphite was found and this compound had no effect upon further production of sulphite (Heinzel and Trüper, 1978).

## F. Hydrogen Sulphide Production

Formation of hydrogen sulphide by yeasts during fermentation of wine, cider or beer is a problem as old as the process of brewing. Hydrogen sulphide, the classic odour of rotten eggs, may be acceptable at low concentrations in beer but in high concentrations, is a highly undesirable product of any fermentation. There have been numerous reports of hydrogen sulphide production over the past sixty years, the confusion and contradiction in which are a reflection of the several possible routes by which hydrogen sulphide may be formed. For the purpose of the present study, possible sulphide sources will be treated in three sections.

### i. Hydrogen Sulphide Formation from Elemental Sulphur

Elemental sulphur is found in musts after grapes have been sprayed or dusted with sulphur shortly before harvesting (Rankine, 1963). Elemental sulphur may be found in beer through the sulphuring of hops (Maw, 1965) as quoted by Lawrence and Cole,( 1968). It may also be found through the sterilizing of cooperage by incomplete burning of sulphur (Rankine, 1963) or on the surface of the pitching yeast. It has been observed that old stationary-phase yeast may oxidize hydrogen sulphide to form globular deposits of elemental sulphur on the cell surface (Skerman et al., 1957).

Hydrogen sulphide was produced from elemental sulphur late in the fermentation (Schutz and Kunkee, 1977), and as in an earlier study, its production did not appear to be related

to the rate of cell multiplication (Acree et al., 1972). Hydrogen sulphide may also be released by stationary-phase cells in the lees at the end of fermentation (Rankine, 1963). Direct contact of the yeast with sulphur particles was necessary for reduction to occur, and the amount of sulphide produced was inversely proportional to the sulphur particle size (Rankine, 1963; Schutz and Kunkee, 1977). This surface reaction gives rise to the possibility that elemental sulphur may be reduced to sulphide in a non-enzymatic chemical reduction caused by contact between elemental sulphur and reducing compounds in the yeast wall. Elemental sulphur will, in vitro, spontaneously react with reduced glutathione to produce hydrogen sulphide at pH 5.4 (Kaji and McElroy, 1959). This non-enzymatic reduction was suggested by Wainwright (1971[b]) and Schneyder (1973) and recently this hypothesis received support from Schutz and Kunkee (1977) who concluded, after work with heated extracts of yeast, that this was a non-enzymatic chemical reaction. Cysteine was also found to reduce elemental sulphur. Nevertheless, the participation of an enzymatic reduction in the production of sulphide from elemental sulphur cannot be ruled out (Roy and Trudinger, 1970).

Yeast strains are found to vary considerably in their ability to reduce elemental sulphur (Acree et al., 1972) and generally, sulphide production by yeast strains from elemental sulphur was much greater than sulphide production from sulphite (Rankine, 1964).

ii. Hydrogen Sulphide from Organo-Sulphur Compounds

Whilst methionine has been noted for its ability to suppress hydrogen sulphide production from other sources, Saccharomyces cerevisiae has been shown capable of forming hydrogen sulphide and other thiols from methionine (Hernandez, 1964; Leppänen et al., 1980). However, the other naturally occuring sulphur-containing amino-acid, cysteine, has been reported to be a far more potent source of hydrogen sulphide (Hernandez, 1964; Kikuchi, 1965; Eschenbruch, et al.,1973; Eschenbruch and Bonish, 1976). Other workers have failed to obtain hydrogen sulphide from cysteine (Rentschler, 1951; Rankine, 1963). Wainwright (1971b) speculated that this contradiction may have arisen through variation in the pantothenic acid content of the medium, and that sulphide is released from cysteine in pantothenic acid-deficient medium (Wainwright, 1970b) although this contradicts his earlier work (Wainwright, 1962b). This hypothesis has received support from Eschenbruch and Bonish (1976) who found that pantothenate diminished hydrogen sulphide production from cysteine although different yeast strains varied considerably in their response.

The enzyme assumed to be responsible for this release of hydrogen sulphide is called cysteine desulphydrase, and it catalyses degradation of L-cysteine into pyruvate, ammonia and hydrogen sulphide. Pyridoxal phosphate is required as a cofactor (Kumagai et al., 1975). Cysteine desulphydrase is inhibited by methionine and S-adenosylmethionine (Kodaira and Uemura, 1960, 1961) as quoted by Lawrence and Cole (1968).

Pantothenic acid is not found to inhibit cysteine desulphydrase activity (Kodaira and Uemura, 1960) but Wainwright (1970b) suggested that pantothenic acid deficiency prevents conversion of cysteine to methionine and that the consequent lowering of the methionine concentration allows derepression of the cysteine degradative enzyme, cysteine desulphydrase.

The relevance of such work has been called into question by a recent survey of the distribution of cysteine desulphydrase in micro-organisms (Ohkishi et al., 1981). This revealed that, while cysteine desulphydrase was widespread in bacteria, notably strains of Enterobacteriaceae, it was not found in any of the 30 yeasts examined, including four strains of Saccharomyces. Earlier work by Binkley (1943) demonstrated cysteine desulphydrase in brewer's and baker's yeast. It is possible that cysteine desulphydrase was repressed under the conditions of the survey of Ohkishi et al. (1981) but, if this enzyme is not present in yeast, a new route for production of sulphide from cysteine would have to be established.

With regard to practical applications, this route to sulphide formation may be irrelevant since cysteine is present in grape musts in only low concentrations (Polo and Llaguno, 1971; Llaguno, 1972; Eschenbruch et al., 1973). It has been speculated by Eschenbruch and Bonish (1976) that yeast proteolytic action upon proteinaceous substances in musts (Feuillat et al., 1972; Radola and Richter, 1972) could release cysteine and thence cause sulphide production. However, cysteine is not a

stable molecule and, unless rapidly used, would become oxidized to cystine and thereby rendered unusable by yeast (Maw, 1963 a)

Cysteine and cystine may be present in beer worts in moderate concentrations due to the addition of soybean flakes (Lawrence and Cole, 1968). This has led to the production of sulphuryl flavour in beer (Kleyn, 1966; Kleyn and Vacano, 1966). It has been concluded (Lawrence and Cole, 1968) that the major organic source of hydrogen sulphide in beer is cysteine in the presence of cysteine desulphydrase. A recent study of hydrogen sulphide production in beer (Nagami et al., 1979; Takahashi et al., 1980) found sulphide to be produced in distinct peaks co-incident with cell maturation in synchronous culture at low temperatures. The possible sources of sulphur were not defined but, if this were derived from cysteine, it is necessary to postulate alteration of cysteine desulphydrase activity or cysteine permeation during the cell cycle.

iii. Hydrogen Sulphide from Sulphate and Sulphite

Hydrogen sulphide from sulphate and sulphite arises as a result of the activity of sulphite reductase, and is presumably caused by overactivity of this enzyme or the suppression of activity of methionine biosynthetic enzymes. Numerous reports have been published citing production of hydrogen sulphide from sulphate (Wainwright, 1962b; Anderson et al., 1971; Eschenbruch et al., 1973; Anderson and Howard, 1974) and from sulphite (Schanderl, 1959; Rankine, 1963; Kikuchi, 1965; Maw, 1965; Eschenbruch et al., 1973). The

situation may have been somewhat confused by oxidation of sulphite to sulphate in the media, or by production of sulphite by yeast action early in the fermentation and its subsequent utilization in sulphide production. Wainwright (1971b) suggested that little sulphide is produced from sulphate unless the culture was methionine limited, and that, in general, sulphite gives rise to hydrogen sulphide more readily than sulphate.

The strain of yeast has been found to have considerable influence upon the amount of hydrogen sulphide formed under given conditions. Selection and use of pure-cultures of yeast have so far been the most successful way of limiting excessive hydrogen sulphide production (Rankine, 1964, 1968).

Reduction of sulphate to sulphite requires ATP and further reduction to sulphide requires NADPH. It follows that any aspect of yeast growth and metabolism that affects the energetic competance of the cell will indirectly affect hydrogen sulphide production. Generally hydrogen sulphide production is favoured by fast fermentation and aerobic conditions (Ricketts and Coutts, 1951a; 1951b; Rankine, 1963; Wainwright, 1971a; Dott and Trüper, 1976).

It has been suggested that must nitrogen content could be related to the hydrogen sulphide formed (Vos, 1966; Blake, 1970). More recent work by Vos and Gray (1979) found a correlation between the free amino-nitrogen content of musts and their hydrogen sulphide-producing potential. However, this

picture was confused by the presence of soluble protein in the musts and possible protease activity by the yeast. Wainwright (1971c) reported, however, that nitrogen limitation did not by itself cause much hydrogen sulphide production but may exacerbate this condition, when caused by other factors. Extensive tests upon the nitrogen content of grape musts could not confirm the correlation with sulphide production noted earlier (Eschenbruch, 1974b) and it was concluded that neither a low nor a high amino acid content determines the potential of a must for hydrogen sulphide production.

Methionine and cysteine reduce or completely suppress formation of sulphite and sulphide (Eschenbruch, 1972 a,b; Eschenbruch et al., 1973) in grape musts. Wainwright (1970b, 1971a) examined the effect of these and other amino acids on sulphide formation and found that, while methionine and cysteine lowered the amount of sulphide formed, other amino acids, notably threonine, increased it. A theory that this could be the result of competition by methionine and threonine for the general amino acid permease (Ramos-Jeunehomme et al., 1979), was not supported by experimental evidence. Suppression of sulphide production by methionine and cysteine is consistent with repression of several enzymes of the sulphate-reduction sequence by methionine, as discussed earlier.

Hydrogen sulphide production in many strains of Saccharomyces cerevisiae can be induced by a deficiency of pantothenic acid or pyridoxine (Wainwright, 1962b; Maw, 1965; Lawrence and Cole,

1968; Wainwright, 1970a,b; 1971a,b). Such a deficiency, it has been suggested, may lead to a lack of methionine and consequently to derepression and uncontrolled activity of the sulphate-reduction sequence (Wainwright, 1970a). However an examination of the activities of the enzymes involved revealed that the sulphate-reduction sequence was usually derepressed during exponential yeast growth whilst sulphide was not produced (Heinzel and Trüper, 1978; Dott and Trüper, 1978, 1979). It is evident that production of hydrogen sulphide does not rest solely upon derepression of the sequence and that other factors are involved. Hydrogen sulphide is not usually produced throughout the fermentation. Hernandez (1980) reported its formation late in grape fermentations while Wainwright (1971c) recorded that sulphide was produced only after a 50% attenuation had been achieved. This timing of sulphide production argues against the theory that sulphide is produced by lack of methionine leading to the sulphate-reduction enzymes becoming derepressed, since the activities of ATP-sulphurylase and sulphite reductase have been found to decline late in the fermentation (Heinzel and Trüper, 1978; Dott and Trüper, 1979).

More direct effects of pantothenic acid upon hydrogen sulphide formation are possible. It has been demonstrated that the isotopic selectivity of the sulphate-reductive pathway, and the evolved hydrogen sulphide are altered by pantothenic acid deficiency (McCready and Din, 1974; McCready, 1975). Furthermore pantothenic acid deficiency altered the cellular lipid and fatty acid content of Saccharomyces cerevisiae and increased sulphate diffusion through the membrane (McCready et al., 1979).

iv. Influence of Metals upon Sulphide Formation

One mechanism of hydrogen sulphide formation by metals has been attributed to attack by acids present in wine, or especially cider, producing nascent hydrogen (Rankine, 1963). This then reduces sulphite to sulphide without involvement of yeast. This cannot, however, explain the involvement of heavy-metal ions in sulphide production. Various metal ions have been reported to increase sulphide production by yeast, including copper, zinc and tin (Eschenbruch, 1971; Wainwright, 1971b; Dittrich and Staudenmayer, 1972; Eschenbruch and Kleynhans, 1974). Most metal ions in high concentrations, being toxic to yeast, lower sulphide formation (Ricketts and Coutts, 1951b). However, it has been proposed that sulphide production in the presence of mercury or copper could detoxify these ions by precipitation of the insoluble metal sulphides (Zambonelli, 1958; Amaha and Takeuchi, 1961; Ashida et al., 1963) as quoted by Lawrence and Cole (1968). Discolouration of yeast cells by metallic sulphides so formed has been observed for some time (White, 1954). Examination of some copper-resistant strains of Saccharomyces cerevisiae found them to be prolific producers of hydrogen sulphide and to deposit copper sulphide in the cell wall (Ashida et al., 1963; Kikuchi, 1965). This correlation does not hold good for all strains of copper-resistant yeasts, however (Seno, 1963). It seems likely that heavy metals influence sulphide production from sulphite (Zambonelli, 1958; Kikuchi, 1965) and that metal ions do not influence formation of sulphide from cysteine. It is probable that the major effect of metal ions is to act as a selective

pressure favouring strains of yeast which produce copious amounts of hydrogen sulphide. With increased use of zinc- and manganese-containing fungicides in vineyards (Eschenbruch, 1974a), this selection of sulphide-producing resistant yeast strains is likely to become common.

## G. The Formation of Volatile Thiols and Mercaptans

Volatile organic sulphur compounds are frequently found associated with formation of hydrogen sulphide in fermentations. Although usually present in small concentrations the very low taste and odour thesholds of these compounds have promoted more interest in them than their concentration would suggest.

A range of thiol complexes may be formed by yeast-mediated degradation of methionine, (Leppänen, et al, 1980) or by a variety of chemical methods (Wainwright, 1971c) including reaction with sulphite and the action of sunlight. Wainwright (1971c) found that supplementing the medium with methionine caused an increase in the concentration of thiols produced, but Rankine (1963), using grape musts, concluded that it was unlikely that thiols were formed from methionine or cysteine.

A more common source of volatile thiols is hydrogen sulphide. This reactive compound has been implicated in chemical reactions with components of the fermentation to form thiols (Rentschler, 1951; Rankine, 1963; Rankine, 1968). Efficient removal of hydrogen sulphide would result in greatly decreased amounts of thiol being formed. Conversely, Wainwright (1971c) found that

addition of sulphide increased formation of thiols. There appears little evidence of a direct reaction between hydrogen sulphide and ethanol, and Rankine (1963) considered the thiols probably arose by the reaction of hydrogen sulphide with aldehydes *via* various homologous cyclic trithioaldehyde intermediates (Neuberg and Grauer, 1952). Hydrogen sulphide is a reactive molecule and its presence in a fermented medium for an extended length of time would undoubtedly result in formation of a variety of complex thiol-containing compounds. Thiols are more difficult to remove than hydrogen sulphide and Rankine (1963) suggested that prompt removal of hydrogen sulphide, by precipitation of elemental sulphur by sulphite, was the best method of controlling production of volatile thiols.

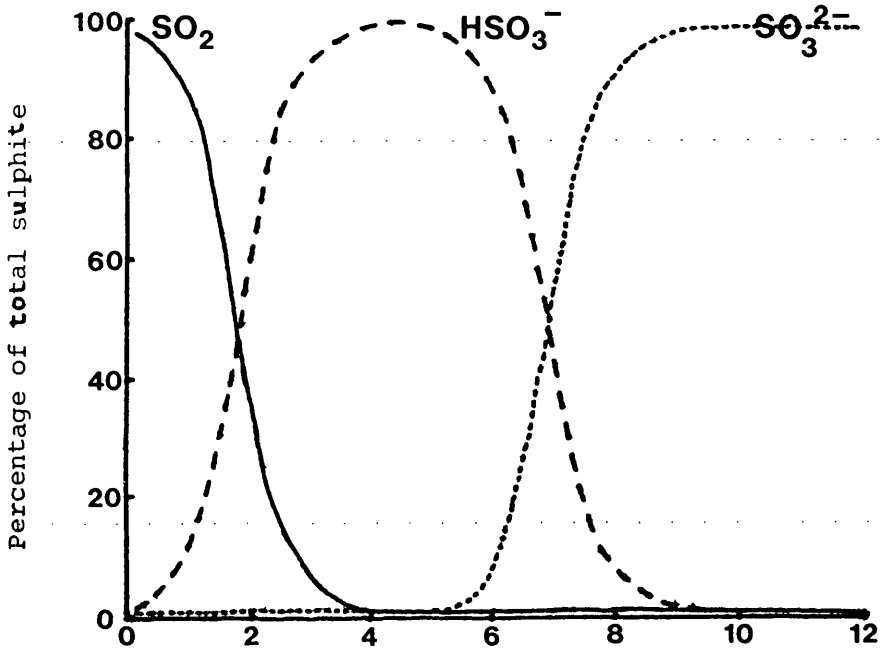
## SULPHITE TOXICITY

### A. Ionization of Sulphite in Aqueous Solution

Sulphur dioxide is used as a preservative, antioxidant and suppressor of enzymatic and non-enzymatic browning in a wide range of foods and beverages. The antimicrobial activity of sulphur dioxide is strongly dependent upon the pH value which influences the degree of ionization of sulphur dioxide.

When sulphur dioxide is dissolved in water, it was thought that sulphurous acid ($H_2SO_3$) was formed. Confirmation of the existence of sulphurous acid, by ultraviolet and infrared spectroscopy, was not forthcoming (Ley and Konig, 1938; Falk and Gigure, 1958; Jones and McLaren, 1958). Raman spectroscopy revealed the presence of molecular $SO_2$ and bisulphite ions in aqueous sulphur-dioxide solutions (Simon and Kriegsmann, 1956), and pyrosulphite ($S_2O_5$) in very concentrated sulphur dioxide solutions (Simon and Waldmann, 1956). In dilute solutions three possible states of ionization of sulphur dioxide exist; namely molecular sulphur dioxide, bisulphite ions and sulphite ions in pH-dependent equilibria. Hydration of sulphur dioxide is one of the most rapid reactions known (De Meyer and Kustin, 1963). There seems general agreement that the first dissociation constant, $K_1$, of molecular $SO_2/HSO_3^+$ is $1.7 \times 10^{-2}$ (King et al., 1981). However, widely different values of the second dissociation constant, $K_2$, of $HSO_3^-/SO_3^{2-}$ have been reported. King (1981) has put forward a value of $6.31 \times 10^{-8}$ and has suggested that the work of Vas and Ingram (1949), based on a value of $5 \times 10^{-6}$, may be in error. The effect of this upon work with sulphur dioxide in alcoholic

beverages is minimal since the pH value of these beverages is far below the $pK_2$ of 7.2 and the active antimicrobial agent being molecular sulphur dioxide.




$$SO_2 \rightleftharpoons HSO_3^- \rightleftharpoons SO_3^{2-}$$

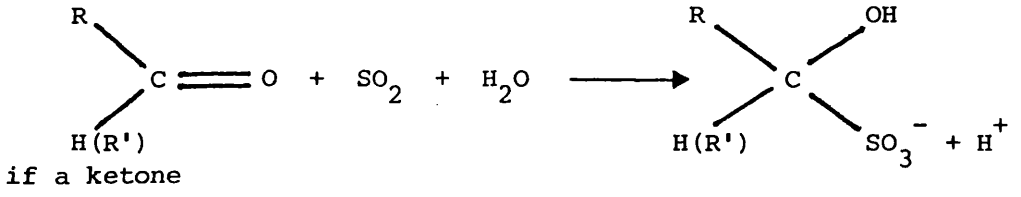
$pK_1 = 1.77$ $pK_2 = 7.2$ (From King et al.,1981)

Figure 4. Percentage distributions of molecular sulphur dioxide, bisulphite and sulphite forms as a function of pH in aqueous solutions of sulphur dioxide.

B. Sulphite Addition Compounds

Sulphur dioxide, in aqueous solution, readily combines with any available carbonyl groups present, to form a class of compounds called α-hydroxysulphonates. These are more commonly termed 'bound sulphite'. The reaction of sulphur dioxide with

aldehyde groups is rapid while that with ketones is very much slower. Generally smaller carbonyl-containing compounds react faster than larger ones. These reactions do not proceed to completion but a series of dynamic reversible equilibria is set up (Burroughs and Sparks, 1964b) each depending upon temperature, pH value and active concentrations of the reagents. The binding of sulphur dioxide to form α-hydroxysulphonates can be summarised as follows:




Aldehyde (or ketone) α-hydroxysulphonate

Many sugars, e.g. glucose, will react slowly with sulphur dioxide, while others, e.g. fructose, will not react to an appreciable extent. Sulphur dioxide may also react with olefinic double bonds in carbon chains and with amines (Joslyn and Braverman, 1954) as quoted by Hammond and Carr (1976).

Binding of sulphur dioxide is not rigid and stable; changes may occur through the fermentation. Binding of sulphite in a must will depend on the carbonyl composition of the must. Binding compounds have been examined in cider (Burroughs and Sparks, 1964b) and were found to usually consist of sugars, acetaldehyde, pyruvic acid, xylosone, galacturonic acid and 2-oxoglutaric acid. Damage to apples resulted in additional sulphite-binding

compounds, as was found with damaged and diseased grapes (Peynaud and Sapis, 1972). However, a fermenting yeast culture will remove some components from these binding equilibria, notably sugars, and will secrete other carbonyl-containing compounds into the medium such as pyruvic acid, acetaldehyde and 2-oxoglutaric acid (Weeks, 1969; Graham, 1979; Lie et al., 1979). Such changes disturb the sulphite-binding equilibria, especially by production of acetaldehyde. The binding reaction of sulphite with acetaldehyde is fast and further towards reaction completion than the other equilibria present (Burroughs, 1964). Thus, secreted acetaldehyde tends to scavenge sulphite from other sulphite-binding compounds. The strain of yeast employed has been shown to influence considerably the binding compounds produced (Rankine and Pocock, 1969).

### C. Action of Sulphite upon *Saccharomyces cerevisiae*

Many reports have been published concerning the reactions of sulphite with various chemical constituents of the yeast cell. Regrettably, the relevance of much of this work must be called into question, as regards the toxic effect of sulphite on the intact yeast cell. Sulphur dioxide is a reactive compound, able both to reduce or oxidize, and probably most compounds found in yeast cells could react with sulphur dioxide, if challenged by it in non-physiological conditions and concentrations. Therefore, for the purpose of this introduction, the literature concerning toxic effects of sulphite have been divided into those upon intact cells at meaningful concentration, and those which can be broadly summarised as *in vitro* effects.

i. Inhibition and Death of Yeast by Sulphite

Sulphite has been shown to inhibit growth of yeasts at low concentrations and to cause cell death when the sulphite concentration is increased. The active antimicrobial agent has been shown to be free, molecular sulphur dioxide (Rahn and Conn, 1944; Ingram, 1948). Bound sulphite showed little antimicrobial activity (Neuberg, 1929; Rehm, 1964), and the little activity present was probably due to the fraction of free sulphite in the equilibrium (Hammond and Carr, 1976). Charged sulphite and bisulphite ions have been shown to be 500-fold less effective than molecular sulphur dioxide against yeast (Rehm and Wittmann, 1962). Consequently, the pH value, which determines the molecular sulphur dioxide concentration, has a profound effect upon the antimicrobial activity of sulphite (Oka, 1960). Adjustment of pH value while maintaining the molecular sulphur dioxide concentration (Rahn and Conn, 1944; Schimz, 1980) demonstrated equal antimicrobial action, and showed that neither the pH value nor bisulphite ion concentration have direct influence upon this action.

Cultures of yeast have been shown to be decimated by molecular sulphur dioxide concentrations of the order of 1.5 μM, 1 p.p.m., (Hammond and Carr, 1976; Schimz, 1980) and that small increases in concentration would kill almost all yeast present. A careful examination by Schimz (1980) revealed that a short period of sulphite-tolerance preceeded inhibition of colony formation and cell death. Stationary-phase cells exhibited a prolonged period of insensitivity while higher temperatures and an active physiological status decreased this period.

After this period, cell viability was found to drop sharply. Sulphite damage was found to be irreversible after some 60 minutes (Schimz and Holzer, 1979).

ii. Sulphite Transport into Yeast

As previously mentioned, uncharged molecular sulphur dioxide is the antimicrobial agent present in sulphite solutions. This had been explained in terms of diffusion of molecular sulphur dioxide into the cell through the hydrophobic interior of the plasma membrane (Jacobs, 1940: Ingram et al., 1956). as quoted by Hammond and Carr (1976). However experiments carried out by Macris (1972) showed that sulphite uptake in Saccharomyces cerevisiae was by an active, carrier-mediated system. It was blocked by the use of metabolic inhibitors which prevented formation or utilization of high-energy phosphate bonds. The kinetics of sulphite uptake were uncharacteristic of passive diffusion and showed a saturation of uptake by high concentrations of sulphur dioxide, indicating that transport was carrier-mediated with a limited number of carriers available (Macris and Markakis, 1974).

The sulphite permease appeared to be specific for undissociated molecular sulphur dioxide (Macris, 1972). This specificity in itself was not evidence for a carrier since non-ionized molecules of weak electrolytes penetrate microbial cells more readily than ionic species. Since the proportion of molecular sulphur dioxide increased with lowered pH value there was a vastly increased rate of transport at lower pH values (Macris and Markakis, 1974). The sulphite permease appeared to show the unusual

characteristic of not displaying a pH optimum. If the pH was varied and the free molecular concentration was maintained, the rate of transport appeared not to alter (Macris, 1972).

The mode of uptake of sulphur dioxide in yeast was unusual in that the transport became inhibited and the rate of uptake declined from its initial maximum, to zero within a few minutes. As the pH value was lowered, the initial rate became greater and up to 60% of the total sulphur dioxide was absorbed before transport ceased.

Macris (1972) found that sulphite taken up remained in the small-molecule fraction. However other work has shown that sulphite may be metabolised as a sulphur source (Schultz and McManus, 1950). This was the result of far smaller concentrations of free sulphite being present and the avoidance of sulphite toxic effects.

iii. Sulphite Effects upon Central Metabolism

Perhaps the most significant finding concerning the effect of sulphite upon Saccharomyces cerevisiae was the discovery that the ATP content of intact cells rapidly decreased after incubation with low concentrations of sulphite (Schimz and Holzer, 1979). This decline occurred very shortly after addition of sulphite and would lead to a sudden interruption of all processes that are ATP-dependent. It was suggested that this process caused irreversible damage and eventual cell death. The decline in ATP content was reversible within 60

minutes but this period was shortened by lowering the pH value and raising the temperature. To refer back to sulphite transportation, after a few minutes sulphite transport was inhibited (Macris and Markakis, 1974), which was indirect evidence that the transport was active and that the consequent decline in ATP content caused cessation of further transport.

It has been known for some years that one reaction of Saccharomyces cerevisiae, treated with sulphite, was to produce moderate amounts of glycerol. This was first discovered in Germany during World War I in an endeavour to produce explosives from beet sugar (Neuberg and Reinfurth, 1918, 1919). Sulphite was not used to kill yeast cells but the process involved the use of sulphite as a 'steering reagent' to induce formation of glycerol by successive sublethal additions of sulphite (Freeman and Donald, 1957). It has been speculated that glycerol arose from glyceraldehyde-3-phosphate as a result of blockage of glycolysis (Gancedo et al., 1968).

Rehm (1964) reported that respiration in yeast, measured by oxygen uptake, was strongly inhibited by sulphite, while fermentation, measured by carbon dioxide evolution, was little affected. He went further in investigating in vivo inhibition of $NAD^+$-dependent steps in fermentation, by sulphite, and concluded that the observed inhibition was caused by formation of an addition complex between NAD and sulphite. These data must now be regarded with caution in the light of new evidence,

since Rehm (1964) added ATP to sulphite-inhibited cultures together with the metabolites under investigation.

D. In vitro Examination of Potential Sites of Sulphite Action

i. Sulphite Reactions with Cellular Constituents

As mentioned earlier, sulphite has an affinity for carbonyl groups. It has been suggested that sulphite reacted with sugars and aldehydes of glycolysis, that is glucose, 3-phosphoglycerate, 3-phosphohydroxyacetone phosphate, pyruvate, acetaldehyde, oxaloacetate, and 2-oxoglutarate (Hammond and Carr, 1976). The addition complexes formed would be effectively removed from metabolism. This has received support from Macris (1972) who found much of the transported sulphite attached to such fractions.

The secondary and tertiary structure of proteins is contributed to by formation of disulphide bridges between the thiol groups of cysteinyl residues. Disulphide bridges have been shown to be cleaved by sulphite (Clarke, 1932). This leads to altered protein structure and possible loss of enzyme function (Cole, 1967). Many enzymes have been examined and found to be inhibited *in vitro* by sulphite (Pfleiderer *et al.*, 1956).

It has been demonstrated that thiamin, is irreversibly cleaved into two inactive fragments by sulphite (Williams, 1935). A derivative of this vitamin, thiamin pyrophosphate, has been implicated as a co-enzyme in many reactions and destruction of thiamin pyrophosphate by sulphite would inhibit all thiamin-dependent reactions (Haisman, 1974).

Reactions of sulphur dioxide with folic acid (Vanderschmitt et al., 1967) and glutathione (Massey and Williams, 1965) have been reported. Furthermore pyridoxal phosphate (Adams, 1969) and isoalloxazine (Müller and Massey, 1971; Hevesi and Bruice, 1973) were reported to react with sulphite. An interaction between $NAD^+$ and sulphur dioxide has been reported (Mayerhof et al., 1938). Rehm (1964) believed that this interaction was responsible for observed inhibition of $NAD^+$-dependent steps in glycolysis. Finally peroxidation of lipids by sulphur dioxide has been demonstrated (Utsumi et al., 1973). This effect could induce major changes in membrane structure and function. Sulphite addition to olefinic double bonds (Hammond and Carr, 1976) of membrane lipids could also modify the activity of yeast cell membranes (Keenan and Rose, 1979).

ii. Mutagenic Effects of Sulphite

In view of the modern preoccupation with mutagenic and carcinogenic agents, it is not altogether surprising that sulphur dioxide has come in for scrutiny. In vitro, several reactions of sulphite with nucleic acids have been discovered. Cytosine-to-uracil conversion has been reported to be caused by the action of sulphite (Hayatsu et al., 1970) and the 5:6 double bonds of uracil and cytosine has been shown to be susceptible to sulphite-addition reactions (Shapiro et al., 1970). Furthermore, sulphur dioxide catalyses a reaction between cytosine derivatives and amines to form 4-aminocytosine (Shapiro and Weisgras, 1970). It has been suggested that this reaction could result in cross linkage of the two strands in DNA.

Mutations, induced by sulphite, have been reported in bacteriophages (Hayatsu and Miura, 1970; Summers and Drake, 1971), bacteria (Mukai et al., 1970), and yeast (Dorange and Dupuy, 1972). It has been suggested that sulphite could only attack single-stranded nucleic acids in vivo (Shapiro et al., 1973). Recent work by Shortle and Nathans (1978) seems to support this hypothesis.

However, the concentrations of sulphite used in some of this work were more than a thousand-fold greater than that encountered in beverages, disregarding any further reduction of sulphite concentration by binding. While bacteriophage nucleic acids are relatively exposed, those in bacteria and yeast are buffered by the presence of sulphite-binding compounds in the cytoplasm, whose affinity with sulphite is very large, and also by the nuclear membrane in eukaryotes. Thus it is very unlikely that large concentrations of sulphite could interact with the genetic material without first causing irreversible damage and cell death. If low concentrations of sulphite were effective in transforming nucleic acid as suggested by Inoue et al. (1972), it is possible that genetic damage would be caused by the sublethal consequences of sulphite poisoning rather than by direct interaction of sulphite upon nucleic acids. Sulphite, in very low concentrations, is usually present in yeast cells as a part of sulphur metabolism without causing apparent harm. Finally, recent work has attributed sulphite with abolishing the mutagenic activity of other compounds, namely those present in chlorinated water (Cheh et al., 1980). Sulphite sharply decreased mutagenic activity as detected by the Ames

salmonella test, of compounds, as yet not identified, formed by chlorine in drinking water.

## E. The Detoxification of Sulphite

Sulphite may be detoxified by three possible routes: (1) oxidation to sulphate; (2) reduction to sulphide; (3) lowering the sulphite concentration by binding components. In mammals, sulphite is rapidly converted to sulphate by the action of sulphite oxidase (Fridovich and Handler, 1956). This enzyme is not present in yeast but there is some evidence for an analogous low-activity system. Eschenbruch et al. (1973) reported that sulphite oxidation in a yeast culture was increased four- to five-fold by methionine and cysteine. It was suggested that this was not a purely chemical reaction on the grounds of variation between yeast strains. Adenosine diphosphate sulphurylase, an enzyme catalysing formation of sulphate from APS, may have a role in sulphite oxidation as it has been reported in sulphur bacteria (Peck, 1960, 1962; Thiele, 1966). In yeast, this enzyme is stimulated by methionine (Heinzel and Trüper, 1978).

Sulphite reductase has been shown to be present and active in yeast, as mentioned earlier. While some sulphite may be detoxified by reduction to sulphide, this is unlikely to be able to operate sufficiently fast to significantly reduce sulphite toxic effects.

Sulphite may be reduced in concentration by secretion of binding compounds by yeast. This would be unlikely to occur

quickly enough to overcome the sulphite after a culture of yeast has been so treated, but production of sulphite-binding compounds into the medium, prior to addition of sulphite, may be a significant mechanism of sulphite resistance by yeast strains.

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The aim of the research reported in the present thesis was to investigate the origins and causes of hydrogen sulphide production by yeast, with especial relevance to cidermaking, and to examine the mechanisms of sulphite toxicity in yeast and the resistance of certain organisms to this.

# Methods and Materials

## MATERIALS AND METHODS

### Organisms

The yeasts employed in this study were Saccharomyces cerevisiae NCYC 366, X 2180lAα, TC 8, TC 16, TC 17, and Saccharomycodes ludwigii TC 10. The strains of yeast prefixed TC were kindly supplied by T. Cowland of the Taunton Cider Company. Yeast cultures were maintained at 3°C upon slopes of MYGP medium (Wickerham, 1951) and were subcultured monthly. The medium contained per litre of water: malt extract (lab m, 3 g), yeast extract (lab m, 3 g), glucose (10 g), mycological peptone (lab m, 5 g) and agar (lab m, No. 2, 20 g). The medium was sterilized by autoclaving at 120°C for 15 min.

### Experimental Cultures

During the study of hydrogen sulphide production, cultures were grown in a defined yeast carbon-base medium and in enriched apple juice medium. The yeast carbon-base medium (pH 4.0) contained per litre of water; glucose (125 g), $(NH_4)_2SO_4$ (0.5 g), yeast carbon-base (Difco, 11.7 g), pyruvic acid (200 mg), thiamin (1 mg), sodium metabisulphite (150 mg; 1.57 mM sulphite), and trisodium citrate and citric acid buffer (30 mM, pH 4). Sodium metabisulphite was added to the medium as a freshly prepared solution after the medium had been sterilized at 115°C for 10 minutes.

Enriched apple juice medium (pH 4.0) contained per litre of water: apple juice concentrate (125 ml), $(NH_4)_2SO_4$ (0.5 g), thiamin (1 mg) and sodium metabisulphite (150 mg; 1.57 mM sulphite).

Apple juice concentrate (specific gravity 1.360) was supplied by the Taunton Cider Company. This was industrially prepared by application of vacuum to heated juices, followed by the re-addition of lost volatile fractions. Concentrate was kept as a homogeneous stock concentrate at $3^{o}C$. Sulphite was added, as with the yeast carbon-base medium, after the medium had been sterilized at $121^{o}C$ for 15 minutes.

Sulphite toxicity was studied using cultures grown aerobically by the method of Patching and Rose (1969), and anaerobically by the method described by Alterthum and Rose (1973). The aerobic medium contained per litre of water: glucose (20 g), $(NH_4)_2SO_4$ (3 g), $KH_2PO_4$ (3 g), yeast extract (Oxoid, 1 g), $MgSO_4.7H_2O$ (25 mg), $CaCl_2.2H_2O$ (25 mg), and trisodium citrate and citric acid buffer (30 mM, pH 4). This medium was sterilized at $115^{o}C$ for 10 minutes. The anaerobic medium contained per litre of water: glucose (100 g), $(NH_4)_2SO_4$ (3 g), $KH_2PO_4$ (3 g), yeast extract (Oxoid, 1 g), $MgSO_4.7H_2O$ (25 mg), $CaCl_2.2H_2O$ (25 mg), ergosterol (5 mg), linoleic acid (30 mg).

The last two constituents were stored as chloroform solutions under nitrogen gas (at $-20^{o}C$) and were added to the medium prior to sterilization at $115^{o}C$ for one minute. High purity nitrogen, after passing through a Jencons Nilox oxygen scrubbing system, was sparged over the medium whilst hot and throughout growth of the culture. Control cultures lacking ergosterol were incubated with each batch of experimental cultures and, if growth in the control reached 0.10 mg dry wt $ml^{-1}$, the experimental cultures were discarded.

One litre portions of media were sterilized by autoclaving in two-litre round flat-bottomed flasks. Non-sulphited media were inoculated with 1 mg dry weight equivalent, and sulphited media were inoculated with 30 mg dry weight equivalent of yeast from a 30 h. liquid culture. Flasks were incubated at 30°C with the medium stirred magnetically at 250 - 300 rev. $min^{-1}$ (4 cm bar). Growth was estimated by measuring extinction at 600 nm and the values related to a dry weight calibration curve. Viable cells were estimated by serial dilution and plating out of the culture onto MYGP medium (Wickerham, 1951).

Analytical Methods

A. Sulphate

Sulphate was measured by a nephelometric technique described by Vogel (1978) based upon quantitative precipitation of barium sulphate. The following reagents were used: Reagent A; NaCl (Analar, 60 g) in 200 ml water and HCl concentrated (5 ml) made up to 250 ml with water. Reagent B; glycerol (Analar, 1 volume) and ethanol 100%, 2 volumes.

Samples (10 ml) of culture were centrifuged at 2000 g for 5 minutes. To these were added 10 ml reagent A and 20 ml reagent B. Samples were made up to 100 ml with water. Portions (3 ml) were removed, as the nephelometric blanks. Sieved barium chloride (0.3 g; 20 - 30 mesh size) was added to each sample and mixed until dissolved. After 5 minutes samples were mixed again and the extinction at 600 nm measured against the appropriate sulphate-free blanks. Extinction values were related to a standard sulphate curve.

B. Total Sulphur Dioxide

Total sulphur dioxide was measured by the method of Burroughs and Sparks (1963). Samples (5 ml) were gently boiled and refluxed with orthophosphoric acid (5 ml; 25%, v/v) in a 250 ml round two-necked flask fitted with a condenser. A steady stream of nitrogen (100 ml $min^{-1}$) was bubbled through the refluxing liquid. The sulphur dioxide gas liberated was bubbled through, and absorbed by, potassium iodide solution (10 ml; 5%, w/v) containing iodine (0.01 m). The gas was then passed through two portions of potassium iodide solution (5 ml; 5%, w/v). Addition of a few drops of starch solution (1%, w/v) in the second portion indicated that no loss of iodine occurred. After 15 minutes, heating of the flask was stopped and the contents of the first two tubes were removed and mixed whilst the carrier-gas flow was maintained. To the mixture was added sodium phosphate (5 ml; 0.2 M) and sulphuric acid (2 ml; 0.5M). This was then titrated against standardized sodium thiosulphate solution (10 mM) using starch indicator. The sample titre was subtracted from the reagent-blank titre leaving a measurement proportional to the original sulphur dioxide content of the sample. Sodium thiosulphate (1 ml; 10 mM) was equivalent to 0.32 mg of sulphur dioxide in the original sample.

C. Free Sulphur Dioxide

Free sulphur dioxide was assayed by the method described by Burroughs and Sparks (1964 a). Samples (5 ml) were placed in a 250 ml round two-necked flask with orthophosphoric acid (5 ml; 25%, v/v). The flask was connected to a series of two boiling tubes fitted with dreschel heads. The pressure in the apparatus was lowered by 80 mm Hg using a vacuum pump whilst a steady stream of

air was bubbled through the contents of the flask. Sulphur dioxide gas was carried by the stream of air and bubbled through a dilute trapping reagent, (5 ml) in each of the two boiling tubes. The trapping agent contained per 100 ml of water; 3.3 ml 100 volume $H_2O_2$ (Analar) in 90 ml water, and 1 ml Tashiro indicator (0.2%, w/v methyl red + 0.1%, w/v methylene blue in 95% ethanol). The mixture was then neutralized with sodium hydroxide (10 mM) to a give a grey colour and made up to 100 ml with water. After 15 min, the vacuum was gradually shut off and the contents of the first tube were back-titrated with standardized sodium hydroxide (1 mM), from a red colour to the original grey end-point. The reagent-blank titre was subtracted from the value and the free sulphur dioxide equivalent was determined. Sodium hydroxide (1 ml; 1 mM) is equivalent to 0.032 mg of sulphur dioxide. Any sulphur dioxide not retained in the first tube was readily detected by a colour change in the second tube.

D. Hydrogen Sulphide

Hydrogen sulphide was measured by the method of Gustafsson (1960). A steady stream of gas (100 ml $min^{-1}$) was bubbled through growing cultures. Air was used in aerobic and high-purity nitrogen gas in anaerobic cultures. The gas was then bubbled through a series of two tubes, each containing 10 ml zinc acetate solution (zinc acetate 0.25 M and sodium acetate 0.1 M) to retain hydrogen sulphide. The zinc acetate traps were changed periodically (20 or 30 minutes) and the sulphide-containing liquid was made up to a standard volume (25 ml) with zinc acetate solution. A portion (1 ml) of this was taken and diluted with 9 ml deionized water in a

quickfit test tube. To this was added 1 ml of acidic p-amino-NN-dimethyl-analine (5 mM $NH_2C_6H_4N(CH_3)_2$ in 3.5 M $H_2SO_4$). Ferric reagent (0.2 ml; 0.25 M $NH_4Fe(SO_4)_2$ in 0.5 M $H_2SO_4$) was immediately added. The tube was then stoppered and shaken for 30 sec. It was incubated at 22°C for 15 min and the methylene blue formed was estimated by measuring the extinction at 667 nM against reagent blanks. The extinction value was related to sulphide concentration by a calibration curve, prepared from a standard sulphide solution (50 mM) formed from large crystals of sodium sulphide ($Na_2S.9H_2O$).

E. Volatile Thiols

Volatile thiols were measured as described by Sinclair et al., (1969). After hydrogen sulphide had been removed, the gas stream from the growing culture was passed through 10 ml of mercuric acetate solution (0.16 M mercuric acetate in 5%, v/v acetic acid), to retain volatile thiols. Every 2 h, the thiol absorption tube was changed and the thiol-containing reagent was adjusted to 10 ml with mercuric acetate solution. The tube was then cooled in ice for 30 min. To this was added 1.5 ml of acidic p-amino-NN-dimethyl-aniline solution (37 mM $NH_2C_6H_4N(CH_3)$ in 36%, w/v hydrochloric acid) and this was immediately followed by 0.5 ml of ferric reagent (0.25 M $FeCl_3.6H_2O$ in 0.8 M nitric acid). The tube was stoppered, shaken vigorously, and incubated at 20°C for 30 min. The red pigment formed was partitioned into nitrobenzene (5 ml) by shaking in a separating funnel. The red nitrobenzene phase was run off and dried by passing it through anhydrous sodium sulphate (0.3 g) in a fluted filter paper (Watman No. 1; 5 cm diam.). The extinction of the solution at 500 nM was measured against a

nitrobenzene reagent blank and the original thiol content determined by reference to a calibration curve. The curve was prepared using ethanethiol (3 mM in 25%, v/v aqueous ethanol).

F. Alpha-Amino Nitrogen

Alpha-amino nitrogen was estimated by reaction with ninhydrin and quantitative measurement of the coloured complex so formed. Using syringes fitted with 150 mm needles, samples of medium (5 ml) were removed aseptically through the suba-sealed sampling ports in the two-litre fermentation vessels. Samples were centrifuged at 2000 g for 5 min. to remove yeast. Portions of supernatant (1 ml) were added to 0.5 ml of citrate buffer (0.8 M; pH 4.8). To this was added 1 ml of ninhydrin reagent (56 mM ninhydrin and 2.3 mM ascorbic acid in methoxyethanol) and the tube was incubated at 100°C for 20 min. The sample was then cooled and made up to 10 ml with aqueous ethanol (70%, v/v). Extinction was measured at 570 nm against a reagent blank and the α-amino nitrogen content estimated using a calibration curve prepared using a standard solution of asparagine.

G. Total Nitrogen

The method used to determine total nitrogen content was based on that described by Howard (1971). Centrifuged yeast-free samples of culture (5 ml) were diluted four times with deionized water. Portions (0.5 ml) were mixed with 0.5 ml acidic selenium catalyst (10 g of $Na_2SO_4Se$ or three Kjeldahl selenium catalyst tablets in 100 ml of concentrated sulphuric acid). A few glass anti-bumping granules were added and the samples were gently

refluxed until clear (this required about 40 min.). They were then cooled,neutralized with sodium hydroxide solution (0.5M) and made up to 25 ml with deionized water. To 0.5 ml of this were added 1 ml of reagent A (0.53 M sodium salicylate in 2 mM sodium nitroprusside) and 1 ml of reagent B (10 mM sodium dichloroisocyanurate in 0.3 M sodium hydroxide), and the samples were mixed and incubated at 37°C for 10 min. After making up the volume to 5 ml with deionized water, the extinction at 660 nm was measured against a reagent blank. The original total nitrogen content was determined by reference to a calibration curve.

H. Ammonia

Ammonium ion concentration was determined using Nessler's reagent (Vogel, 1979). The reagent was prepared by dissolving mercuric iodide (0.22 M) and potassium iodide (0.42 M) in 100 ml of deionized water and adding it to a cooled solution of sodium hydroxide (4 moles) in 700 ml of deionized water. The volume was made up to one litre and the precipitate formed was allowed to settle. The cleared supernatant was racked off and stored in a dark-glass vessel. Samples (1 ml) of yeast-free culture-filtrate were diluted to 10 ml with deionized water. To these were added Nessler's reagent (0.2 ml). The samples were mixed and incubated at 20°C for 10 min. The colour formed was measured at 525 nm against ammonia-free reagent blanks and the ammonia concentration was determined by reference to a calibration curve.

Measurement of rate of solute accumulation

The rate of solute accumulation was measured by a method based upon that described by Keenan (1981). Cells were harvested from late exponential-phase cultures (0.24 - 0.26 mg dry wt $ml^{-1}$) by filtration through a membrane filter (0.45 μM pore size; 50 mm diameter, Oxoid), were washed with cold citrate buffer (20 mM, pH 4.5), and were resuspended in 12 ml of the same buffer in a stoppered tube. The tube was stored in an ice-water mixture and, when cells had been grown anaerobically, the headspace in the tube was flushed through with oxygen-free nitrogen.

The incubation mixture (20 ml) contained citrate buffer (20 mM, pH 4.5), glucose (100 mM), solute (1 - 10 mM) and radioactive tracer (1 - 5 μCi). The solutes used were D-glucose, D-glucosamine, L-lysine and L-arginine. Glucose (100 mM) was excluded from the incubation mixture when glucose and glucosamine accumulations were examined. The radioactive tracers used were D-[6-$^{3}$H]glucose, D-[U-$^{14}$C]glucosamine, L-[U-$^{14}$C]lysine, monohydrochloride, and L-[U-$^{14}$C]arginine monohydrochloride. The incubation mixture was placed in a 100 ml round-bottomed three-necked Quickfit flask and was pre-incubated at 30°C for 15 min. in a water bath, while air, or nitrogen, was flushed through the flask. The contents of the flask were stirred magnetically. The experiment was started by addition of cells in buffer (0.5 mg dry wt organisms $ml^{-1}$) to the incubation mixture. Samples (1 ml) were removed at preselected intervals and were rapidly filtered through membrane filters (0.45 μm pore size; 25 mm diameter; Millipore). The filters, bearing yeast cells, were then immediately washed through with 10 ml cold citrate buffer (20 mM; pH 4.5) containing solute

at the concentration in the incubation mixture. Filters were then placed in scintillation vials containing scintillation fluid (7 ml of Unisolve liquid scintillator No. 1, Koch-Light, Colnbrook, Bucks, England). Radioactivity in the vials was measured in a Packard Tricarb liquid scintillation spectrometer (model 3385) and the values obtained were corrected for counting efficiency.

The rate of loss of solute from cells was examined in the following manner. A portion (30 ml) of incubation mixture was prepared and cells were added in the usual manner. Samples (1 ml) were then removed at intervals. After 30 min., 15 ml of incubation suspension were removed, filtered (0.45 μm pore size; 25 mm Millipore) and the cells washed with 10 ml citrate buffer (20 mM; pH 4.5) at 30°C containing solute at the concentration in the incubation mixture. The loaded cells were resuspended in incubation medium (15 ml), lacking radioactive tracer, in a 100 ml flask at 30°C with gas flushed through it. Samples (1 ml) were removed at one-minute intervals, filtered, washed and the radioactivity was determined in the usual way.

Different techniques were used for measuring rates of uptake of $^{35}SO_4^{2-}$, $^{35}SO_2$ and formation of $H_2{}^{35}S$. Samples of medium (10 ml) were removed from late exponential-phase cultures and were centrifuged at 2000 g for 5 min. to remove yeast, and filtrates were stored deep frozen at -20°C. When required, samples (10 ml) were removed and incubated with sodium [$^{35}$S] sulphate (10 μCi) or sodium[$^{35}$S]sulphite (5 μCi) for 2 hours at 30°C. These solutions were then injected into mid or late exponential-

phase yeast cultures through suba-sealed sample ports. Samples of medium (1 ml) were removed at intervals using syringes fitted with 150 mm needles (Hamilton) and the radioactivity present in the cells and culture filtrate were determined as already described. Gases (air or nitrogen) were bubbled through the medium and any hydrogen[$^{35}$S]sulphide produced was carried through and bubbled through 30 ml zinc acetate solution (zinc acetate, 0.25 M, with sodium acetate, 0.1 M) contained in a 100 ml round-bottomed three-necked Quickfit flask. Its contents were stirred magnetically and samples (0.2 ml) were periodically removed through the two suba-sealed sample ports. These samples were placed directly into vials containing 7 ml scintillation fluid (Unisolve liquid scintillator No. 1) and the radioactivity measured as already described. After passing through the zinc acetate solution, the gas stream was bubbled through two solutions of saturated potassium hydroxide solution as a safety trap.

These experiments were terminated by injection of large volumes (250 ml) of saturated potassium hydroxide solution into the fermentation vessels. The gas was left flowing for 3 h to enable radioactive gas to become absorbed. The systems were eventually opened and washed out in a fume cupboard with the operator wearing a gas mask suitable for removing hydrogen sulphide.

The methods employed for the production and trapping of [$^{14}$C]carbon dioxide were essentially the same. When cells were incubated in suspensions (20 mM citrate; pH 4.5) containing $^{14}$C-labelled sugar or $^{14}$C-labelled pyruvate, the[$^{14}$C]carbon dioxide

formed was conveyed away by a gas stream and was absorbed by bubbling through 25 ml of stirred hyamine hydroxide solution (1.0 M in methanol; Koch-Light, Colnbrook, Bucks, England). This was contained in a 100 ml round-bottomed, three-necked Quickfit flask placed in an ice-water bath. Samples (0.5 ml) of hyamine hydroxide solution were periodically removed through two suba-sealed necks of the flask using syringes fitted with 150 mm needles. These samples were directly added to 7 ml aliquots of scintillation fluid (Unisolve liquid scintillator No. 1) in vials and the radioactivity determined as already described. The gas stream was subsequently passed through two saturated solutions of potassium hydroxide to remove any residual [$^{14}$C]carbon dioxide. These experiments were terminated by the injection of saturated potassium hydroxide solution (50 ml) into the incubation medium, while the gas flow was maintained. After three hours, the system was opened and washed out in a fume cupboard.

Estimation of Sulphite Reductase Activity

Sulphite reductase activity was measured by a method based upon that described by Prabnakararao and Nicholas (1969) which entails estimation of hydrogen sulphide produced. Cells (200 mg dry wt.) were harvested by filtration through a membrane filter (0.45 μ pore size; 50 mm diam., Oxoid), washed with ice-cold buffer (50 ml; 200 mM phosphate buffer; pH 7.4), refiltered, and finally resuspended in buffer (10 ml). Cells were disrupted by shaking in a Braun homogenizer (B. Braun Melsungen, West Germany) for three periods of 30 s at speed 2 (4000 rev. min$^{-1}$) after addition of 30 g glass beads (Glasperlen, B. Braun; 0.45 -

0.5 mm diam.). Glass beads were removed by filtration through a glass sinter.

Cellular debris was removed by centrifugation at 2500 g for 30 min., and the supernatant stored in an ice-water mixture. The reaction mixture contained phosphate buffer (200 mM; pH 7.4), $MgCl_2$ (1 mM), sodium sulphite (0.3 mM), $NADP^+$ (0.1 mM), glucose 6-phosphate (1.7 mM), and glucose 6-phosphate dehydrogenase (166 I.U. $l^{-1}$). Portions (3 ml) were placed into small test tubes (10 ml) which were fitted with suba-seals. Cell extract (0.1 ml - 1 ml) was injected into the tubes which were then incubated at 30°C for 40 min. Acidic p-aminodimethylanaline (0.5 ml; 5 mM $NH_2C_6H_4N(CH_3)_2$ in 3.5 M $H_2SO_4$) was injected, followed by ferric reagent (0.1 ml; 0.25 M $NH_4Fe(SO_4)_2$ in 0.5 M $H_2SO_4$). The contents of the tubes were mixed and incubated at 22°C for 15 min. The methylene blue formed was estimated by measuring the extinction at 667 nm against blanks prepared from reaction mixture lacking sulphite but treated in all other respects as the experimental samples.

Protein in the extracts was determined by the method of Lowry et al. (1951). Standard curves were prepared relating extinction at 750 nm to protein content (within the range 25 - 500 µg) using bovine serum albumin (Sigma).

Measurement of Respiration and Fermentation Rates

Rates of respiration and fermentation were measured using conventional Warburg manometric techniques. Flask constants were determined using saturated hydrazine sulphate solution (0.3 ml)

and standardized potassium iodate solution (2 ml; 2 mM). Cells were harvested from aerobic and anaerobic cultures by filtration, washed in buffer and resuspended as already described. The incubation mixture in the Warburg flasks contained glucose (10 mM), sulphite (0.35 mM) and buffer (trisodium citrate and citric acid buffer 20 mM; pH 4.5) in a volume of 2 ml. The centre well contained saturated potassium hydroxide solution (0.4 ml) or water. The side arm contained a suspension of cells in buffer (0.3 - 7 mg dry wt., 0.2 ml). Where yeast had been grown in anaerobic culture, the Warburg apparatus was flushed through with high-purity nitrogen gas. After pre-incubation at 30°C for 10 min, experiments were started by tipping the contents of the side arms into the flasks.

# Results

# RESULTS

## Hydrogen Sulphide Production

### Factors influencing occurrence of hydrogen sulphide

Strains TC8, TC16, and TC17 of Saccharomyces cerevisiae produced hydrogen sulphide in distinct peaks of activity, late in the fermentation (Figs. 5, 6, 7). Hydrogen sulphide production began suddenly at a time closely coincident with cessation of exponential yeast growth. Hydrogen sulphide production was greater by strain TC8. Saccharomyces cerevisiae NCYC 366 was found to produce hydrogen sulphide throughout the fermentation (Fig. 8) but to produce an excessive quantity of this gas upon cessation of exponential growth. The manner of hydrogen sulphide production in enriched apple juice was very similar to thatin yeast carbon-base medium in all strains examined, namely TC8, TC16, TC17, and NCYC 366. However, both the amount and rate at which hydrogen sulphide was produced was greater in enriched apple juice medium.

For all four strains, both the maximum rate and total production of hydrogen sulphide were closely dependent upon the rate of exponential growth. Factors slowing the growth rate diminished production of hydrogen sulphide both in rate and total formed. Adjustment of rate of agitation and degree of aeration were particularly effective in this respect. Anaerobically grown cultures of strain TC8 produced small amounts of hydrogen sulphide while anaerobic cultures of strains TC16 and TC17 produced none of this gas (Tables 1 and 2). Hydrogen sulphide production, in all strains, was influenced by temperature. As the temperature was lowered, the sharp peak of hydrogen sulphide production by strain TC8, gave way to a flat rate of production over many hours (Fig. 9). The rate of decay of hydrogen sulphide production was slowed such that

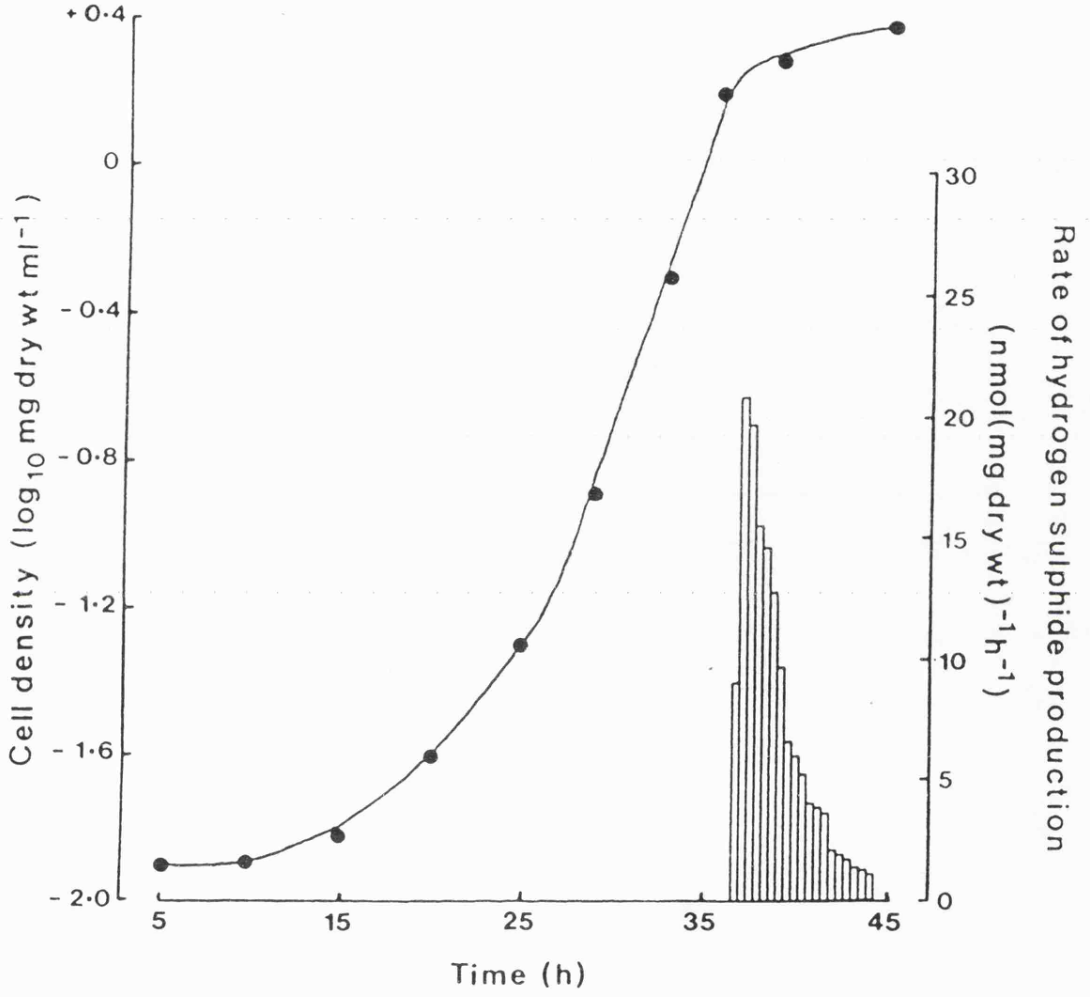



Figure 5. Hydrogen sulphide production and growth (●) of Saccharomyces cerevisiae TC8 at 30°C in yeast carbon-base medium. Hydrogen sulphide production, over periods of 30 min, is indicated by histograms.

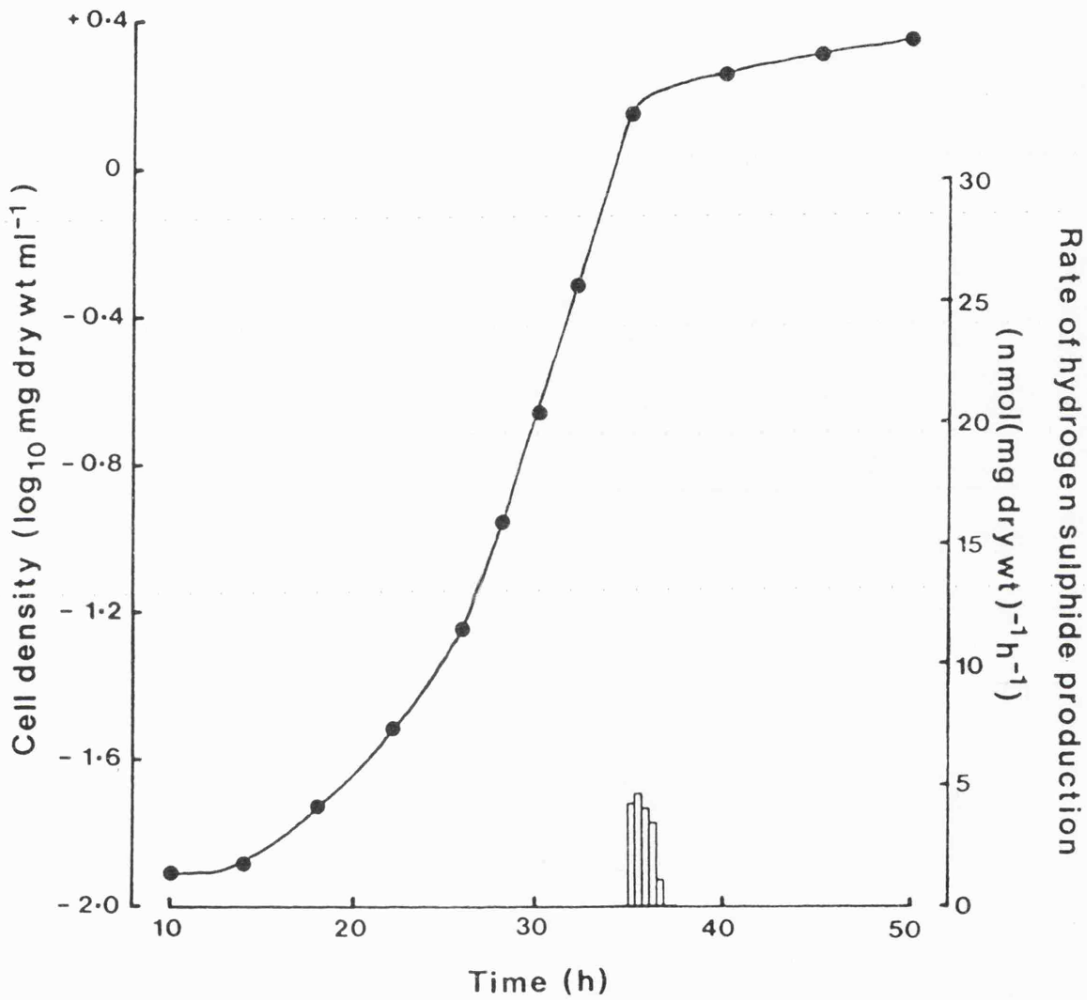



Figure 6. Hydrogen sulphide production and growth (●) of Saccharomyces cerevisiae TC16 at 30°C in yeast carbon-base medium. Hydrogen sulphide production over periods of 30 min, is indicated by histograms.

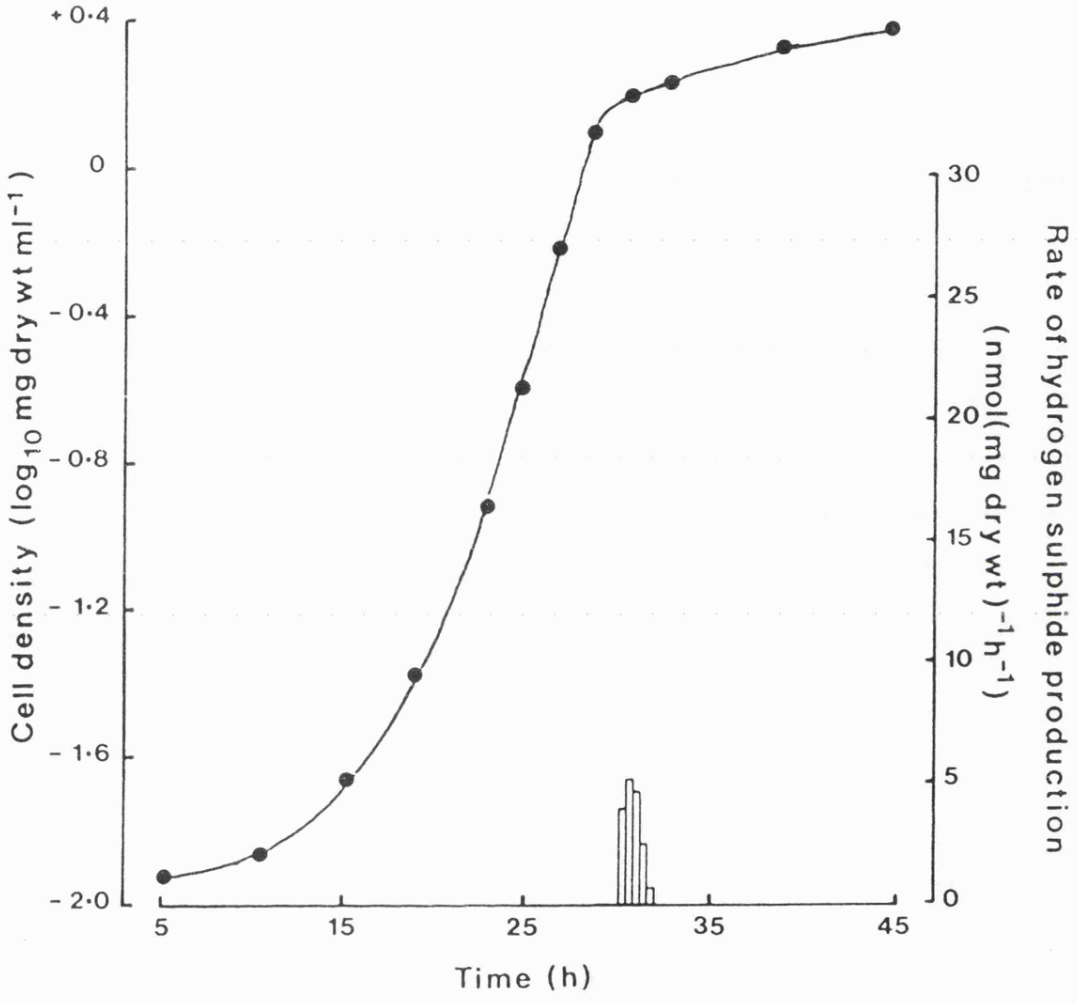



Figure 7. Hydrogen sulphide production and growth (●) of Saccharomyces cerevisiae TC17 at 30°C in yeast carbon-base medium. Hydrogen sulphide production over periods of 30 min is indicated by histograms.

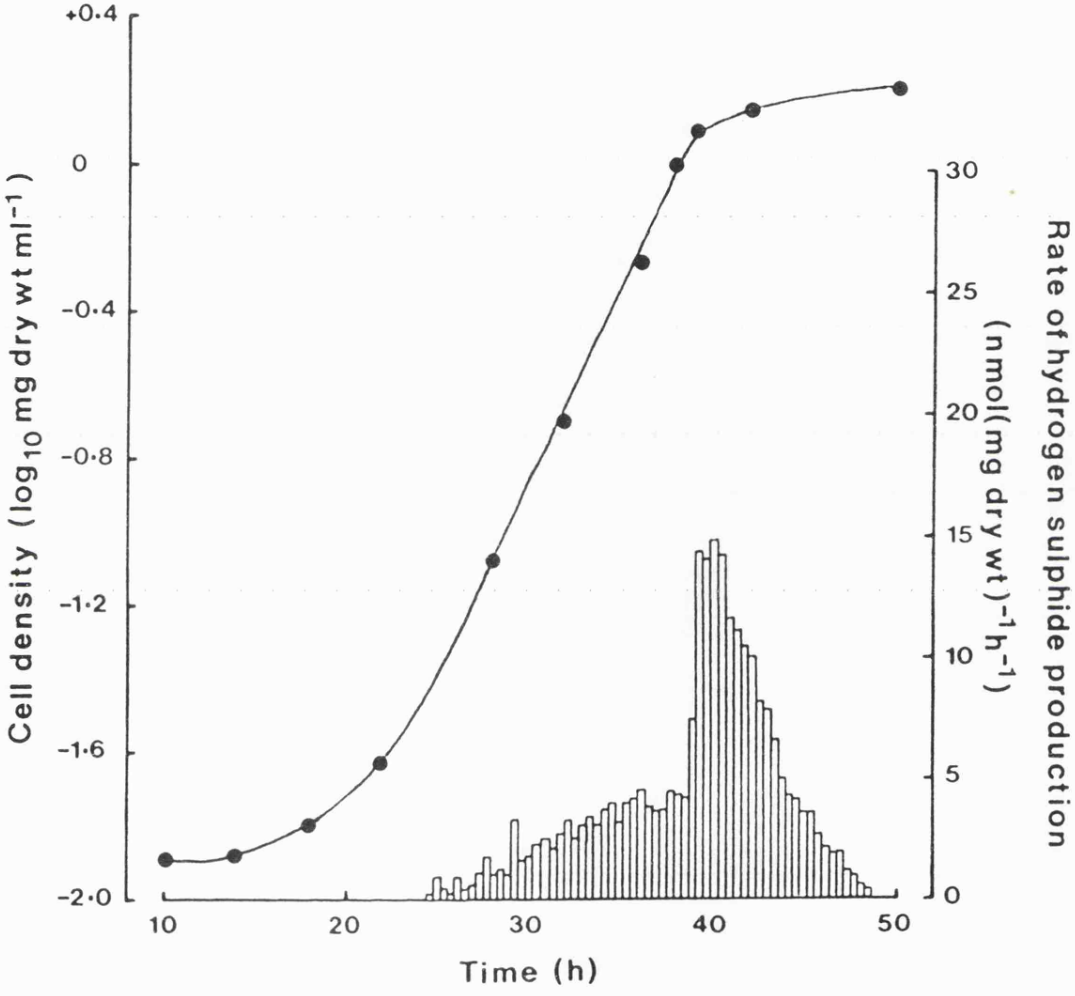



Figure 8. Hydrogen sulphide production and growth (●) of Saccharomyces cerevisiae NCYC 366 at 30°C in yeast carbon-base medium. Hydrogen sulphide production, over periods of 30 min, is indicated by histograms.

Table 1. Total hydrogen sulphide production, by strains of Saccharomyces cerevisiae, grown in enriched apple juice at 25°C. Values quoted are the average of at least 5 independent determinations ± S.E.M.

| | Total hydrogen sulphide production ($\mu mol\ L^{-1}$) | | |
|---|---|---|---|
| Yeast Strain | TC8 | TC16 | TC17 |
| Aerobic | 260 ± 16 | 11 ± 2.5 | 15 ± 3.1 |
| Anaerobic | 25 ± 4.9 | 0 | 0 |

Table 2. The maximum rate of hydrogen sulphide production by strains of Saccharomyces cerevisiae, grown in enriched apple juice at 25°C. Values quoted are the average of at least 5 independent determinations ± S.E.M.

| | Maximum rate of hydrogen sulphide production (nmol (mg dry wt)$^{-1}$h$^{-1}$) | | |
|---|---|---|---|
| Yeast Strain | TC8 | TC16 | TC17 |
| Aerobic | 33 ± 5.1 | 3.9 ± 1.0 | 4.7 ± 0.7 |
| Anaerobic | 5 ± 0.8 | 0 | 0 |

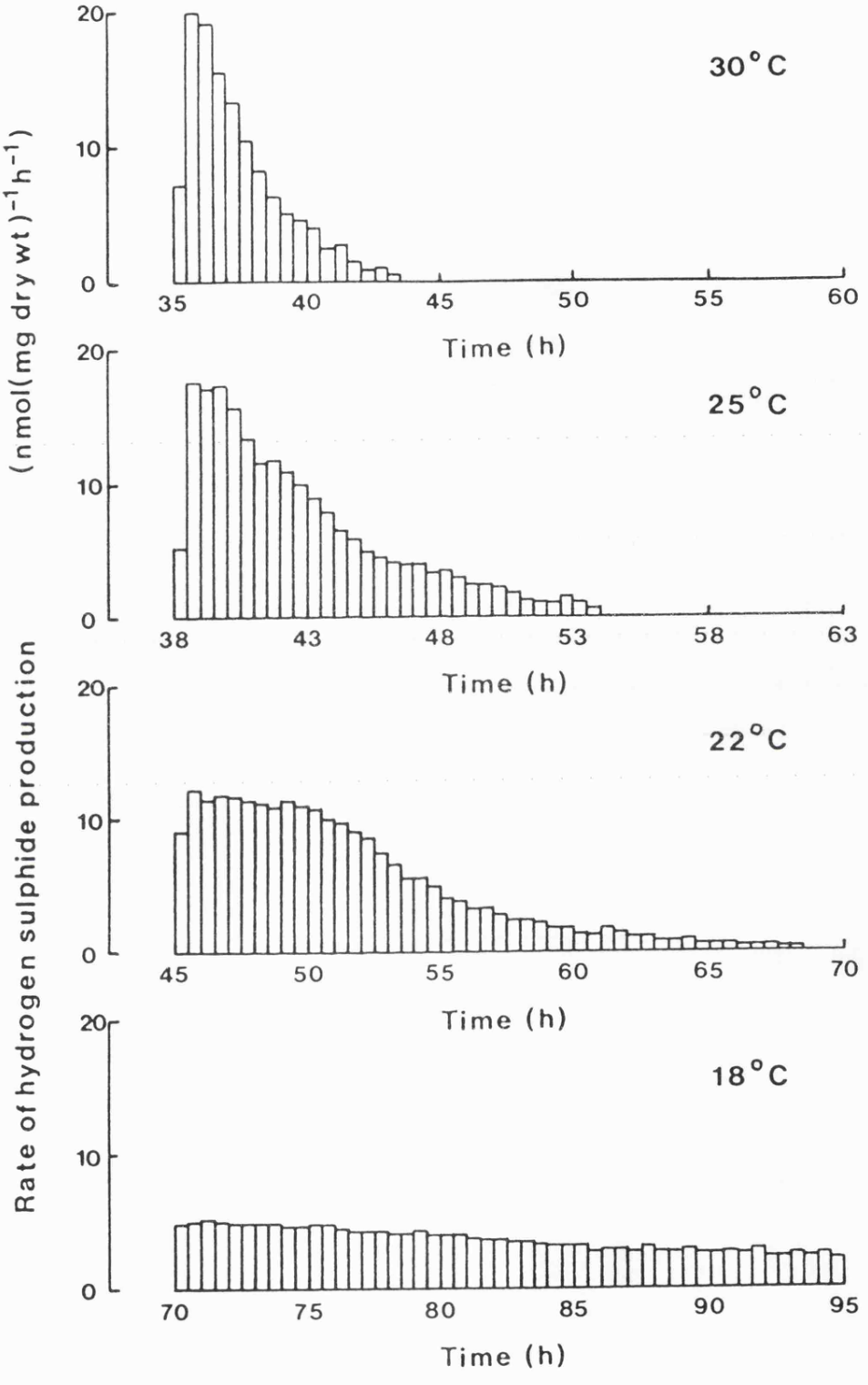



Figure 9. The effect of temperature upon the rate of production of hydrogen sulphide by Saccharomyces cerevisiae TC8 grown in yeast carbon-base medium.

the half-life of 2.5 hours at 30°C was prolonged to approximately 25 hours at 18°C.

Neither growth of cultures of any of the strains nor the production of hydrogen sulphide was influenced by supplementing the medium with pantothenic acid (1 mg $l^{-1}$), pyridoxine (1 mg $l^{-1}$), or thiamin (1 mg $l^{-1}$). Addition of methionine (0.2 mM), however, resulted in an amelioration of hydrogen sulphide production, to about 50% of its usual rate of production.

Origin of the sulphur evolved as hydrogen sulphide

The total amount of sulphur evolved as hydrogen sulphide by strain TC8 in enriched apple juice was found to be close to 0.25 nmol $l^{-1}$. Only sulphate and sulphite were present in the medium, in sufficient concentration to accommodate this. Medium lacking sulphate but containing sulphite supported normal growth of strains TC8, TC16 and TC17 and the rates of production and amounts of hydrogen sulphide produced were unchanged (Fig. 10). In contrast, from medium containing sulphate but lacking sulphite, no hydrogen sulphide was formed by strain TC8 (Fig. 11) and only traces of this gas were formed by strains TC16 and TC17. Cells of strain TC8 grown in media containing successively greater concentrations of sulphite formed correspondingly increased amounts of hydrogen sulphide (Figs. 12, 13, 14). Similar results were obtained using apple juice medium (Tables 3 and 4).

Figure 15 shows that the sulphate concentration was not found to change appreciably during growth of strain TC8. Free sulphite was not detected in the medium after 25 hours when growth was entering

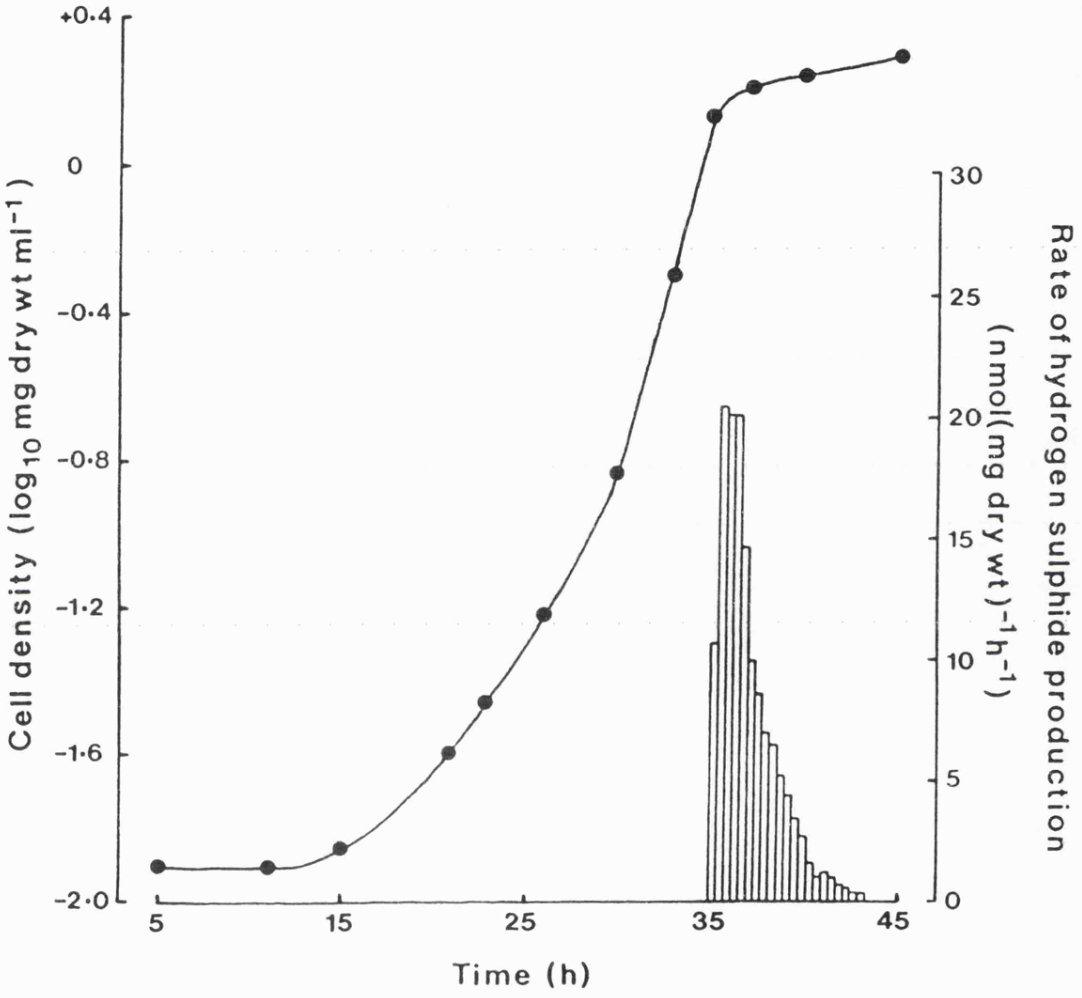



Figure 10. Hydrogen sulphide production and growth (●) of Saccharomyces cerevisiae TC8 in yeast carbon-base medium lacking sulphate at 30°C. Hydrogen sulphide production, over periods of 30 min, is indicated by histograms.

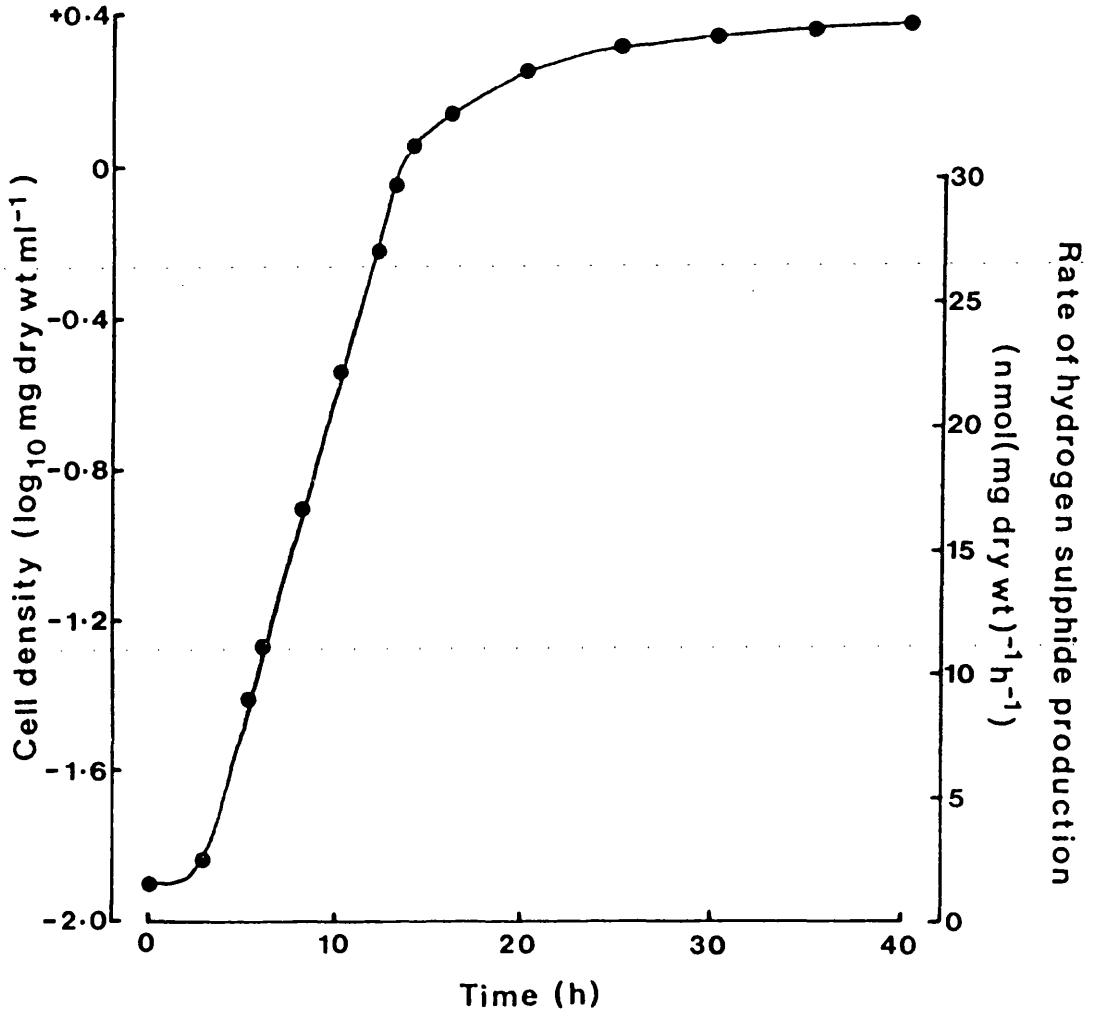



Figure 11. Hydrogen sulphide production and growth (●) of Saccharomyces cerevisiae TC8 in yeast carbon-base medium lacking sulphite at 30°C. Hydrogen sulphide production, over periods of 30 min, is indicated by histograms.

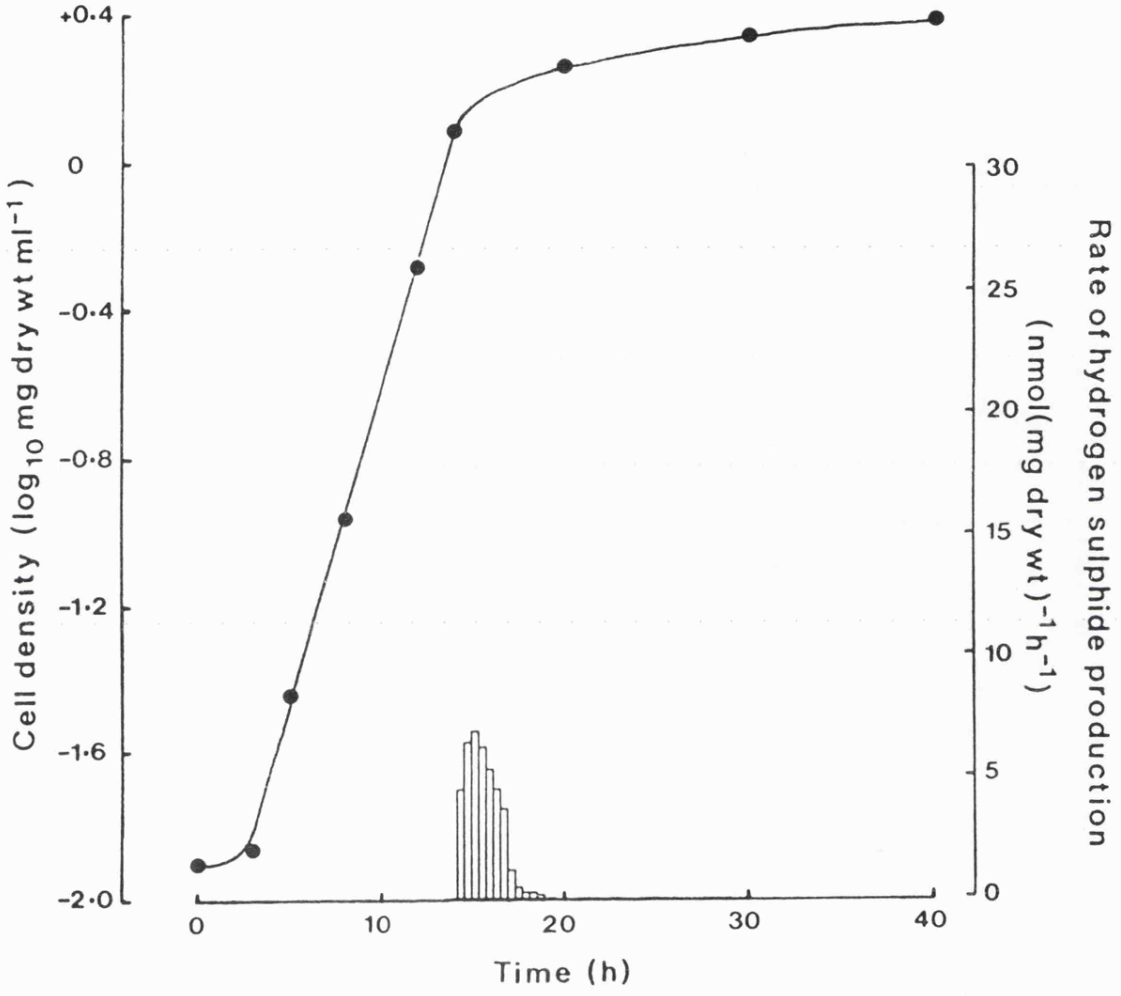



Figure 12. Hydrogen sulphide production and growth (●) of Saccharomyces cerevisiae TC8 at 30°C in yeast carbon base medium containing sulphite (0.37 mM). Hydrogen sulphide production, over periods of 30 min, is indicated by histograms.

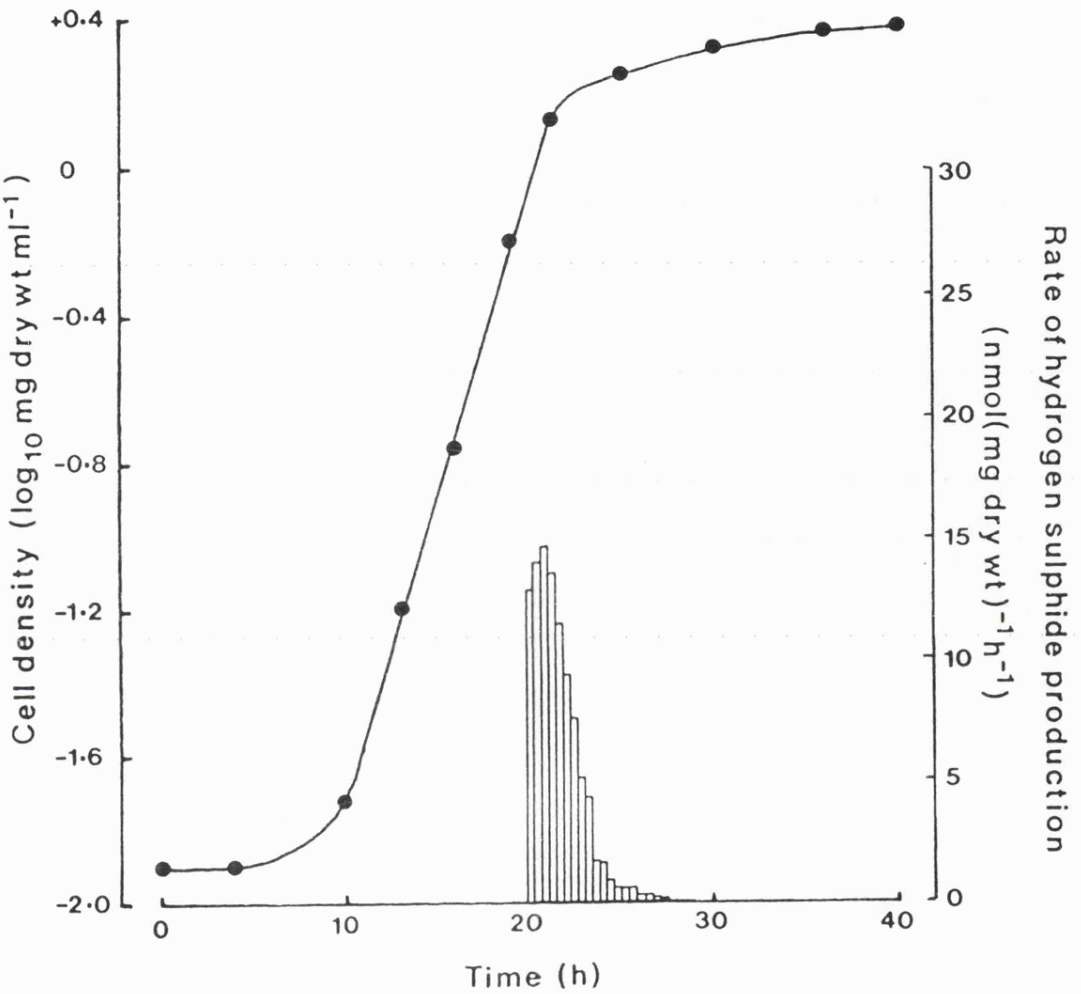



Figure 13. Hydrogen sulphide production and growth (●) of Saccharomyces cerevisiae TC8 at 30°C in yeast carbon-base medium containing sulphite (0.75 mM). Hydrogen sulphide production, over periods of 30 min, is indicated by histograms.

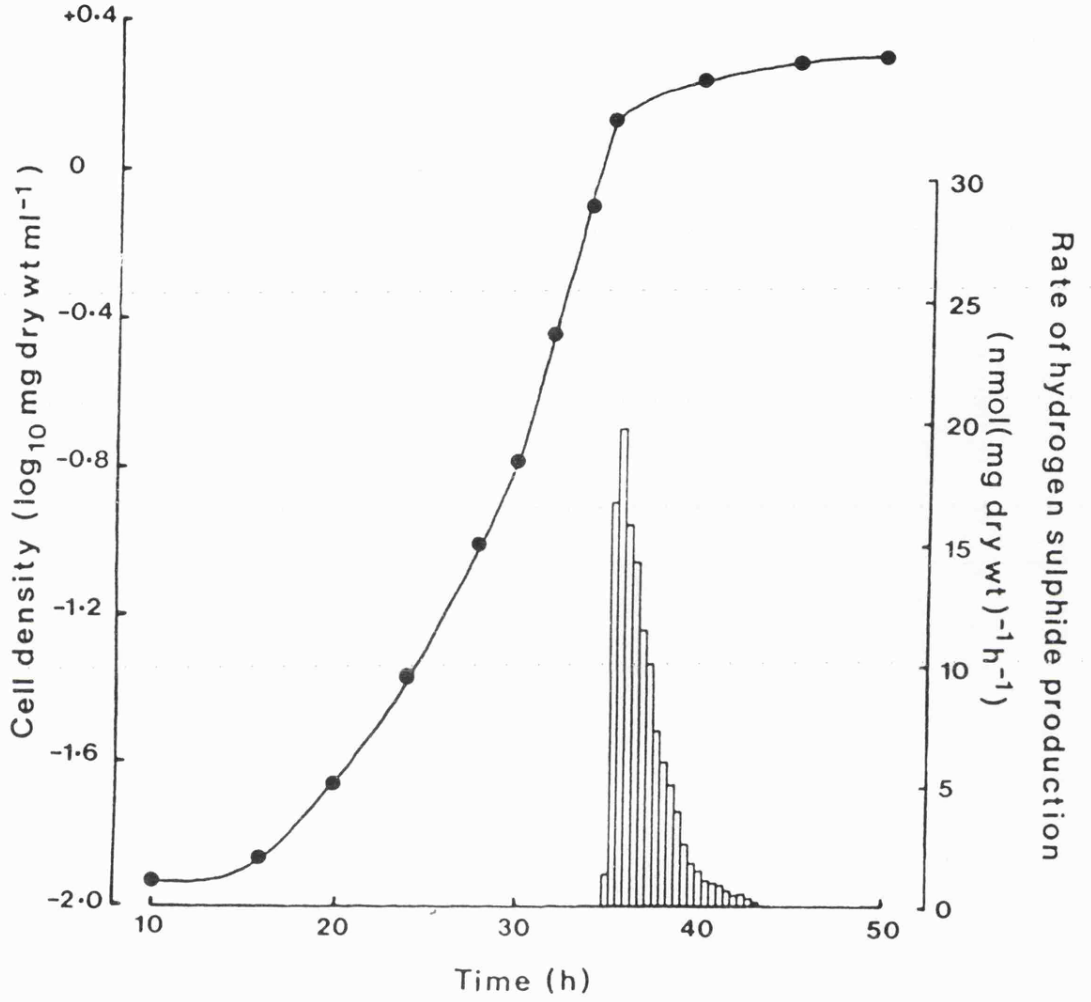



Figure 14. Hydrogen sulphide production and growth (●) of Saccharomyces cerevisiae TC8 at 30°C in yeast carbon-base medium containing sulphite (1.5 mM). Hydrogen sulphide production, over periods of 30 min, is indicated by histograms.

Table 3. The effect of sulphate and sulphite concentrations upon the total hydrogen sulphide production by strains of Saccharomyces cerevisiae grown at 25°C in enriched apple juice. Values quoted are the average of at least 5 independent determinations ± S.E.M.

| | Yeast strain | TC8 | TC16 | TC17 |
|---|---|---|---|---|
| Sulphate Conc. (mM) | Sulphite conc. (mM) | Total hydrogen sulphide production (μmol $L^{-1}$) | | |
| 6.5 | 1.5 | 260 ± 16 | 11 ± 2.5 | 15 ± 3.1 |
| 0.7 | 1.5 | 265 ± 21 | 10 ± 1.5 | 13 ± 2.8 |
| 6.5 | 0 | 0 | 0.5 ± 0.3 | 0.5 ± 0.4 |
| 0.7 | 0 | 0 | 0.4 ± 0.2 | 0.5 ± 0.3 |

Table 4. The effect of sulphate and sulphite concentrations upon the maximum rates of production of hydrogen sulphide by strains of Saccharomyces cerevisiae grown at 25°C in enriched apple juice. Values quoted are the average of at least 5 independent determinations ± S.E.M.

| Yeast Strain | | TC8 | TC16 | TC17 |
|---|---|---|---|---|
| Sulphate conc. (mM) | Sulphite conc. (mM) | Maximum rate of hydrogen sulphide production (nmol (mg dry wt)$^{-1}h^{-1}$) | | |
| 6.5 | 1.5 | 33 ± 5.1 | 3.9 ± 1.0 | 4.7 ± 0.7 |
| 0.7 | 1.5 | 30 ± 5.7 | 3.4 ± 0.6 | 4.4 ± 1.1 |
| 6.5 | 0 | 0 | 0.1 ± 0.06 | 0.09± 0.06 |
| 0.7 | 0 | 0 | 0.1 ± 0.04 | 0.11± 0.05 |

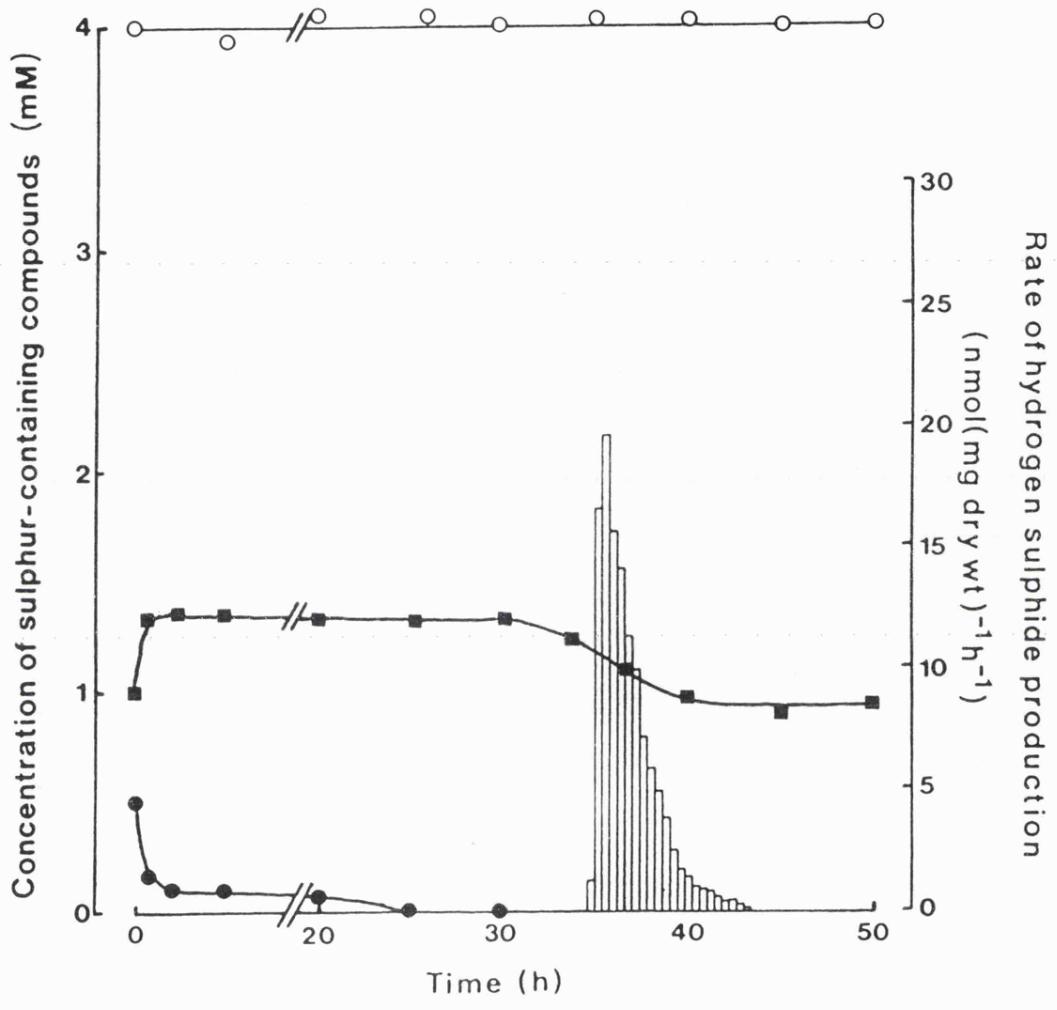



Figure 15. Changes in concentration of sulphate (O), free sulphite (●) and bound sulphite (■) during the growth of Saccharomyces cerevisiae TC8 in yeast carbon-base medium at 30°C. Histograms indicate hydrogen sulphide production.

exponential phase. Bound sulphite concentration declined during exponential growth and during production of hydrogen sulphide. Strains TC16 and TC17 produced minor quantities of bound sulphite (0.2 mM) during exponential growth.

The origin of the sulphur evolved as hydrogen sulphide was confirmed for strain TC8 using $^{35}SO_4^{2-}$ and $^{35}SO_3^{2-}$ Radioactive $H_2{}^{35}S$ from [$^{35}$S]sulphite (Fig. 16) was detected within 11 minutes following introduction of the isotope whilst radioactive hydrogen sulphide was not detectable when [$^{35}$S]sulphate was introduced. Production of radioactive $H_2{}^{35}S$ by strain TC8 from [$^{35}$S]sulphite was preceded by accumulation of label (Fig. 17) by cells. This accumulation continued steadily for up to 2.5 hours after introduction of the isotope. In contrast, label from radioactive sulphate did not penetrate cells under these conditions. Figure 18 shows that a substantial proportion of the radioactive bound sulphite disappeared from the medium while the concentration of radioactive sulphate remained largely unchanged. During the course of the experiments described in Figures 16, 17 and 18, yeast growth and hydrogen sulphide production were similar to that shown in Figure 5.

Permeation of bound sulphite into yeast

An examination was carried out, using strain TC8, upon entry of pyruvate and sulphite from a complex into cells, using [$^{14}$C]pyruvate and [$^{35}$S]sulphite. From the complex (1.5 mM), label from [$^{35}$S] sulphite was accumulated at a rate of 1 nmol (mg dry wt)$^{-1}$ min$^{-1}$, while label from [$^{14}$C]pyruvate was accumulated at about 0.3 nmol (mg dry wt)$^{-1}$ min$^{-1}$ (Fig. 19). Entry of the [$^{14}$C]label was largely abolished by pre-treatment of the cells with 2-deoxy-D-glucose and antimycin A (Fig. 20) while entry of the [$^{35}$S]label was unaffected. The rate of [$^{35}$S]

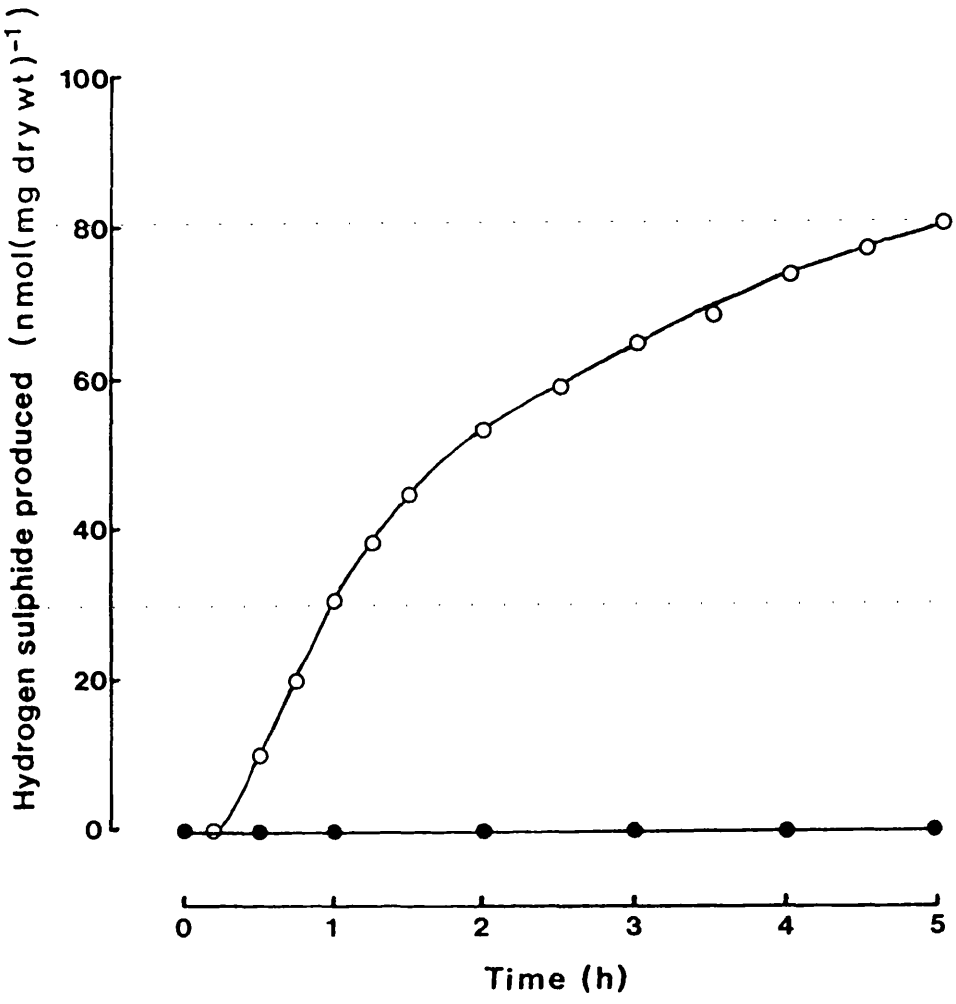



Figure 16. Production of hydrogen sulphide from radioactive sulphite (1.5 mM, O) or sulphate (4 mM, ●) by Saccharomyces cerevisiae TC8, in one-litre portions of yeast carbon-base medium at 30°C. Labelled compounds, [$^{35}$S]sulphite (8.1 µCi) or [$^{35}$S]sulphate (8.2 µCi), were added to 36h-cultures (zero time on abscissa) after the onset of hydrogen sulphide production. Hydrogen sulphide production and growth are as shown in Figure 5.

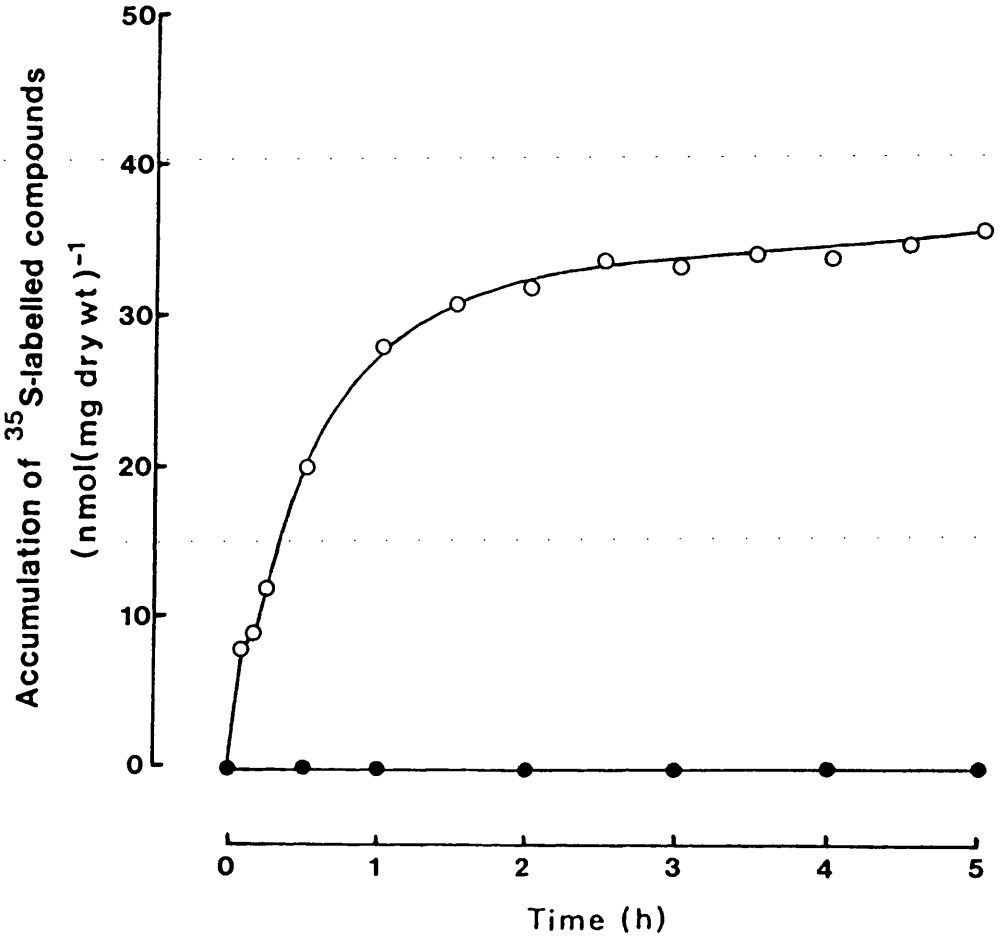



Figure 17. Accumulation of [$^{35}$S]label from radioactive sulphite (1.5 mM, O) or sulphate (4 mM, ● ) by Saccharomyces cerevisiae TC8 in one-litre portions of yeast carbon-base medium, at 30°C. Labelled compounds, [$^{35}$S]sulphite (8.1 µCi) or [$^{35}$S]sulphate (8.2 µCi), were added to 36h-cultures (zero time on abscissa) after the onset of hydrogen sulphide production. Hydrogen sulphide production and growth are as shown in Figure 5.

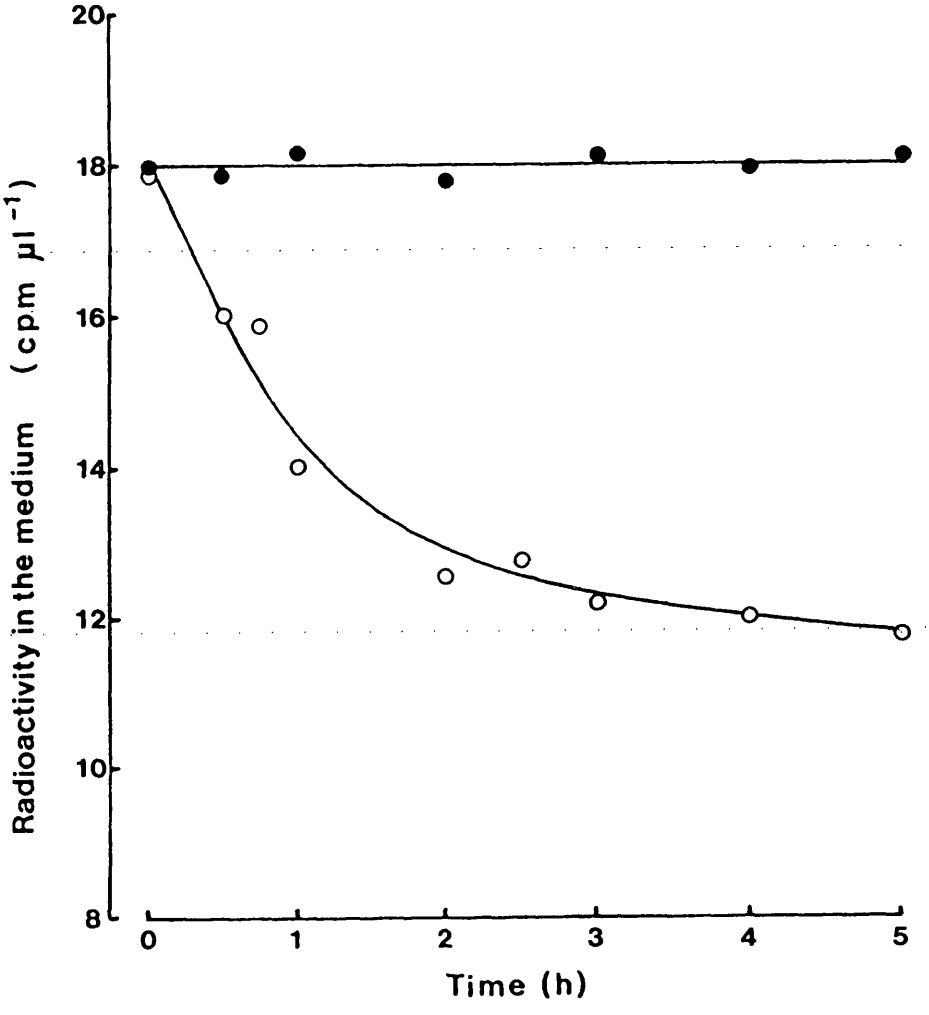



Figure 18. Disappearance of radioactive sulphite (1.5 mM, ○) or sulphate (4 mM, ●) from one-litre portions of yeast carbon-base medium at 30°C during production of hydrogen sulphide by Saccharomyces cerevisiae TC8. Labelled compounds, [$^{35}$S]sulphite (8.1 µCi) or [$^{35}$S]sulphate (8.2 µCi), were added to 36h cultures (zero time on abscissa) after the onset of hydrogen sulphide production.

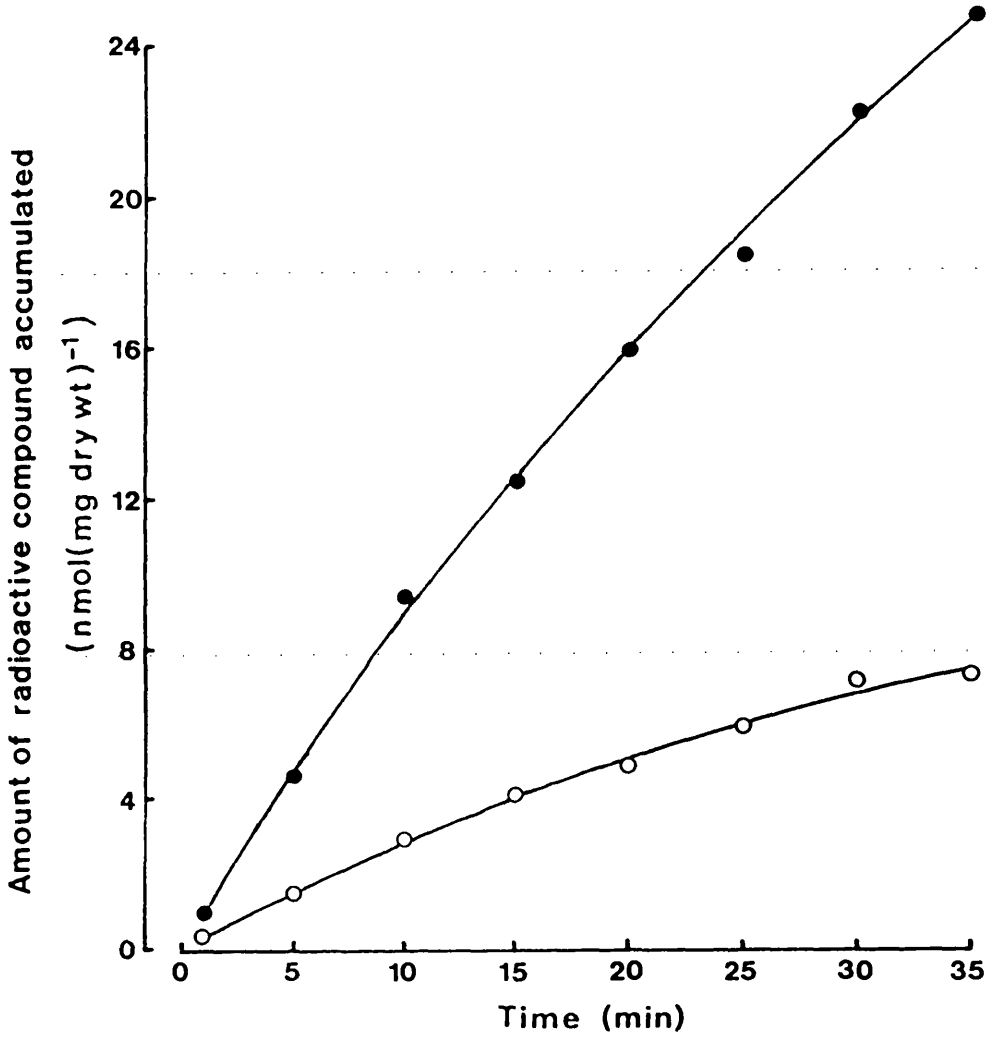



Figure 19. Accumulation of label, [$^{35}$S] (●) or [$^{14}$C] (○), by a suspension of Saccharomyces cerevisiae TC8 in citrate buffer (20 mM, pH 4.0) containing glucose (100 mM) at 30°C, from a radioactive sulphite-pyruvate complex (1.5 mM) containing [1-$^{14}$C]pyruvate (66 mCi mol$^{-1}$) or [$^{35}$S]sulphite (66 mCi mol$^{-1}$).

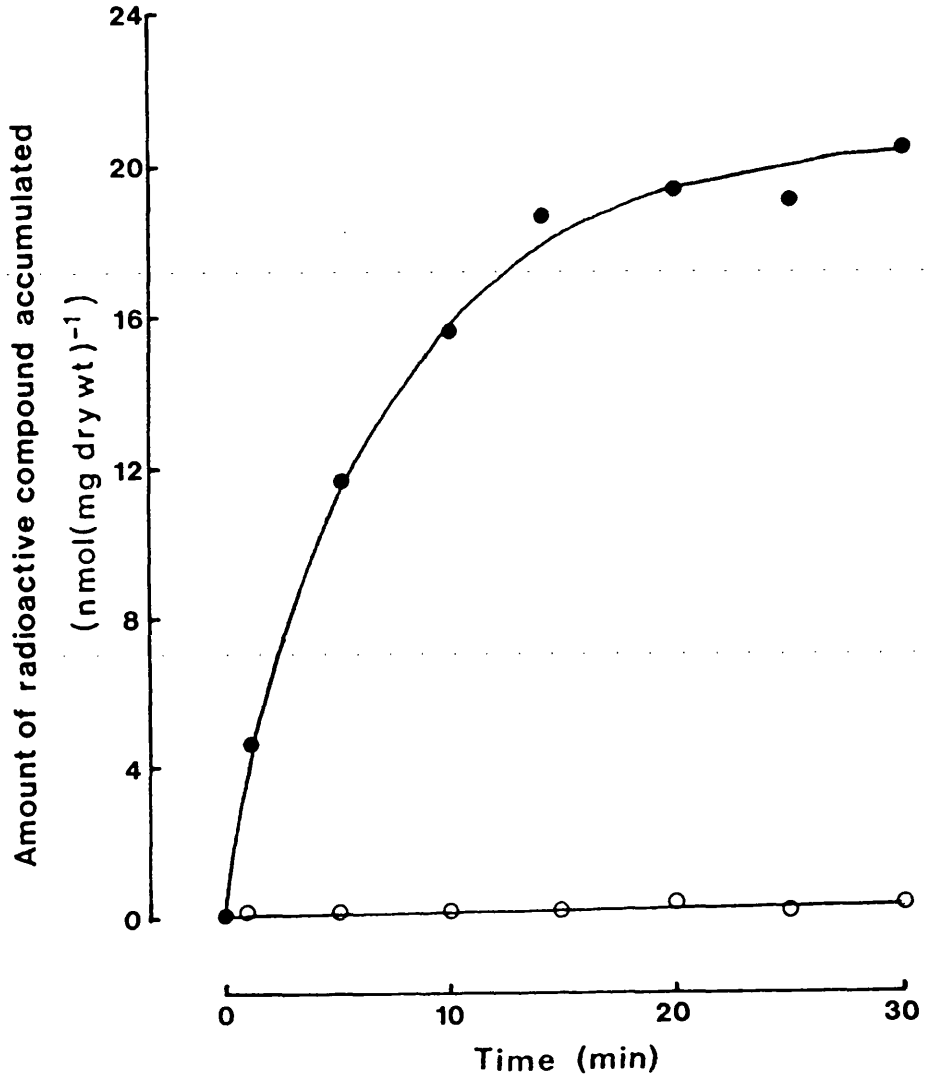



Figure 20. Accumulation of label, [$^{35}$S] (●) or [$^{14}$C] (○) by a suspension of Saccharomyces cerevisiae TC8 in citrate buffer (20 mM), containing glucose (100 mM) at 30°C, from a radioactive sulphite-pyruvate complex (1.5 mM) containing [1-$^{14}$C]pyruvate (66 mCi mol$^{-1}$ or [$^{35}$S]sulphite (66 mCi mol$^{-1}$). Cells were suspended in citrate buffer (20 mM, pH 4.0) containing 2-deoxy-D-glucose (10 mM) and antimycin A (50 mg L$^{-1}$) 30 minutes before the experiment was started. The pH value of the buffer was 4.0.

accumulation was lowered by raising the pH value (Fig. 21). Rates of accumulation (Fig. 22) and production of $CO_2$ (Fig. 23) from pyruvate (1 mM) showed that these rates were only slightly different in the presence or absence of sulphite (0.95 mM). Introduction of 2,4-dinitrophenol, during measurement of accumulation rates increased the rate of accumulation of [$^{35}S$] label from sulphite-pyruvate complexes (1.5 mM) while abolishing further accumulation of [$^{14}C$] label. (Fig. 24).

The rates of hydrogen sulphide production, by strain TC8, from sulphite bound by a variety of carbonyl compounds at several concentrations (Table 5) were very similar. Only when the sulphite concentration was lowered (Table 6) was there any appreciable effect upon the rate of hydrogen sulphide formation.

Causes of hydrogen sulphide production

The ammonium ion concentration in the medium was depleted by the yeast (Strains TC8 and TC16) at a time coincident with production of hydrogen sulphide and cessation of exponential yeast growth (Figs. 25 and 26). This was also found to be true for strains TC17 and NCYC 366. In enriched apple juice medium, this point of hydrogen sulphide formation was found to coincide with depletion of α-aminonitrogen. Onset of hydrogen sulphide production did not coincide with depletion from the medium of total sugars, sulphate, sulphite or total nitrogenous compounds in cultures of the strains examined. If additional ammonium ions were injected into fermentations of strain TC8 after the onset of hydrogen sulphide production, yeast growth was resumed and within a short time hydrogen sulphide production ceased (Fig. 27). However, within a moderately short time, ammonium ions were again

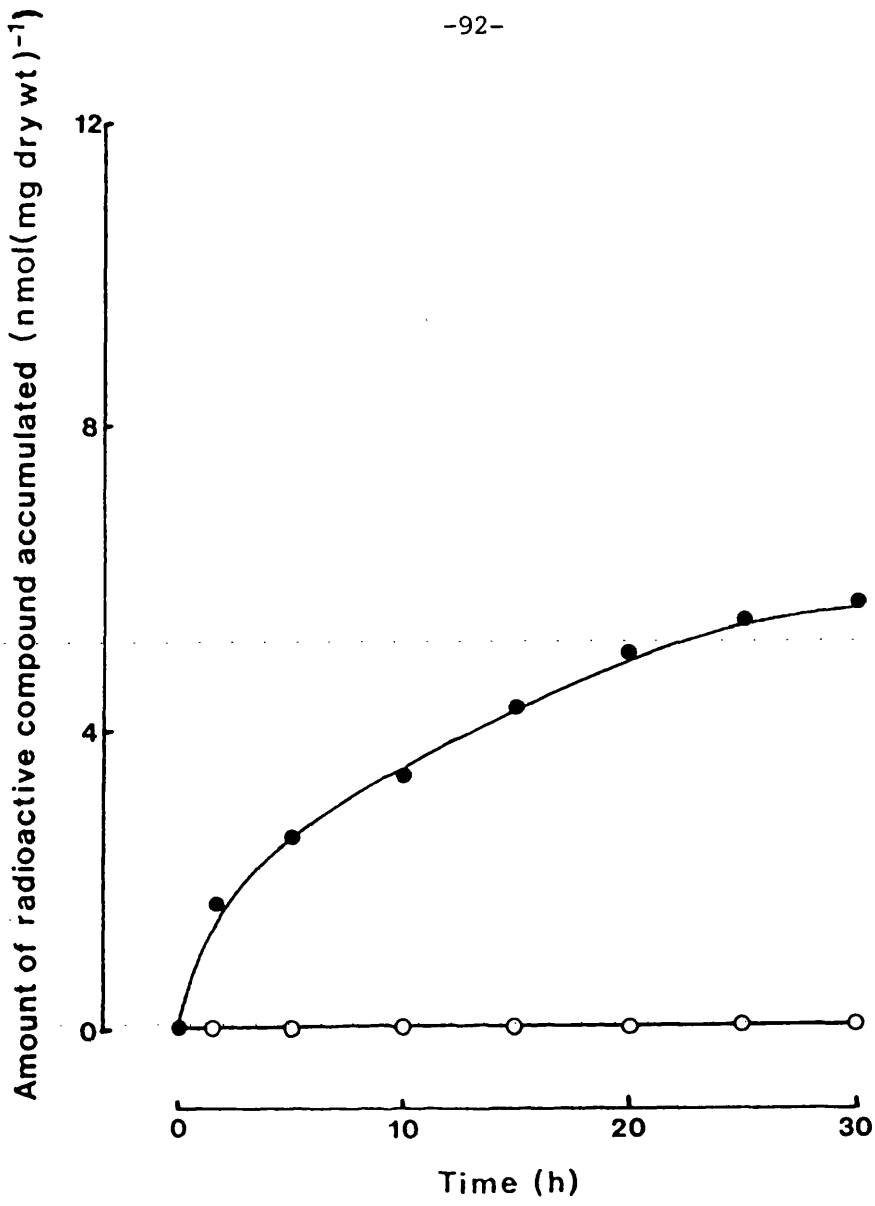



Figure 21. Accumulation of label, [$^{35}$S] (●) or [$^{14}$C] (○), by a suspension of Saccharomyces cerevisiae TC8 in citrate buffer (20 mM) containing glucose (100 mM) at 30°C, from a radioactive sulphite-pyruvate complex (1.5 mM) containing [1-$^{14}$C]pyruvate (66 mCi mol$^{-1}$) or [$^{35}$S]sulphite (66 mCi mol$^{-1}$). Cells were suspended in citrate buffer (20 mM, pH 6.0) containing 2-deoxy-D-glucose (10 mM) and antimycin A (50 mg L$^{-1}$), 30 minutes before the experiment was started. The pH value of the buffer was 6.0.

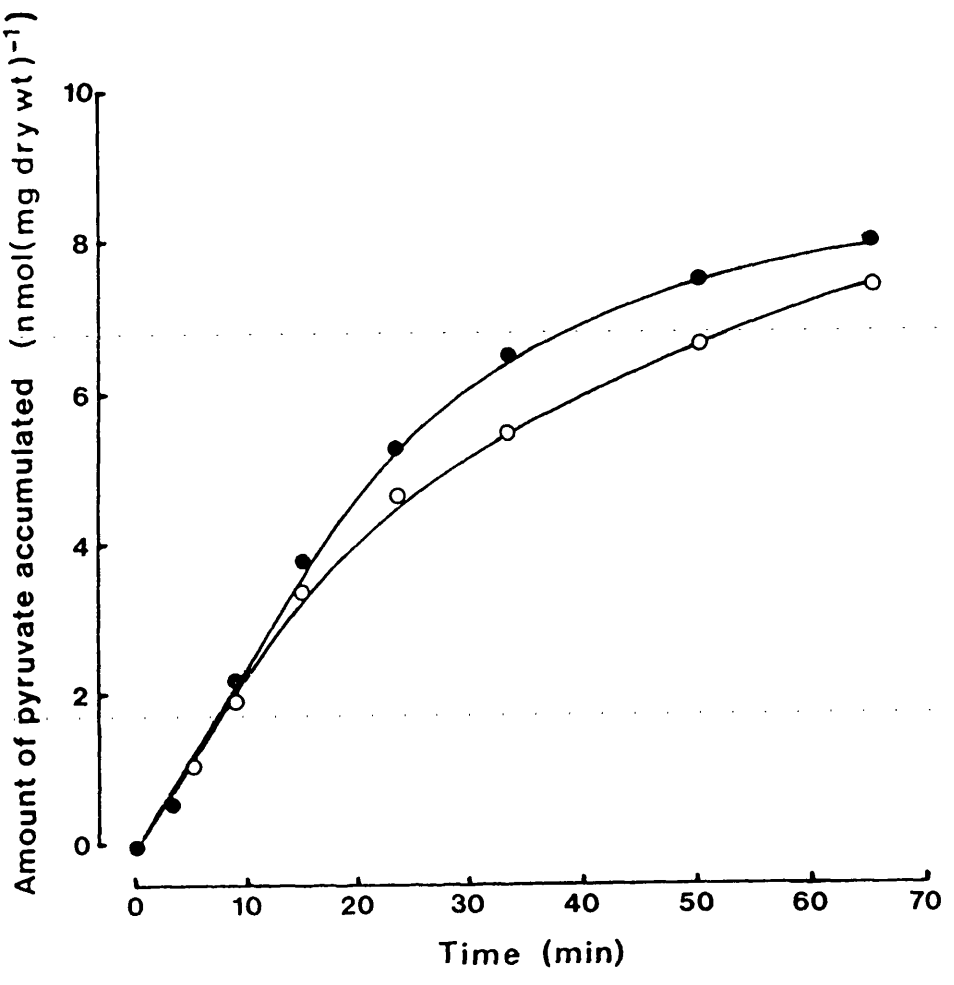



Figure 22. Accumulation of pyruvate (1 mM) labelled with [1-$^{14}$C]pyruvate (50 mCi mol$^{-1}$), in the presence (○) and absence (●) of sulphite (0.95 mM) by a suspension of Saccharomyces cerevisiae TC8 in citrate buffer (20 mM, pH 4.0) containing glucose (100 mM) at 30°C.

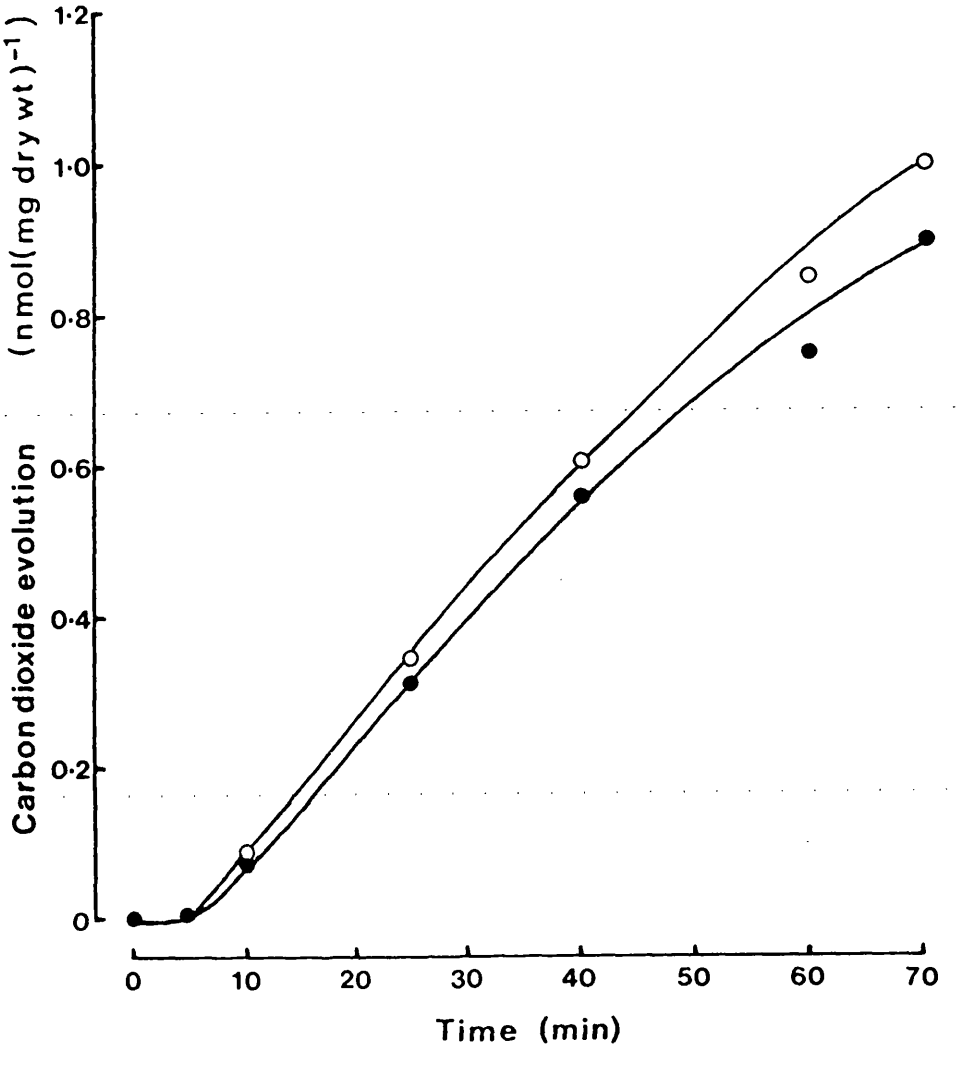



Figure 23. Production of carbon dioxide from pyruvate (1 mM) labelled with [1-$^{14}$C]pyruvate (50 mCi mol$^{-1}$), in the presence (O) and absence (●) of sulphite (0.95 mM) by a suspension of Saccharomyces cerevisiae TC8 in citrate buffer (20 mM, pH 4.0) containing glucose (100 mM) at 30°C.

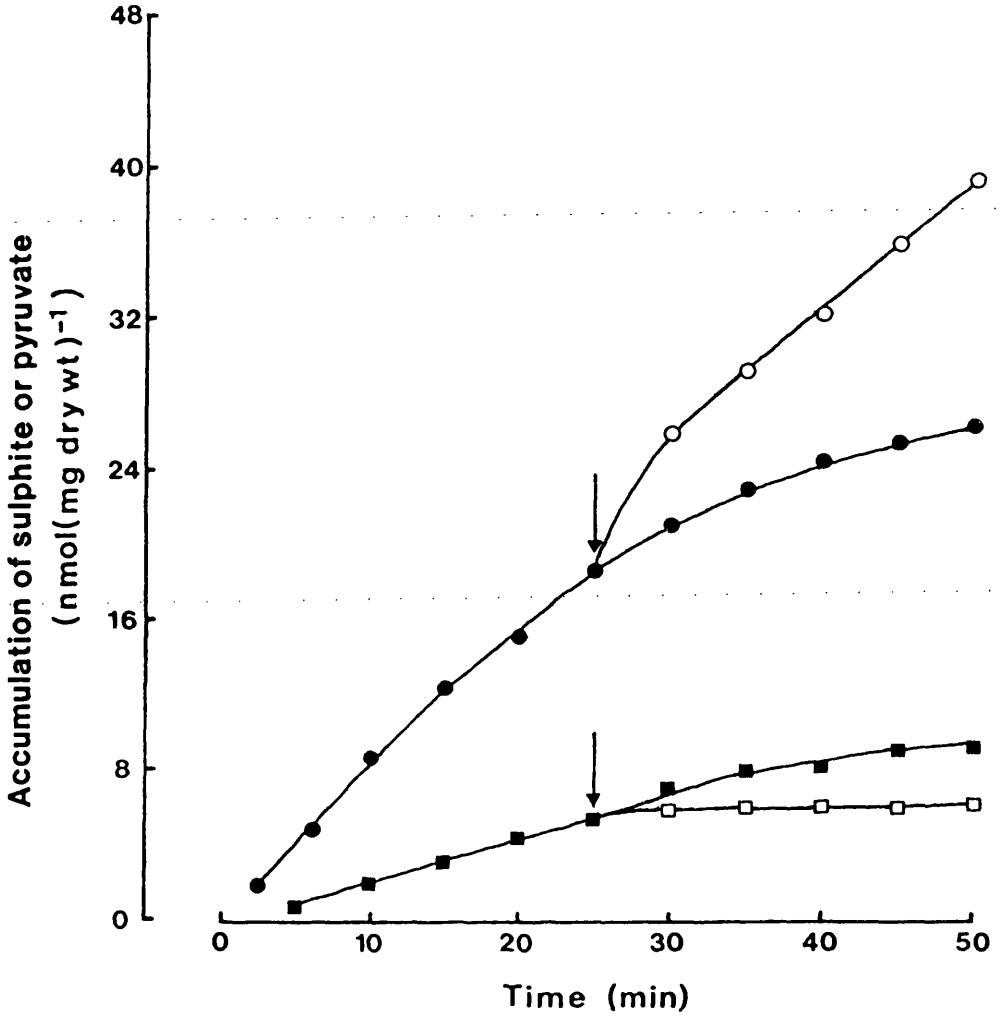



Figure 24. Effect of 2,4-dinitrophenol (0.5 mM, open symbols) upon accumulation of label, [$^{14}$C] (■) or [$^{35}$S] (●), by a suspension of Saccharomyces cerevisiae TC8 in citrate buffer (20 mM, pH 4.0) containing glucose (100 mM) at 30°C, from a radioactive sulphite-pyruvate complex (1.5 mM) containing [1-$^{14}$C]pyruvate (66 mCi mol$^{-1}$) or [$^{35}$S]sulphite (66 mCi mol$^{-1}$). 2,4-Dinitrophenol was added after 25 min incubation.

Table 5. Maximum rates of hydrogen sulphide production by Saccharomyces cerevisiae TC8 grown in yeast carbon-base medium at 30°C and pH 4.0. Cells were harvested at 33 hours, washed and resuspended in 20 mM fructose buffered to pH 4.0 at 30°C containing unless otherwise stated sulphite (1.5 mM) and various sulphite-binding compounds. Values quoted are the average of at least three independent determinations.

| Treatment of cultures and sulphite-binding agents employed | Maximum Rate of hydrogen sulphide production (nmol(mg dry wt)$^{-1}$ h$^{-1}$) |
|---|---|
| Control - yeast absent | 0 |
| Control - sulphite absent | 0 |
| No sulphite-binding agent | 18 |
| Glucose 10 mM | 19.5 |
| Glucose 100 mM | 18.6 |
| Pyruvate 0.51 mM | 17.9 |
| Pyruvate 5.1 mM | 20.7 |
| Pyruvate 0.51 mM + 2,4-dinitrophenol (0.5 mM) | 19.5 |
| Pyruvate 5.1 mM + 2,4-dinitrophenol (0.5 mM) | 21.2 |
| Acetaldehyde 0.125 mM | 20.1 |
| Acetaldehyde 1.25 mM | 22.3 |
| Acetaldehyde 6.1 mM | 17.6 |

Table 6. Maximum rates of hydrogen sulphide production by Saccharomyces cerevisiae TC8 grown in yeast carbon-base medium at 30°C and pH 4.0. Cells were harvested at 32.5 hours, washed and resuspended in 20 mM fructose buffered to pH 4.0 at 30°C and containing various concentrations of acetaldehyde and sulphite. Values quoted are the average of at least two independent determinations.

| Acetaldehyde Concentration (mM) | Sulphite Concentration (mM) | Maximum rate of hydrogen sulphide production (nmol (mg dry $wt^{-1}$)$h^{-1}$) |
|---|---|---|
| 1.0 | 0.2 | 2.9 |
| 1.0 | 0.4 | 14.6 |
| 1.0 | 0.75 | 25.1 |
| 1.0 | 1.0 | 25.8 |
| 1.0 | 1.5 | 25.4 |
| 1.0 | 2.0 | 26.3 |
| 0.1 | 0.75 | 25.1 |
| 0.4 | 0.75 | 25.2 |
| 0.75 | 0.75 | 26.5 |
| 1.0 | 0.75 | 26.2 |
| 1.0 | 0.75 | 25.1 |
| 2.0 | 0.75 | 25.1 |
| 3.0 | 0.75 | 23.9 |

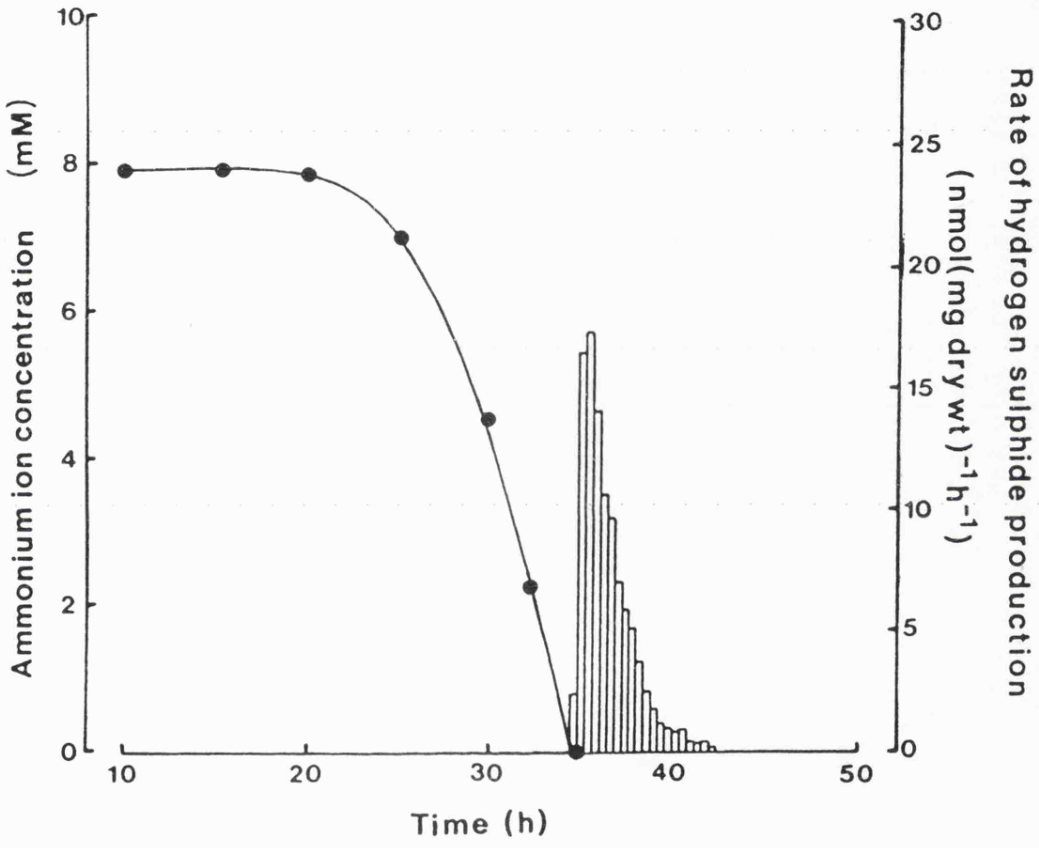



Figure 25. Ammonium ion concentration (●) and hydrogen sulphide production (histograms) by Saccharomyces cerevisiae TC8 in yeast carbon-base medium at 30°C.

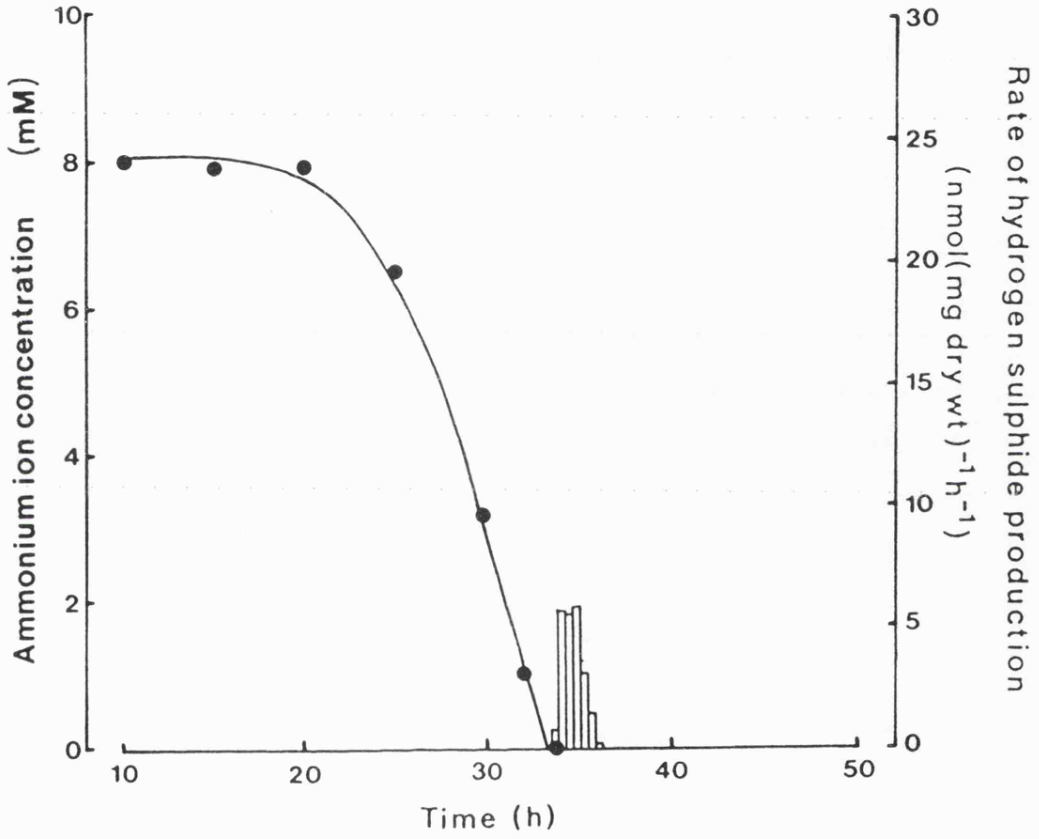
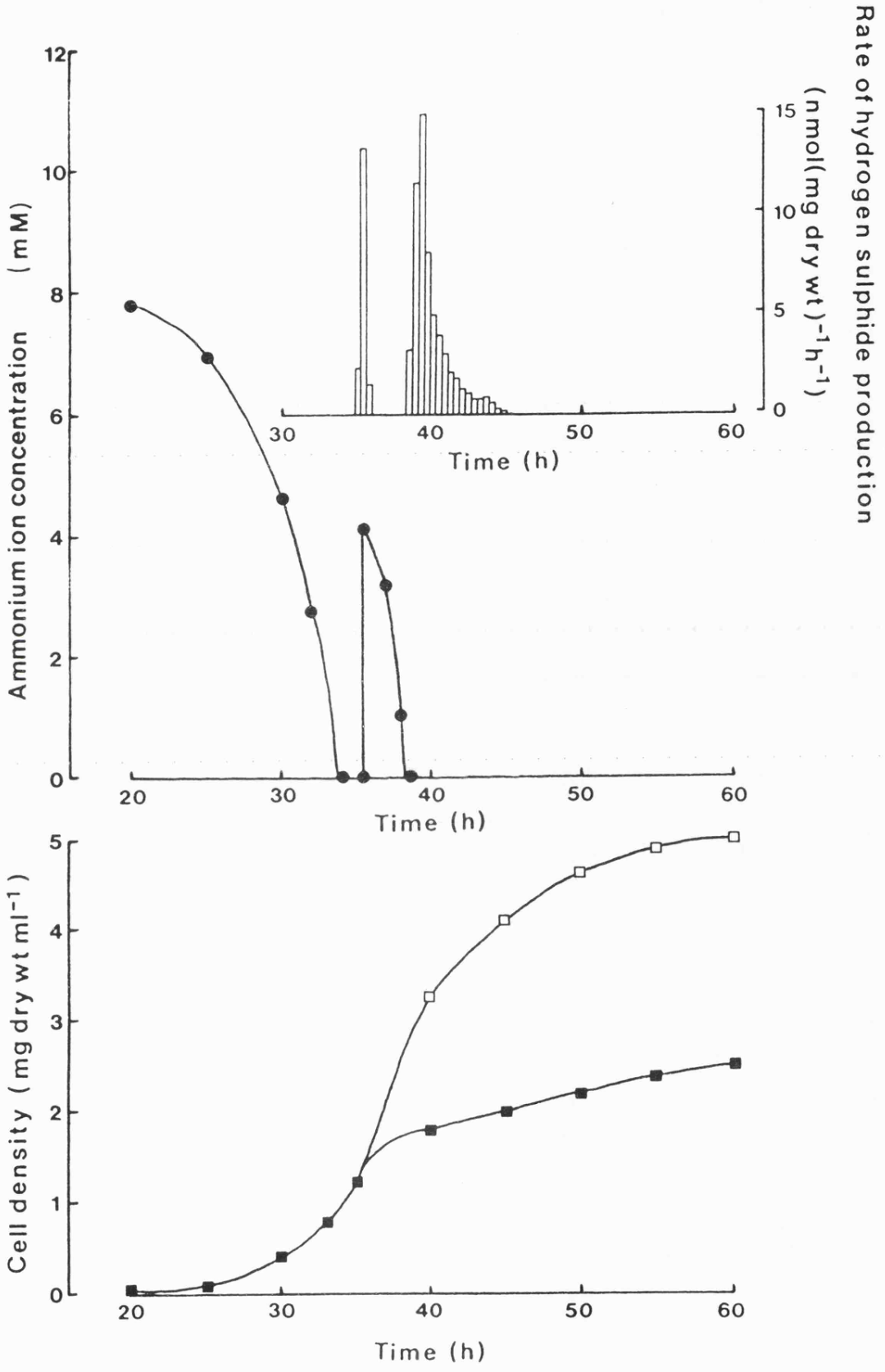



Figure 26. Ammonium ion concentration (●) and hydrogen sulphide production (histograms) by Saccharomyces cerevisiae TC17 in yeast carbon-base medium at 30°C.

Figure 27, Relationship between ammonium ion concentration (●), growth (■) and hydrogen sulphide production (histograms) by Saccharomyces cerevisiae TC8 at 30°C in yeast carbon-base medium. Additional ammonium ions (4 mM) were added at 35.5 hours. Growth following this increment is represented by open symbols.


Ammonium ion concentration (mM)
Rate of hydrogen sulphide production (nmol(mg dry wt)$^{-1}$h$^{-1}$)
Time (h)
Cell density (mg dry wt ml$^{-1}$)
Time (h)

depleted and hydrogen sulphide was again produced. Very similar findings were made when proline or asparagine were employed as an alternative nitrogen source to ammonium ions, in respect of the coincidence of exhaustion of nitrogen source and onset of hydrogen sulphide production. Treatment of rapidly growing cultures of TC8 or TC16 with cycloheximide (10 mg $L^{-1}$) terminated yeast growth and induced hydrogen sulphide production in a very similar manner to that caused by nitrogen starvation (Fig. 28). Iodoacetamide (5 mM) was also found to induce formation of hydrogen sulphide in similar amounts. Actinomycin D was found to have little effect upon strain TC8 while treatment with ethanol (10%v/v) or acid shock (pH drop 3.8 - 2.5) induced formation of only traces of hydrogen sulphide (Table 7).

Potential for hydrogen sulphide production

Induction of hydrogen sulphide production, by nitrogen starvation or addition of cycloheximide, at progressively later stages during the fermentation of cultures (Fig. 29) led to production occurring at progressively greater rates until a peak was reached during mid-exponential growth. This effect was demonstrated using strain TC8 in both enriched apple juice (Fig. 30) and in yeast carbon-base medium (Fig. 31). The presence of methionine (0.2 mM) in the medium resulted in a diminution of rate of hydrogen sulphide production of 40% - 50% at all the stages of growth examined (Table 8).

Activity of NADPH-sulphite reductase was found to rise to a peak during exponential growth (Fig. 32) when measured in strain TC8.

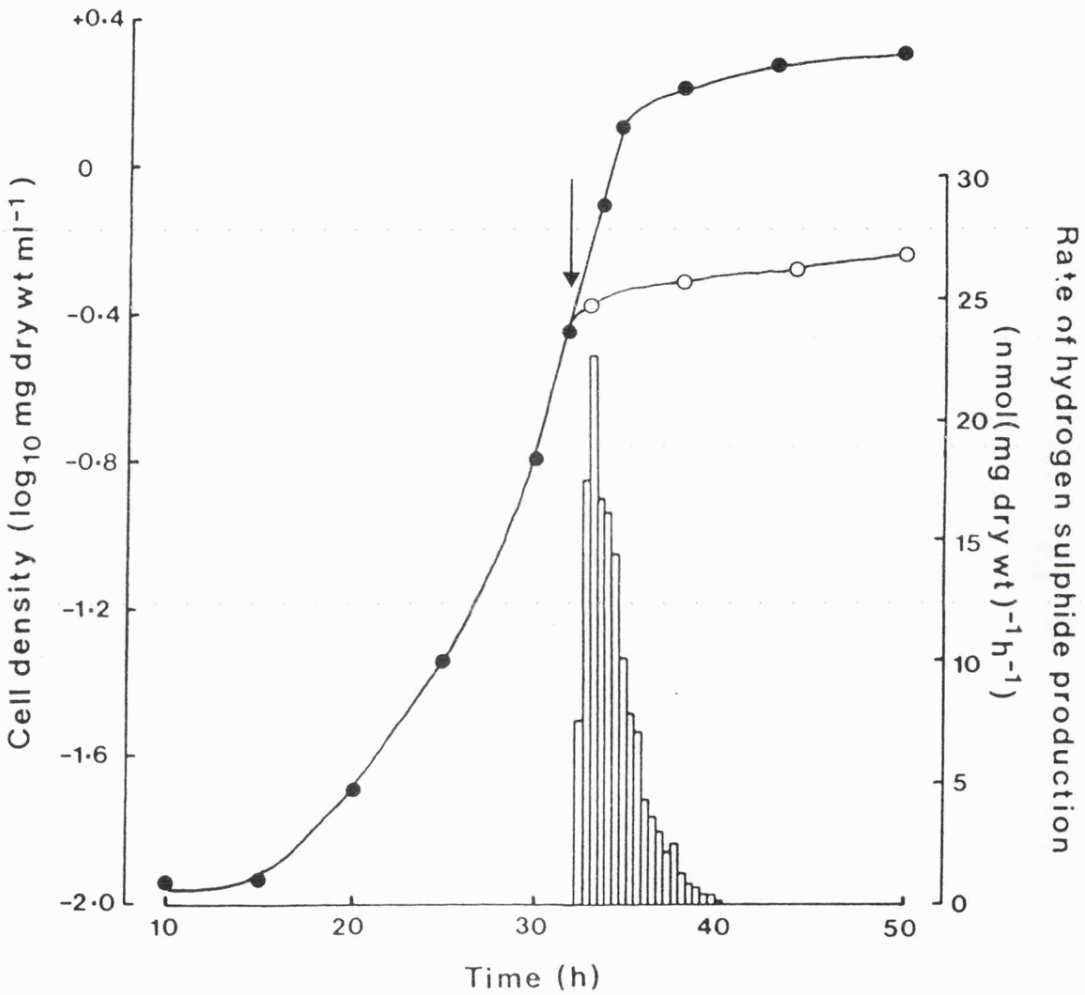
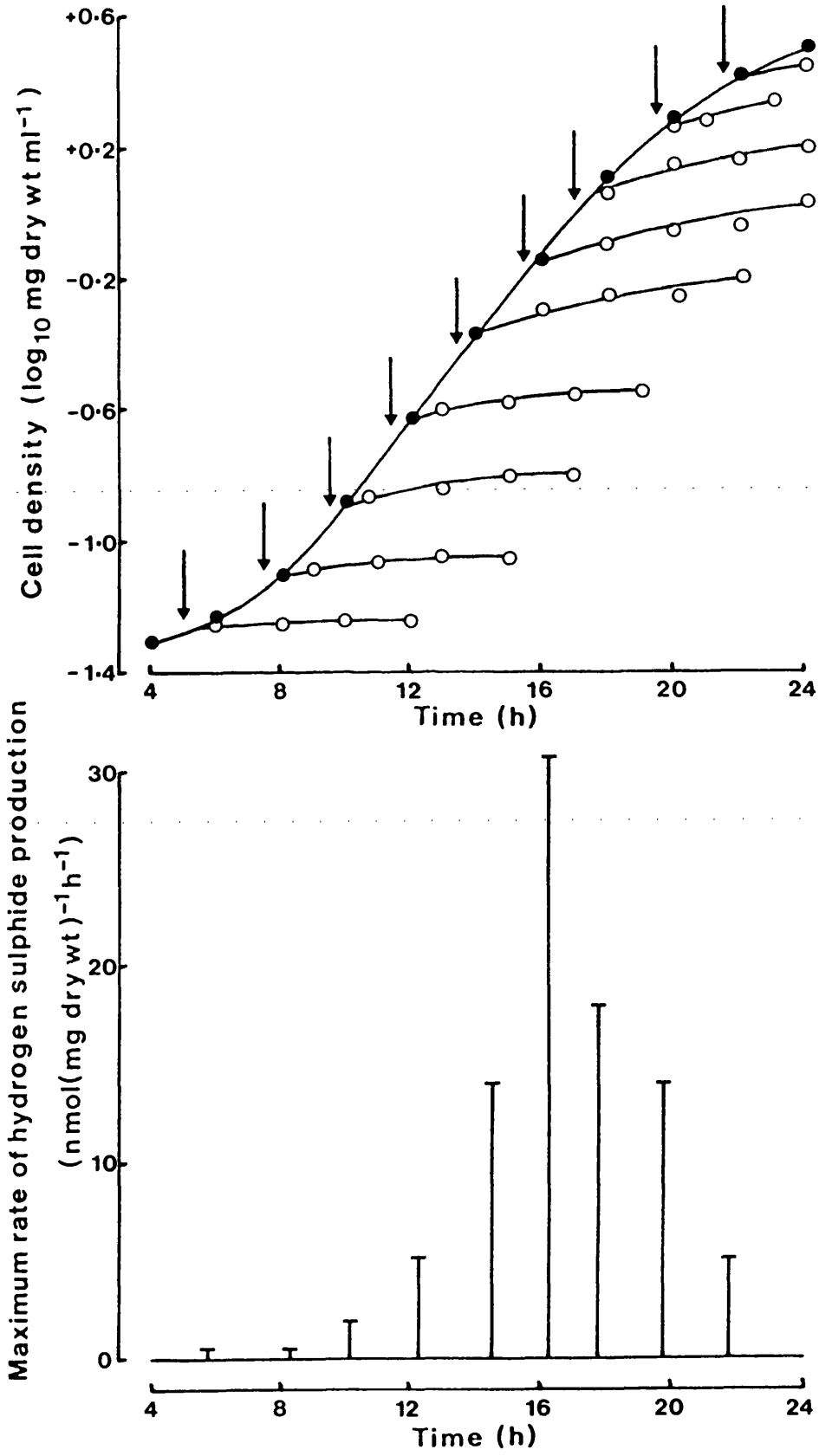



Figure 28. Effect of cyclohexamide (10 mg $L^{-1}$, O ) upon growth (●) and hydrogen sulphide production (histograms) by Saccharomyces cerevisiae TC8 in yeast carbon-base medium. Cycloheximide was added after 32 h.

Table 7. Maximum rates of hydrogen sulphide formation induced by various treatments imposed upon 36 hour cultures of Saccharomyces cerevisiae TC8 grown in yeast carbon-base medium at 30°C. Values quoted are the average of at least two independent determinations.

| Treatment used for the induction of hydrogen sulphide formation | Maximum rate of hydrogen sulphide production (nmol (mg dry wt)$^{-1}$ h$^{-1}$) |
|---|---|
| Nitrogen starvation | 20.2 |
| Cycloheximide (10 mg L$^{-1}$) | 18.5 |
| Iodoacetamide (5 mM) | 19.4 |
| Actinomycin D (100 mg L$^{-1}$) | 0 |
| Ethanol (10% v/v) | 0.9 |
| Acid Shock (pH drop 3.8 to 2.5) | 0.2 |

Figure 29. Effect of cycloheximide (10 mg $L^{-1}$, ○) at progressively later stages of growth (●) of Saccharomyces cerevisiae TC8 in enriched apple juice at 25°C. Maximum rates of hydrogen sulphide production following cycloheximide addition are represented by histograms.


+0·6
+0·2
-0·2
-0·6
-1·0
-1·4
Cell density ($log_{10}$ mg dry wt $ml^{-1}$)
4
8
12
16
20
24
Time (h)
30
20
10
0
Maximum rate of hydrogen sulphide production
(nmol(mg dry wt)$^{-1}h^{-1}$)
4
8
12
16
20
24
Time (h)

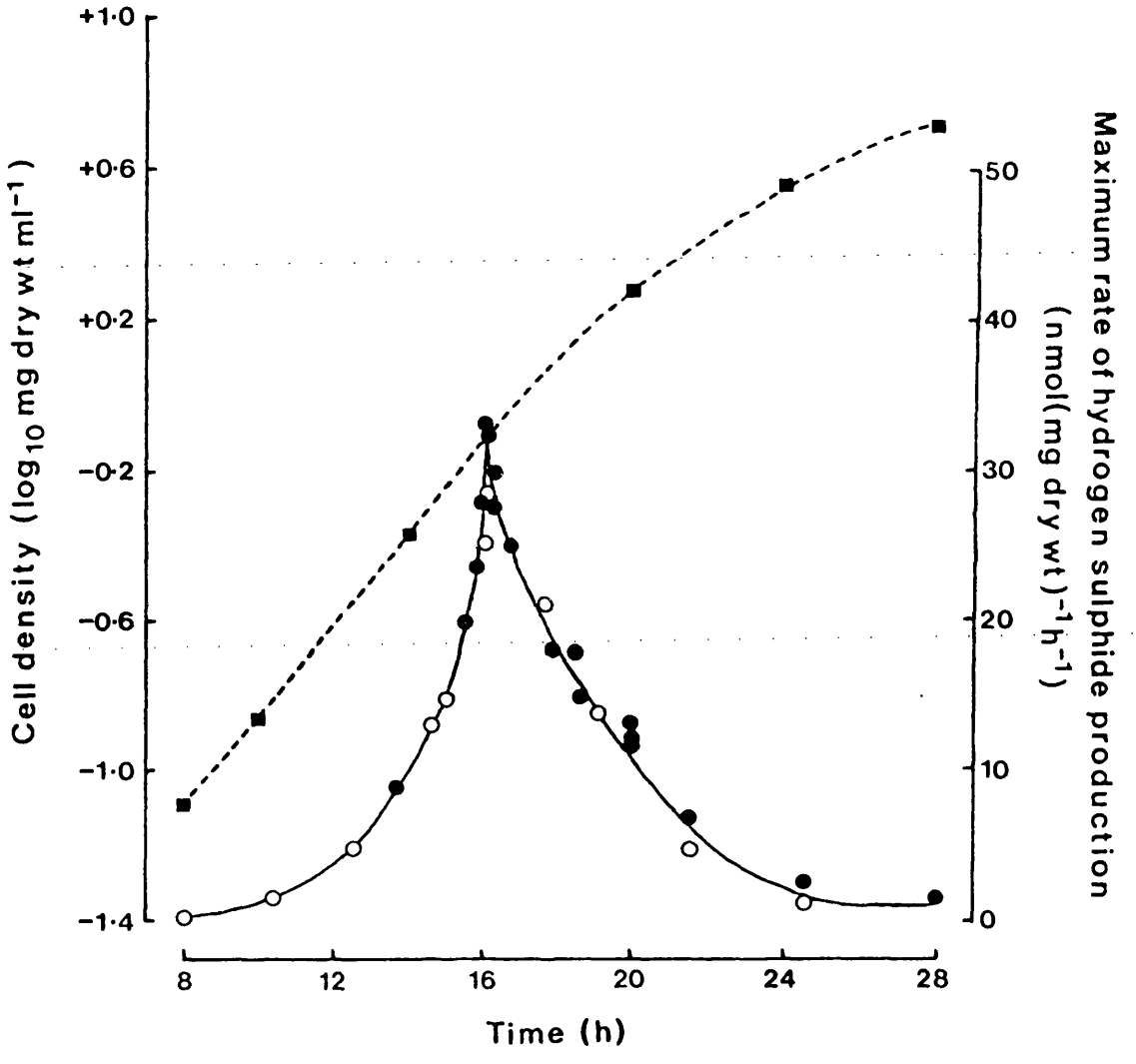



Figure 30. Maximum rates of hydrogen sulphide production at different times throughout the growth of Saccharomyces cerevisiae TC8 in enriched apple-juice at 25°C. Hydrogen sulphide formation was induced by cycloheximide addition (10 mg $L^{-1}$, ○) or nitrogen starvation (●). Growth is indicated by a broken line (■).

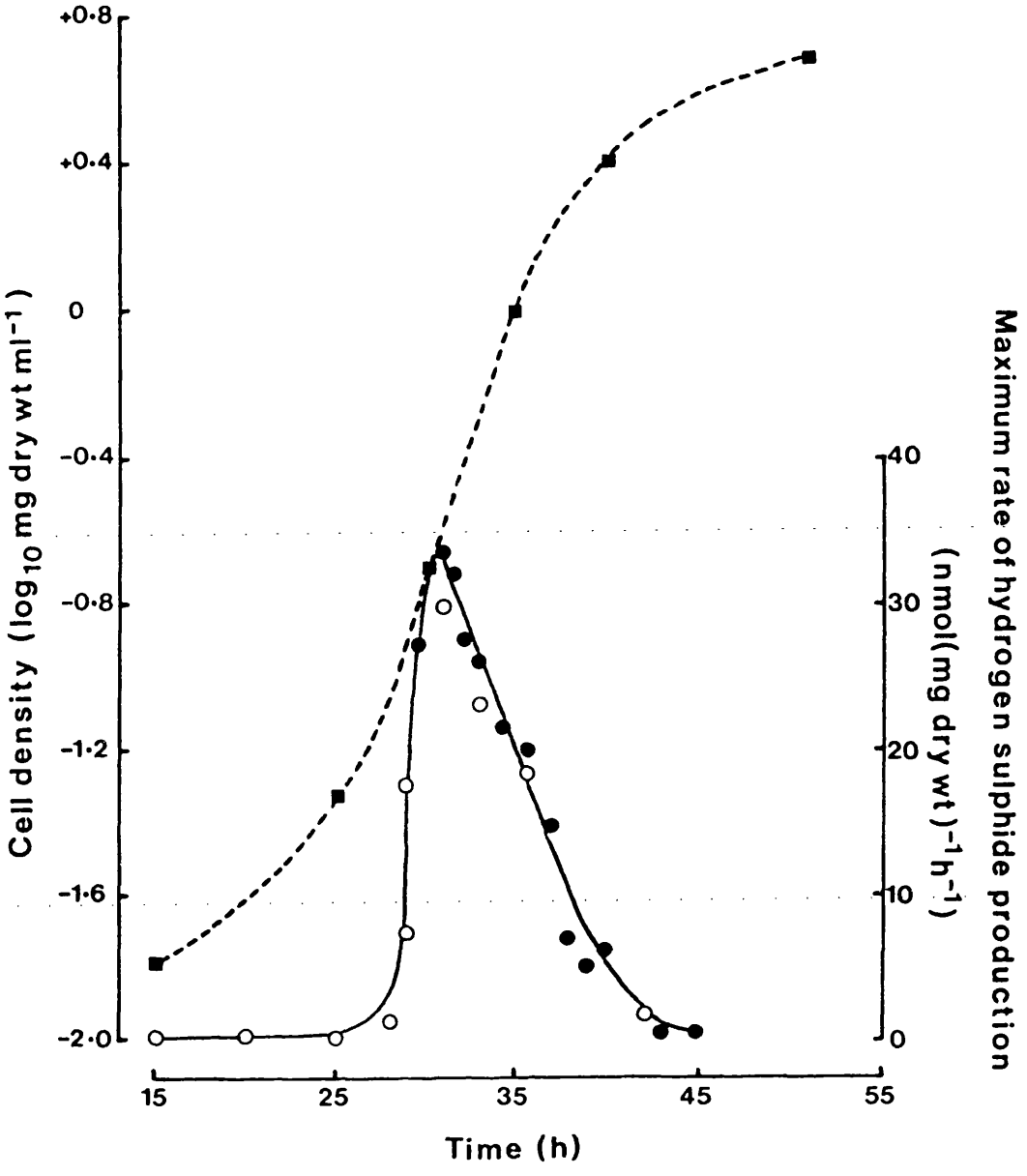



Figure 31. Maximum rates of hydrogen sulphide production at different times throughout growth of Saccharomyces cerevisiae TC8 in yeast carbon-base medium at 30°C. Hydrogen sulphide formation was induced by cycloheximide (10 mM, open symbols) or nitrogen starvation (closed symbols). Growth is indicated by a broken line (■).

Table 8. Effect of methionine (0.2 mM) upon maximum rates of hydrogen sulphide production at various times by cultures of *Saccharomyces cerevisiae* TC8 grown in yeast carbon-base medium at 30°C.

| Age of culture (h) | Maximum rate of hydrogen sulphide production (nmol (mg dry wt)$^{-1}$ h$^{-1}$) | |
|---|---|---|
| | Absence of methionine | Presence of methionine |
| 31.5 | 32.1 | 20.5 |
| 32.0 | 27.5 | 16.7 |
| 33.0 | 26.0 | 15.1 |
| 33.5 | - | 13.6 |
| 34.0 | 21.5 | 9.8 |
| 36.0 | 20.8 | 10.3 |
| 37.0 | 15.5 | 7.4 |

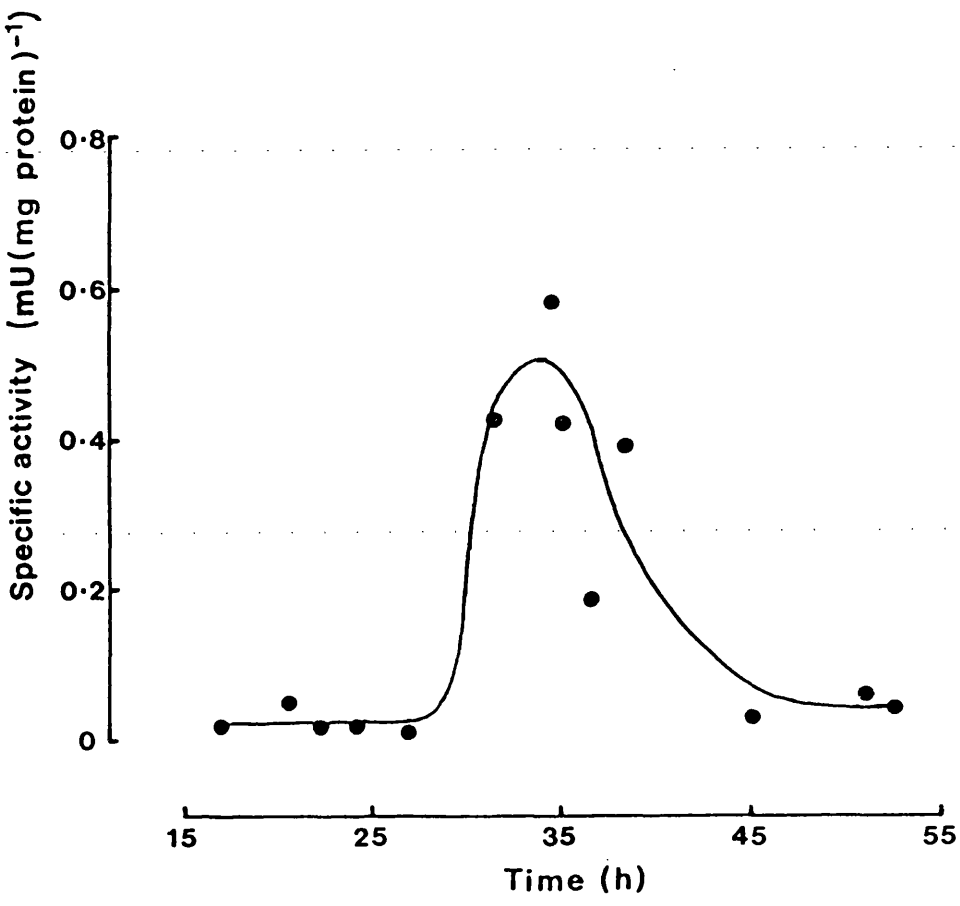



Figure 32. Specific activity of NADPH-sulphite reductase (microunits (mg protein)$^{-1}$) in Saccharomyces cerevisiae TC8 throughout growth in yeast carbon-base medium at 30°C. One unit of enzyme activity is that which forms 1 µmol hydrogen sulphide over 40 min at 30°C.

## Sulphite Toxicity and Transport

### Effect of sulphite on growth and viability

Strain TC8 of Saccharomyces cerevisiae was found to be relatively tolerant to sulphite concentrations which were toxic to strains NCYC 366 and X2180 1Aα. Little loss in viability was observed in strain TC8 exposed to sulphite concentrations less than 10 μM (free $SO_2$) and less than 5 μM (free $SO_2$) in strains NCYC 366 and X2180l Aα. At sublethal concentrations sulphite caused a prolongation of lag-phase of all the yeast strains proportional to the sulphite concentration (Figs. 33, 34). In sulphited cultures growth was initially slow but eventually the exponential rate of growth was similar to that of yeast grown in non-sulphited media. During the lengthy lag-phase, it was noted that the free sulphite concentration declined and reached undetectable levels at approximately the time that yeast growth became fully exponential in strains TC8 and NCYC 366.

### Effect of sulphite on solute accumulation

Sulphite (0.5 mM) treatment was found to interrupt accumulation of L-lysine and L-arginine by Saccharomyces cerevisiae NCYC 366 (Figs. 35,36). A slight delay of 4 minutes occurred before an inhibition of transport of L-lysine or L-arginine became apparent. Accumulation of these amino acids was not totally inhibited in the short term. An examination of yeast cells revealed that little or no leakage of solute out of the cells was occurring and that there was little loss of viability evident over the period of the experiments.

Glucose accumulation was studied using three different radio-labelled compounds, label from D-[U-$^{14}$C]glucosamine, was accumulated relatively slowly (85 nmol(mg dry wt)$^{-1}$min$^{-1}$). Label from D-[6-$^{3}$H]glucose

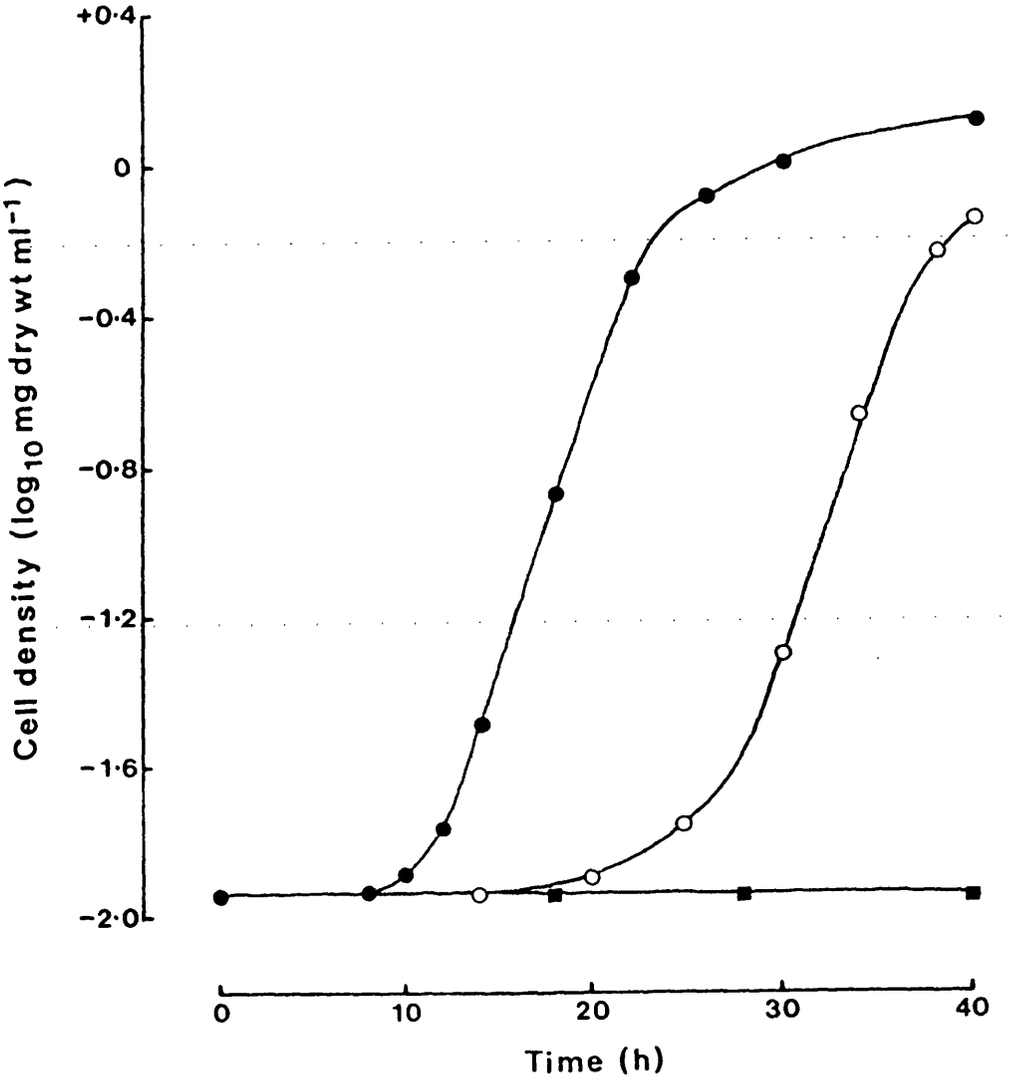



Figure 33. Effect of sulphite concentration ( ● 0 mM, ○ 0.2 mM, ■ 0.5 mM total sulphite) upon growth of Saccharomyces cerevisiae NCYC 366 in aerobic medium at 30°C and pH 4.5.

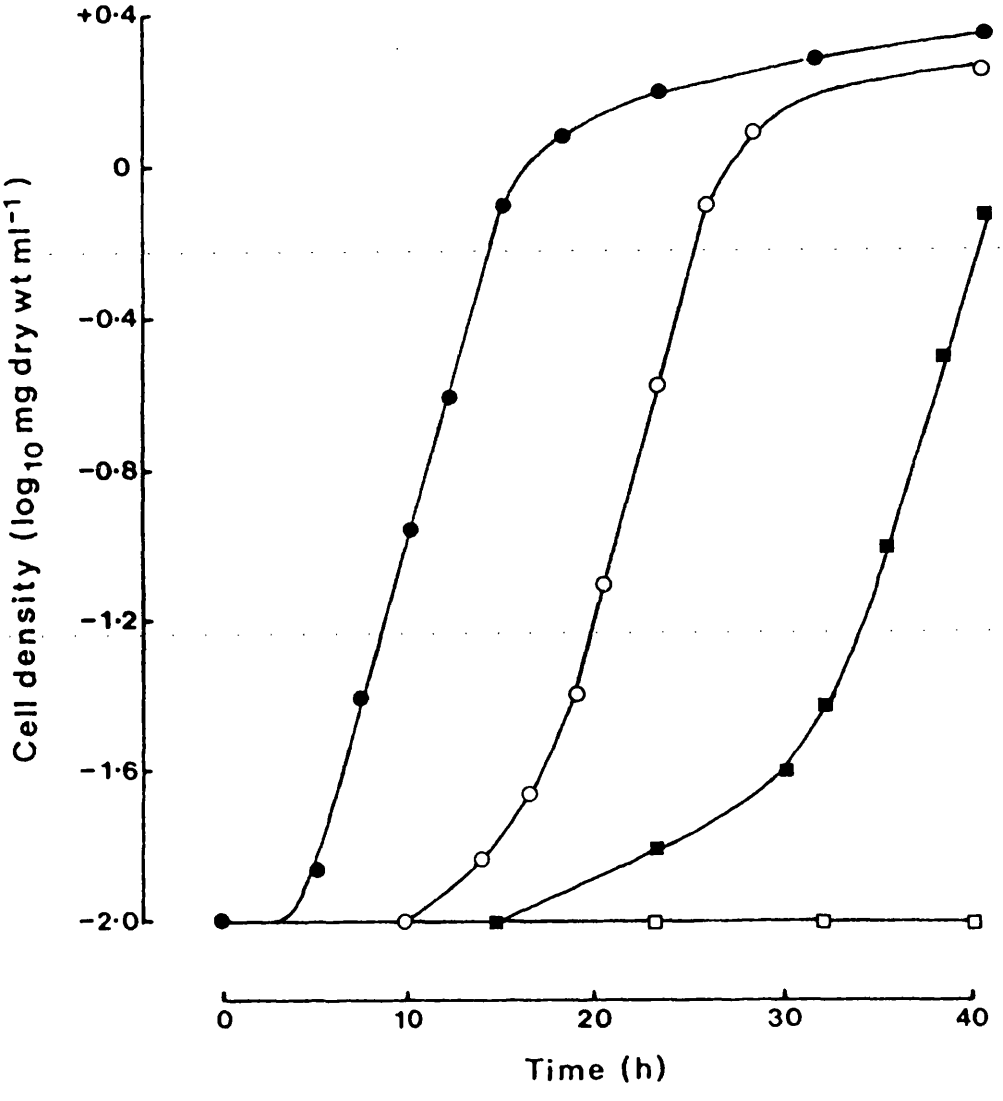



Figure 34. Effect of sulphite concentration (● 0 mM, ○ 0.6 mM, ■ 0.9 mM, □ 1.2 mM total sulphite) upon growth of Saccharomyces cerevisiae TC8 in aerobic medium at 30°C and pH 4.0.

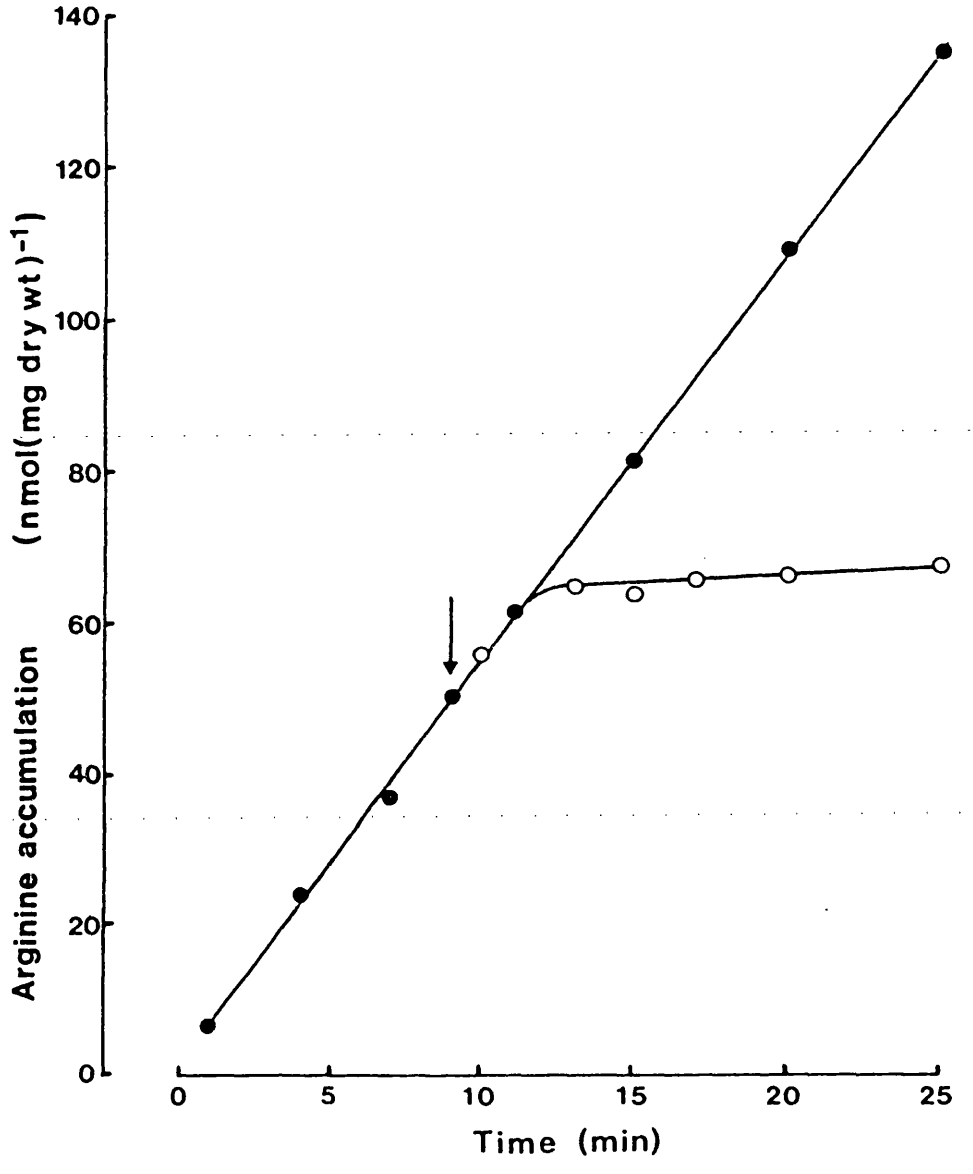



Figure 35. Accumulation of L-arginine (●) by Saccharomyces cerevisiae NCYC 366 at 30°C and pH 4.5 in the presence of 100 mM glucose and the effect of adding sulphite (0.5 mM) after 9 minutes (○). Arginine was present at 1.3 mM .

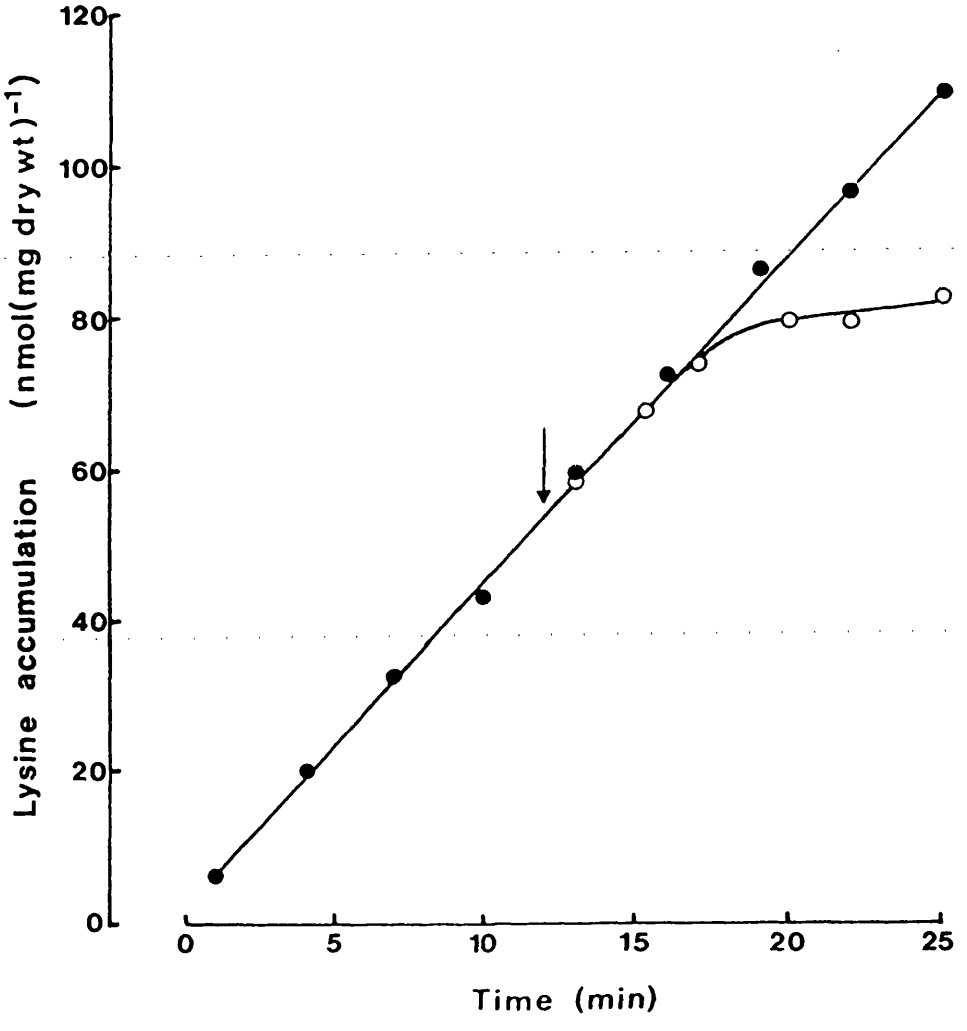



Figure 36. Accumulation of L-lysine (●) by Saccharomyces cerevisiae NCYC 366 at 30°C and pH 4.5 in the presence of 100 mM glucose and the effect of adding sulphite (0.5 mM) after 12 minutes (O). Lysine was present at 1 mM.

was accumulated at a similarly slow rate, and the radiolabel was extremely rapidly lost from yeast cells. Label from D-[U-$^{14}$C]glucose was rapidly accumulated by cultures of strain NCYC 366 and the $^{14}CO_2$ evolved was trapped and the radiolabel so caught was added to the radioactivity present inside yeast cells. Inhibition of transport by sulphite was not detected in any of the systems examined, either aerobically or anaerobically (Fig. 37).

Effect of sulphite upon respiration and fermentation

Rate of evolution of carbon dioxide, as measured using Warburg manometry, by anaerobically grown yeast strain NCYC 366 was approximately halved by the addition of sulphite (Fig. 38). In contrast in aerobically grown cultures, the rate of evolution of carbon dioxide was not significantly altered by sulphite (Fig. 39) although the uptake of oxygen was severely curtailed in these cells (Fig. 40). It was noted during the course of these experiments that the concentration of cells of strain NCYC 366 present in the apparatus had a noticeable effect upon the degree of inhibition imposed by sulphite such that doubling the cell concentration from 0.5 mg dry wt $ml^{-1}$ resulted in a halving of the degree of inhibition of oxygen uptake by aerobic cells.

Using anaerobically grown strain NCYC 366 (Fig. 41) it was found that sulphite inhibited [$^{14}$C] carbon dioxide production from D-[U-$^{14}$C]glucose and from D-[6-$^{14}$C]glucose but not from D-[1-$^{14}$C]-glucose. However in aerobically grown cells sulphite only inhibited [$^{14}$C] carbon dioxide production from D-[6-$^{14}$C]glucose (Fig. 42). Evolution of carbon dioxide from D-[U-$^{14}$C]glucose was only slightly lowered by sulphite addition, while that from D-[1-$^{14}$C]glucose was

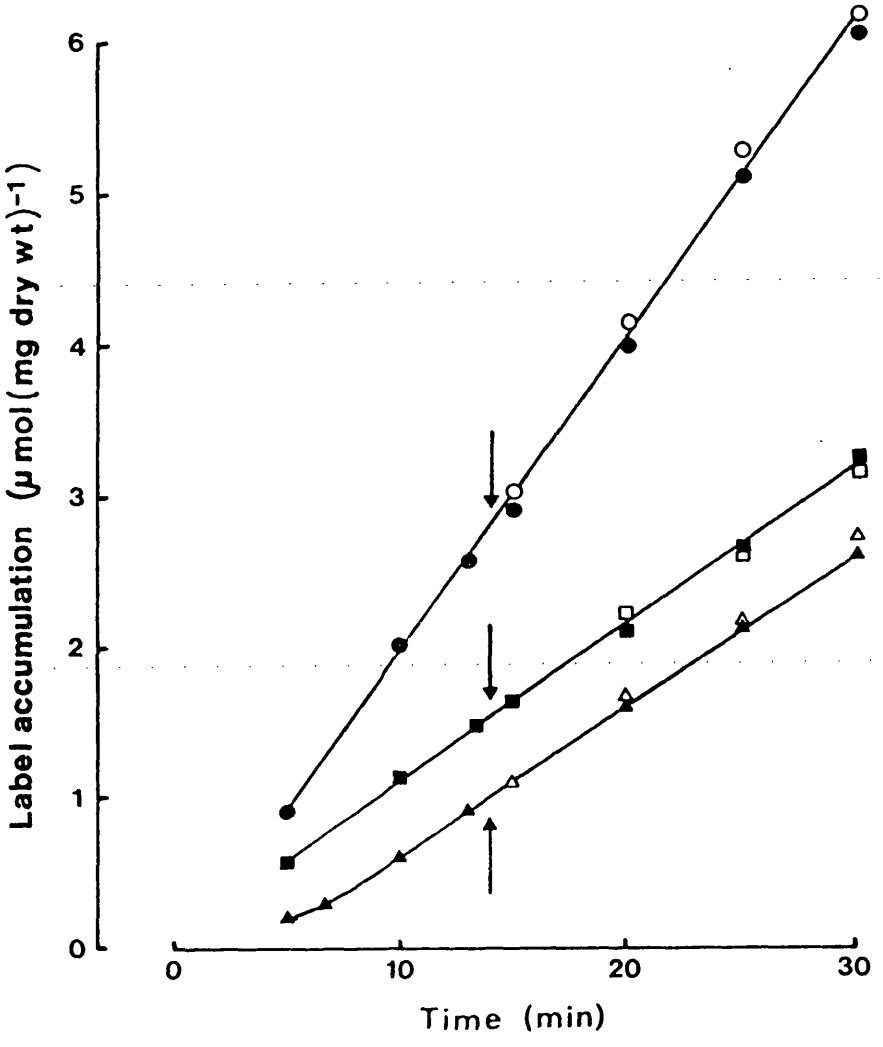



Figure 37. Accumulation of label from D-[U-$^{14}$C] glucose (●), D-[6-$^{3}$H]-glucose (■) and D-[U-$^{14}$C] glucosamine (▲) by Saccharomyces cerevisiae NCYC 366 at 30°C and pH 4.5 and the effect of sulphite (open symbols). Sulphite was added after 14 minutes.

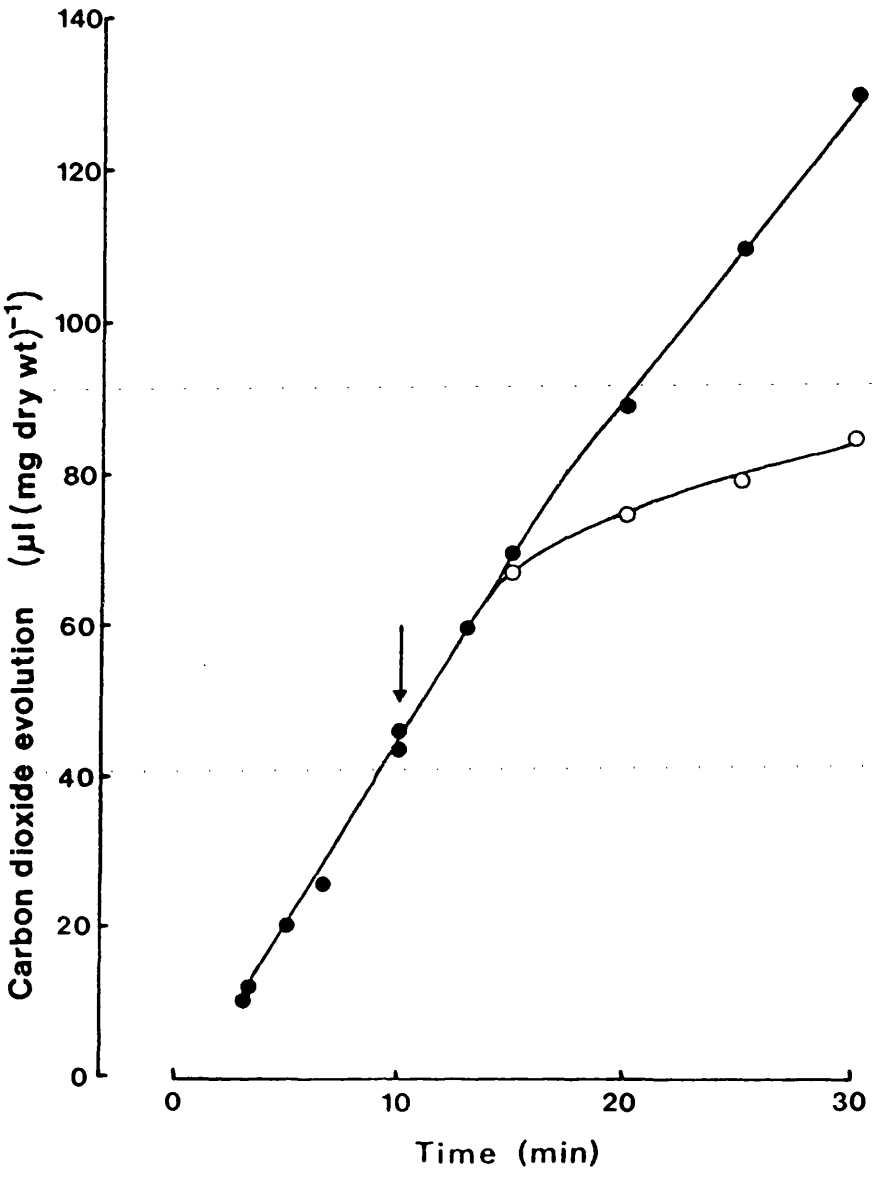
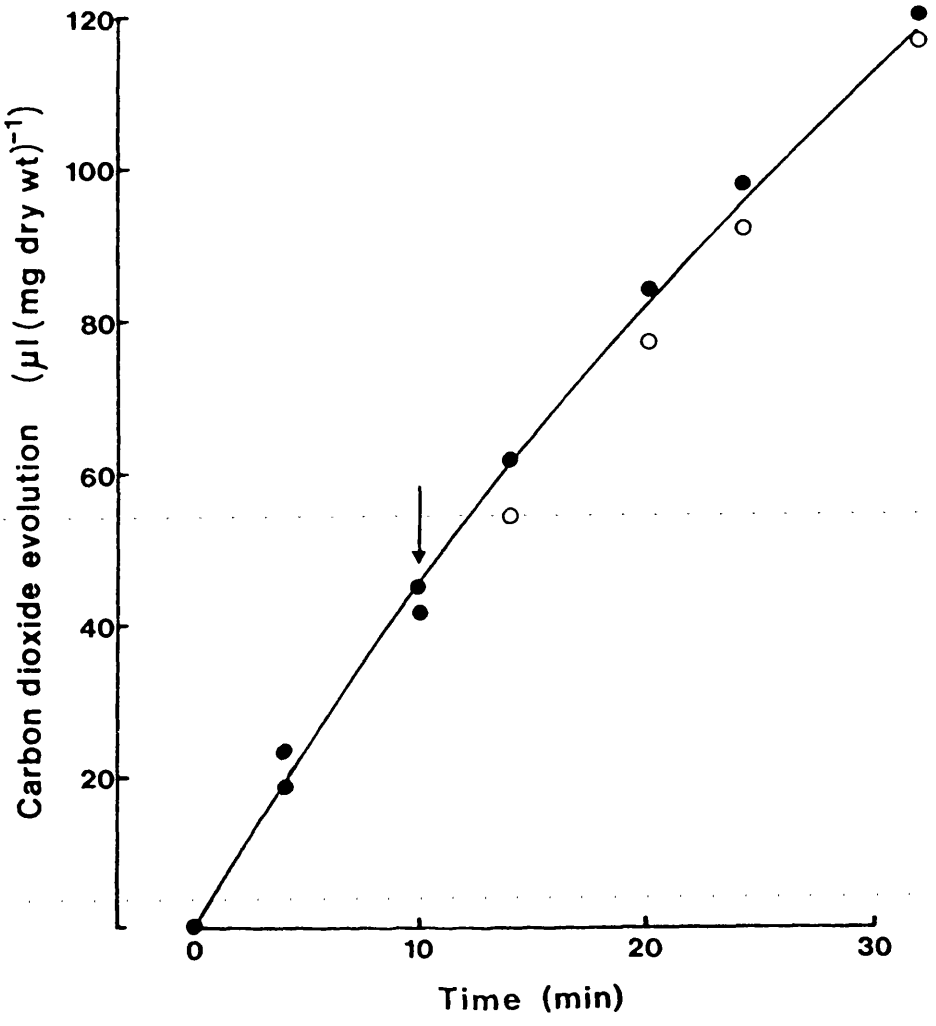



Figure 38. Carbon dioxide formation (●) by an anaerobic culture of Saccharomyces cerevisiae NCYC 366 and the effect of sulphite (0.5 mM) (O). Sulphite was added after 10 min.

Figure 39. Production of carbon dioxide (●) by an aerobic culture of Saccharomyces cerevisiae NCYC 366 and effect of sulphite (0.5 mM) addition after 10 min (O).

Figure 40. Uptake of oxygen (■) by an aerobic culture of Saccharomyces cerevisiae NCYC 366 and the effect of sulphite (0.5 mM) addition after 10 min (□).


Carbon dioxide evolution (µl (mg dry wt)$^{-1}$)
120
100
80
60
40
20
0
10
20
30
Time (min)

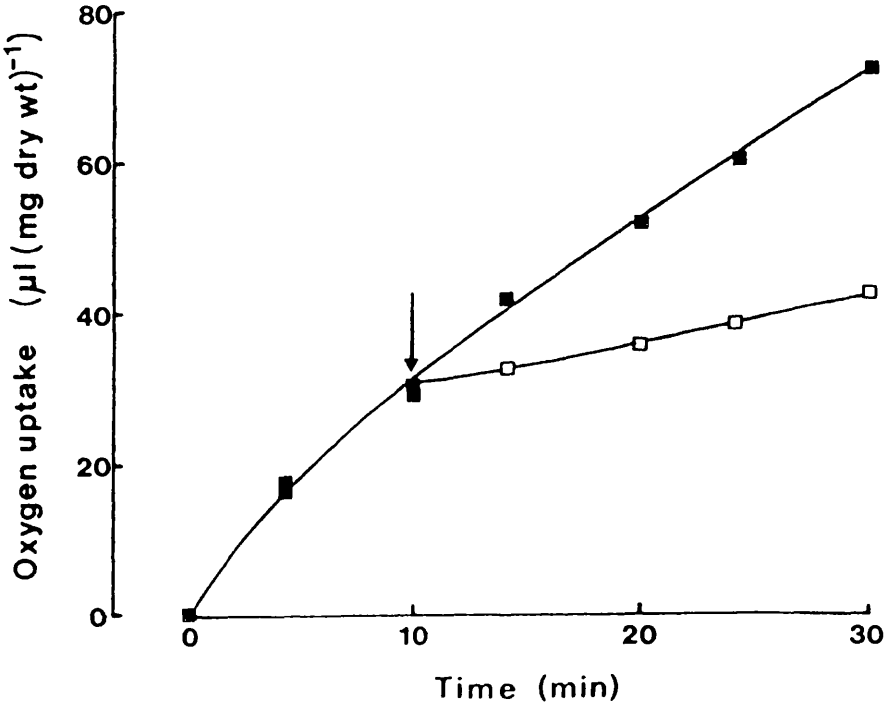

Oxygen uptake (µl (mg dry wt)$^{-1}$)
80
60
40
20
0
10
20
30
Time (min)

Figure 41. Production of $^{14}CO_2$ by anaerobically grown cells of Saccharomyces cerevisiae NCYC 366 from various $^{14}C$ labelled glucoses.

● D-[U-$^{14}$C]glucose

■ D-[1-$^{14}$C]glucose

▲ D-[6-$^{14}$C]glucose

Open symbols indicate production when sulphite (0.5 mM) was added to the suspension after 4 min.

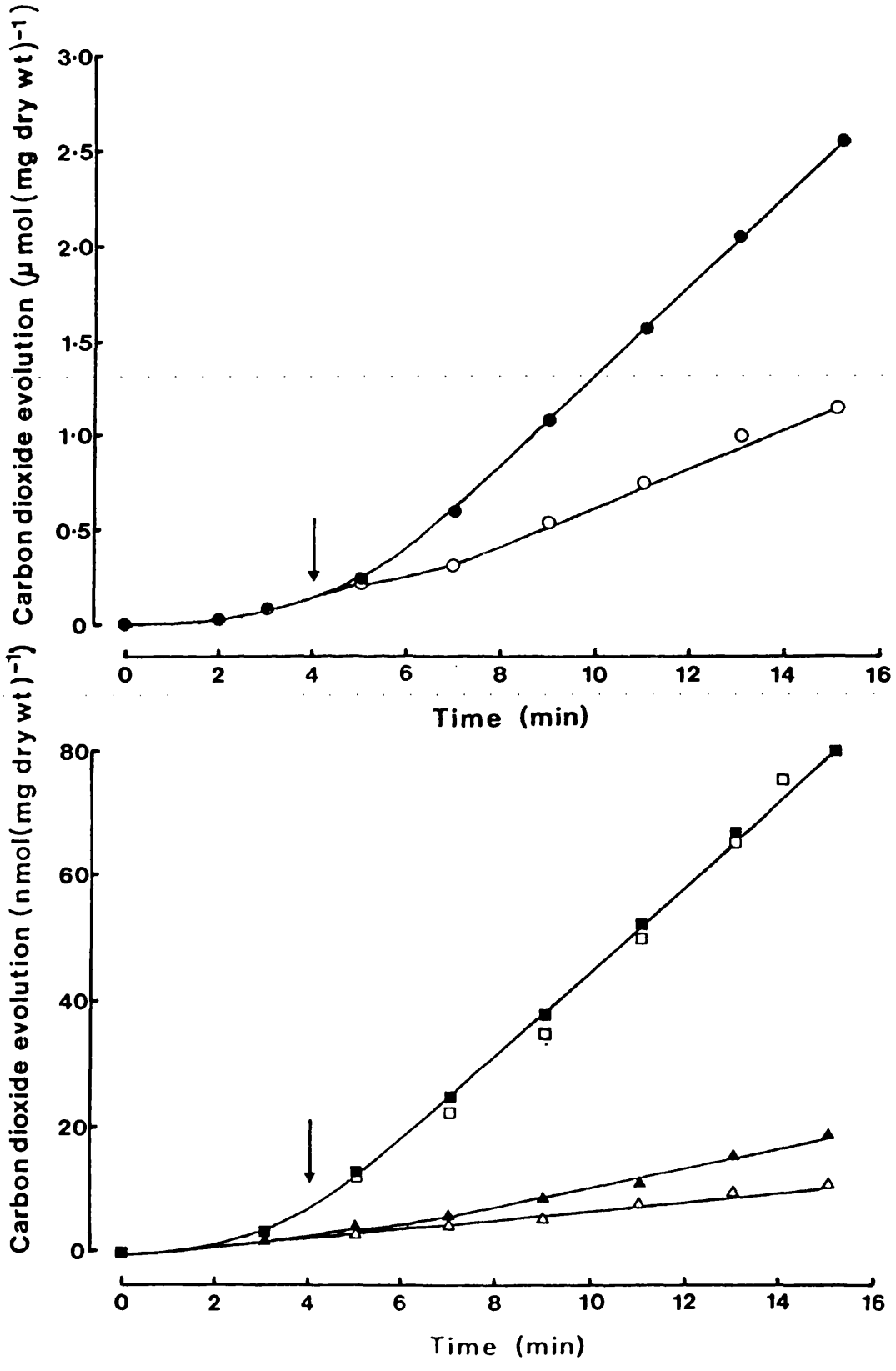

Carbon dioxide evolution (μmol(mg dry wt)$^{-1}$)
3·0
2·5
2·0
1·5
1·0
0·5
0
0
2
4
6
8
10
12
14
16
Time (min)
Carbon dioxide evolution (nmol(mg dry wt)$^{-1}$)
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Time (min)

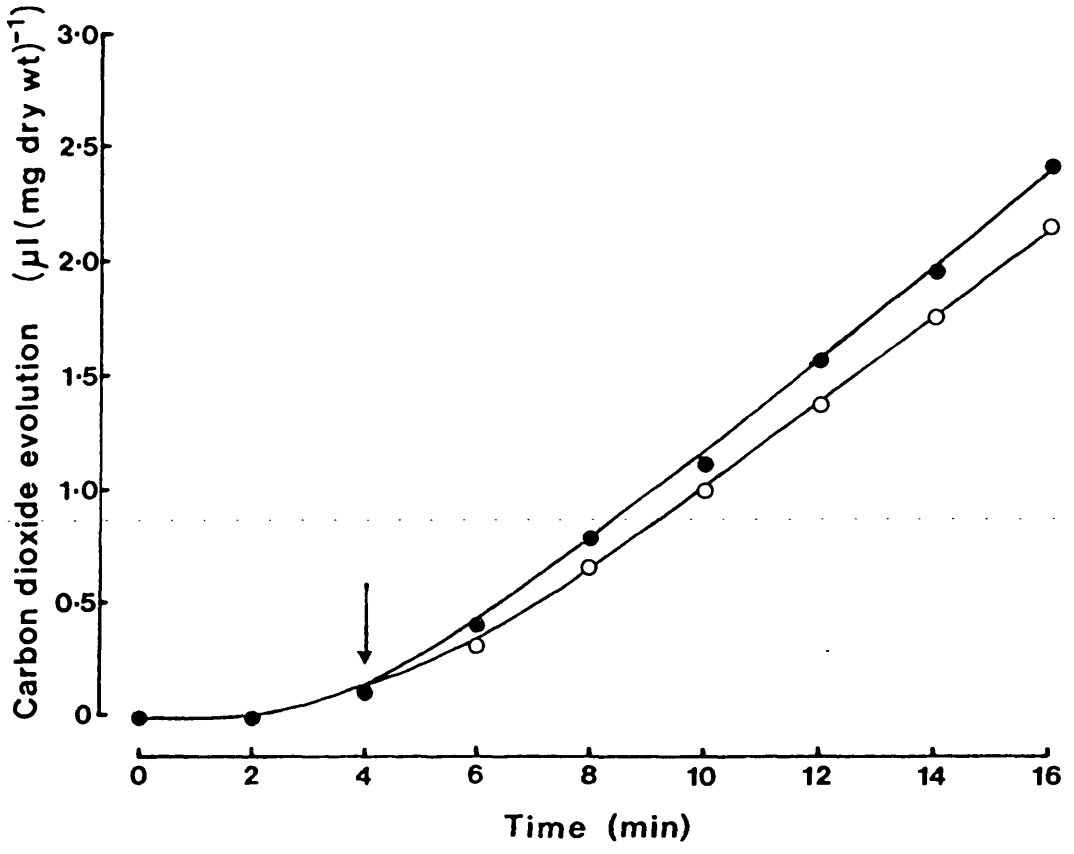
Figure 42. Production of $^{14}CO_2$ by an aerobic culture of Saccharomyces cerevisiae NCYC 366 from various $^{14}C$ labelled glucose sources

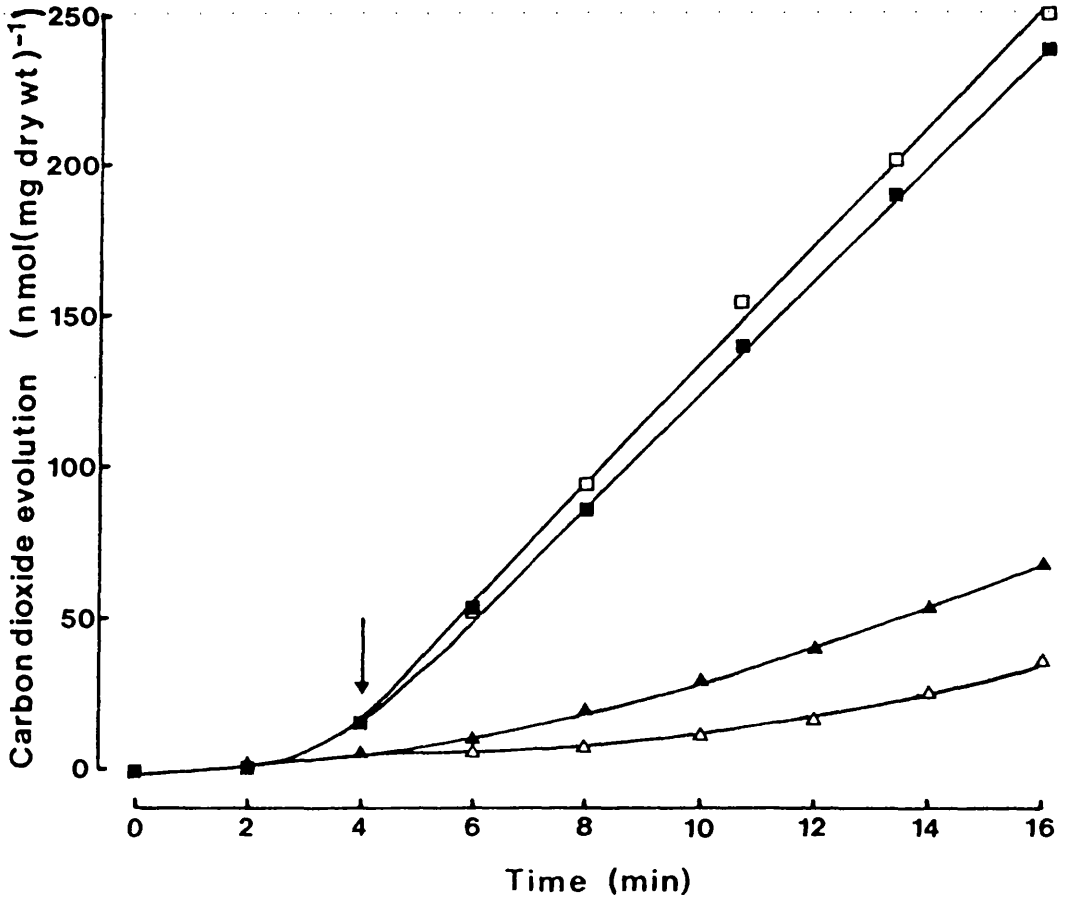
● D-[U-$^{14}$C]glucose

■ D-[1-$^{14}$C]glucose

▲ D-[6-$^{14}$C]glucose

Open symbols indicate production after sulphite (0.5 mM) was added to the suspension after 4 minutes.


Carbon dioxide evolution (µl (mg dry wt)$^{-1}$)
3·0
2·5
2·0
1·5
1·0
0·5
0
0
2
4
6
8
10
12
14
16
Time (min)


Carbon dioxide evolution (nmol(mg dry wt)$^{-1}$)
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
Time (min)

unaffected (Table 9). Table 10 shows the estimated activity of several decarboxylation reactions, namely the pentose phosphate decarboxylations, the pyruvate decarboxylation and the tricarboxylic acid cycle decarboxylations based upon data from Table 9.

Accumulation of label from sulphite (1.5 mM) by Saccharomyces cerevisiae TC8 was initially rapid reaching a peak within three minutes, after which the label was progressively lost from the yeast cells back into the medium. Traces of label were recovered as hydrogen sulphide. 2,4-Dinitrophenol promoted entry of label, even when there had previously been a net loss of label from cells (Fig. 43). Figure 44 shows the initial accumulation of sulphite present at low concentrations by Saccharomyces cerevisiae TC8. At concentrations up to 300 µM, the initial rate of transport was proportional to the sulphite concentration (Fig. 45). A double reciprocal plot of these data was linear and passed close to the origin (Fig. 46).

Resistance of Saccharomycodes ludwigii TC10 to sulphite toxicity

Sulphite at concentrations greater than 60 µM (free sulphur dioxide) caused loss of viability of Saccharomycodes ludwigii while lower concentrations caused prolongation of the lag-phase of growth. During this lag-phase of growth the concentration of free sulphite in the medium was rapidly lowered (Fig. 47). The concentration of total sulphite during this period remained reasonably constant. A small amount of growth occurred while free sulphite was present in the medium but exponential yeast growth began only after the free sulphite had been completely bound.

In the presence of high concentrations of sulphite, this

Table 9. Effect of sulphite (0.5 mM) upon rates of carbon dioxide evolution by cells of Saccharomyces cerevisiae NCYC 366 from various [$^{14}$C]labelled glucoses, at 30°C and pH 4.5. Values quoted are the averages of at least four independent determinations ± S.E.M.

| | | Rate of carbon dioxide production (nmol(mg dry wt)$^{-1}$ min$^{-1}$) | |
|---|---|---|---|
| | | Anaerobic cells | Aerobic cells |
| D-[U-$^{14}$C]Glucose | Control | 262 ± 45 | 251 ± 39 |
| | Sulphited | 139 ± 25 | 234 ± 42 |
| D-[1-$^{14}$C]Glucose | Control | 6.6 ±0.8 | 24 ± 4 |
| | Sulphited | 7.6 ±0.8 | 27 ± 5 |
| D-[6-$^{14}$C]Glucose | Control | 1.6± 0.2 | 6.8 ± 0.9 |
| | Sulphited | 1.0± 0.3 | 4.0 ± 0.6 |

| | | Rate of carbon dioxide production (nmol (mg dry wt)$^{-1}$ min$^{-1}$ | |
|---|---|---|---|
| | | Anaerobic cells | aerobic cells |
| Pentose phosphate pathway decarboxylation | Control | 5.0 ± 1.0 | 17.2 ± 4.9 |
| | Sulphited | 6.6 ± 1.0 | 23.0 ± 5.6 |
| Tricarboxylic acid cycle decarboxylation | Control | 9.6 ± 1.2 | 40.8 ± 5.4 |
| | Sulphited | 6 ± 1.8 | 24.0 ± 3.6 |
| Pyruvate decarboxylation | Control | 248 ± 47 | 193 ± 49 |
| | Sulphited | 126 ± 28 | 187 ± 51 |

Table 10 Effect of sulphite (0.5 mM) upon estimated activities of various decarboxylation reactions liberating carbon dioxide in Saccharomyces cerevisiae, NCYC 366 at 30° and pH 4.5. Activities were estimated using the data in Table 9, as follows:

Pentose phosphate pathway - rate from D-[1-$^{14}$C]glucose minus rate from D-[6-$^{14}$C]glucose,

Tricarboxylic acid cycle - 6x rate from D-[6-$^{14}$C]glucose,

Pyruvate decarboxylation - rate from D-[U-$^{14}$C]glucose minus those activities attributed to the pentose phosphate pathway.

The data (Table 9) from which these values are obtained are the averages of at least four independent determinations, ± S.E.M.

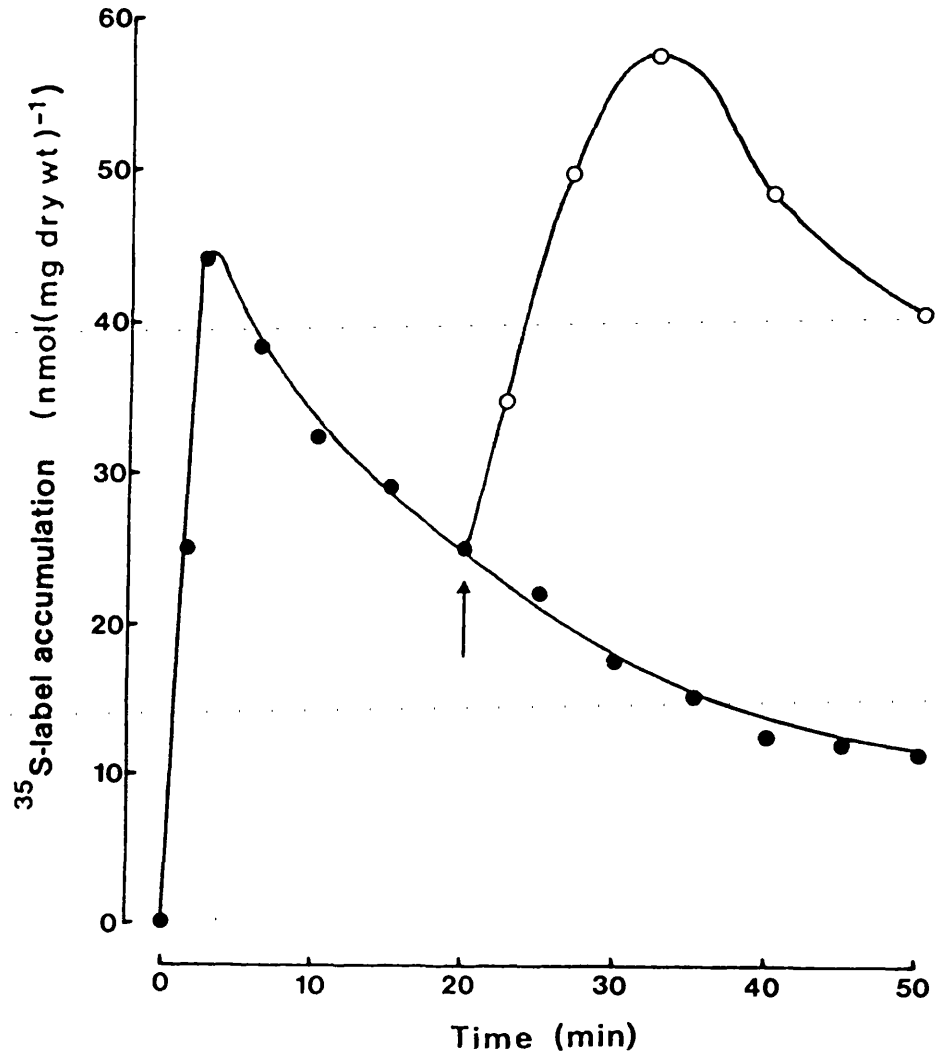



Figure 43. Long-term accumulation of [$^{35}$S] label from sulphite (1.5 mM - 10 µCi, ●) by Saccharomyces cerevisiae TC8 at 30°C and pH 4.0. The effect of 2,4-Dinitrophenol (0.5 mM) addition after 20 min is shown by open symbols.

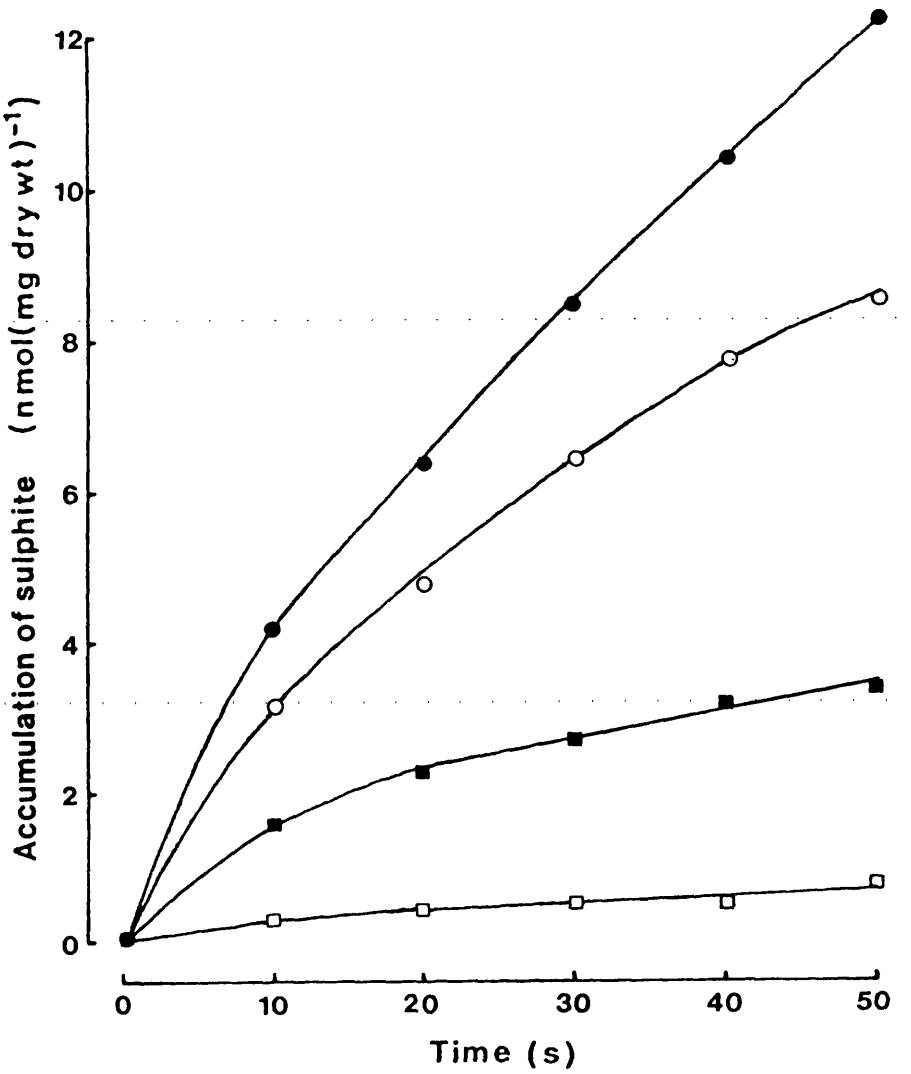



Figure 44. Accumulation of [$^{35}$S] label from sulphite by Saccharomyces cerevisiae TC8 at 30°C and pH 4.0. Concentrations of sulphite present were: 20 μM □ , 101 μM ■ , 203 μM O and 305 μM ● .

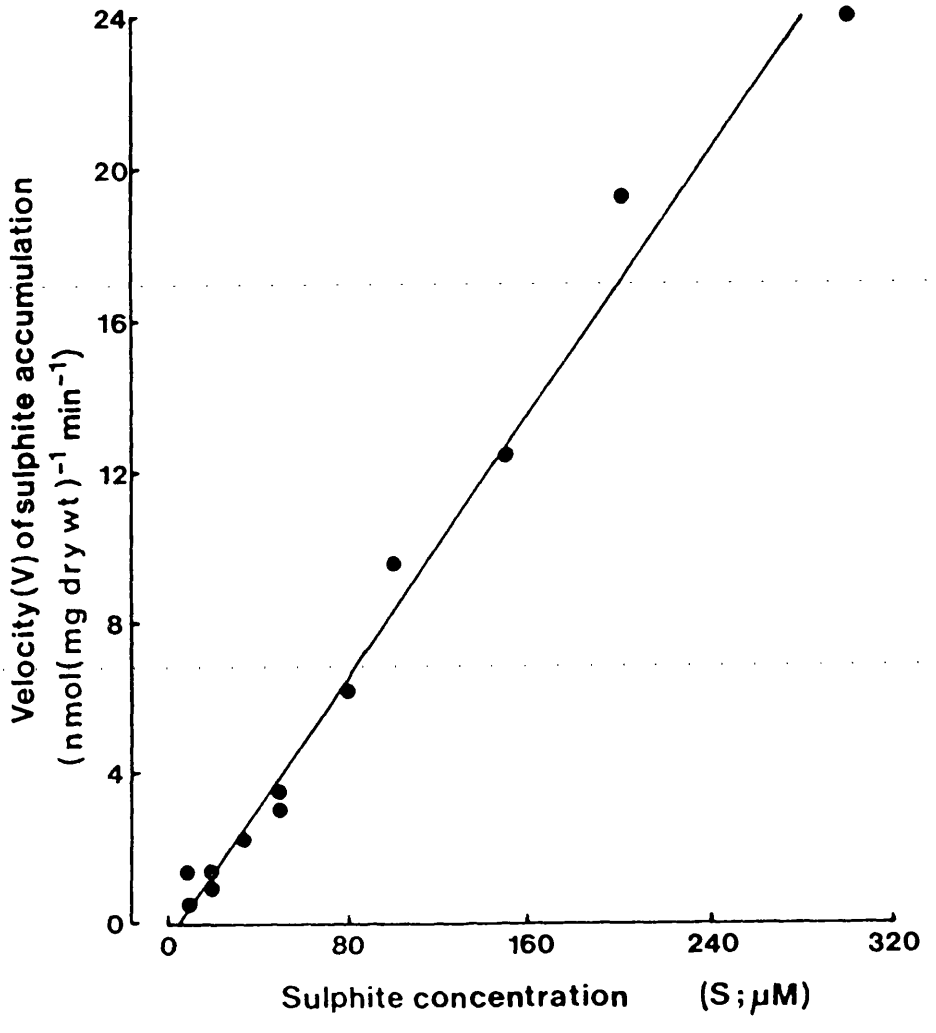



Figure 45. Michaelis-Menten plot of the initial velocity of sulphite accumulation against the sulphite concentration for Saccharomyces cerevisiae TC8 at 30°C and pH 4. Regression analysis $r^2$ = 0.98.

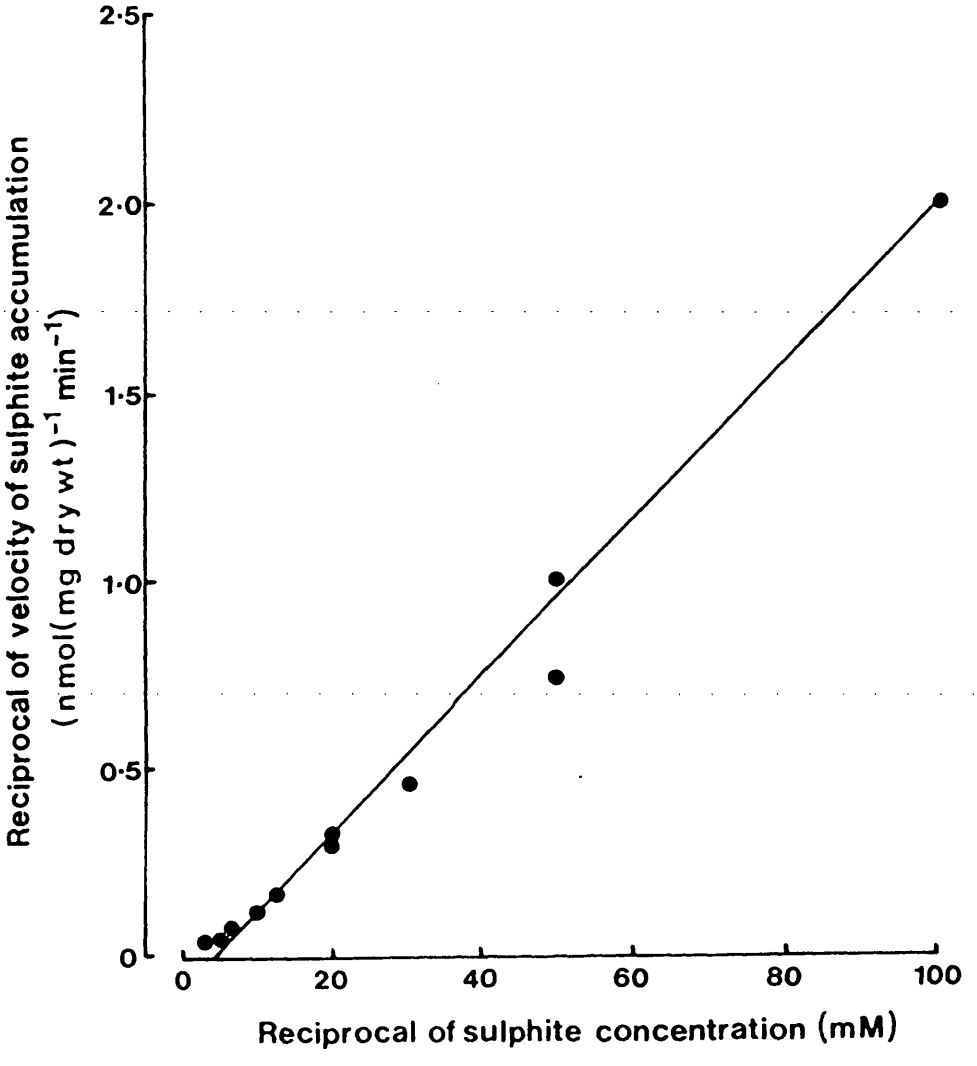



Figure 46. Lineweaver-Burke plot for sulphite accumulation between 0 and 300 μM by Saccharomyces cerevisiae TC8 at 30°C and pH 4.0.

Regression analysis = $r^2$ = 0.98.

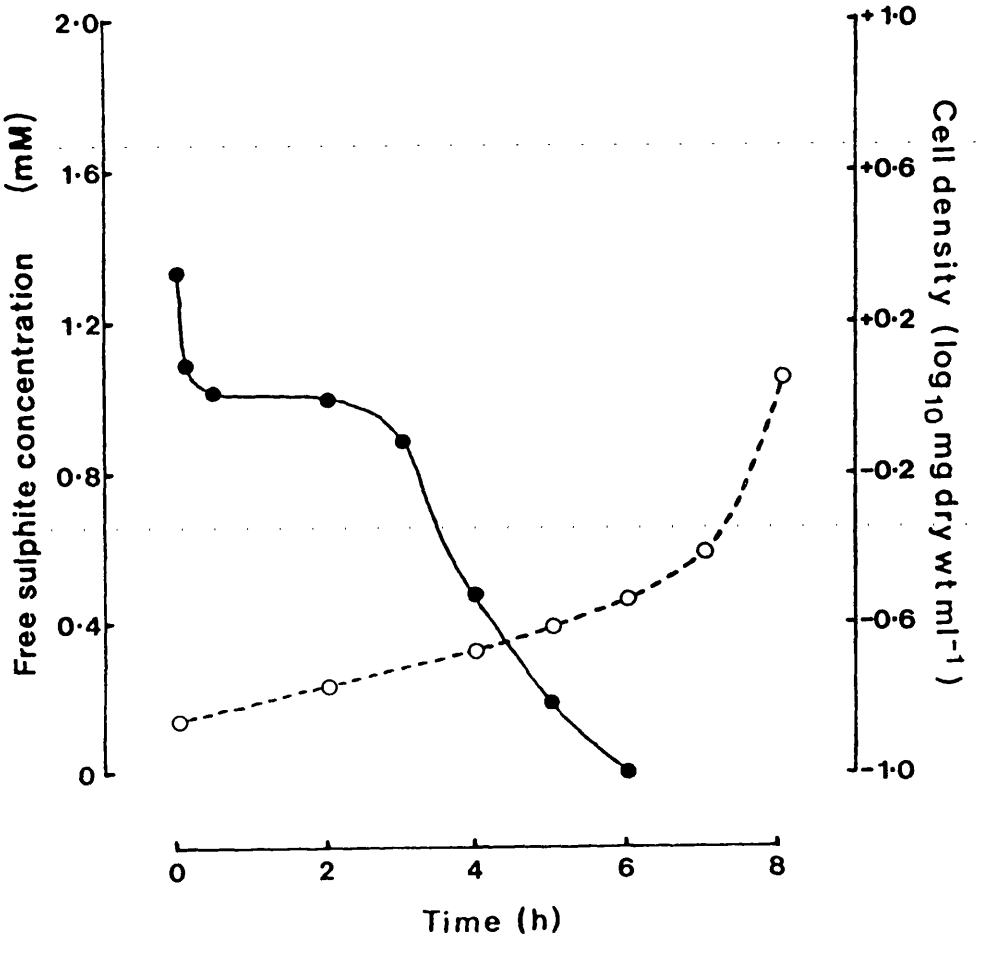



Figure 47. Time-course of growth (O) and of lowering of the concentration of free sulphite (●) in cultures of Saccharomycodes ludwigii.TClO in aerobic medium at pH 4.0 and 30°C.

compound was accumulated rapidly for a few minutes, after which time tracer reappeared in the medium (Fig. 48). At low concentrations (up to 1.5 mM) sulphite was progressively taken up into the yeast cells (Fig. 49). Figure 50 shows that the initial rate of entry was proportional to sulphite concentration. Likewise the double reciprocal plot of these data is linear and passes close to the origin (Fig. 51). Cells of Saccharomycodes ludwigii, either intact or homogenised, contained similar quantities of carbonyl-containing compounds to cells of Saccharomyces cerevisiae strain TC8.

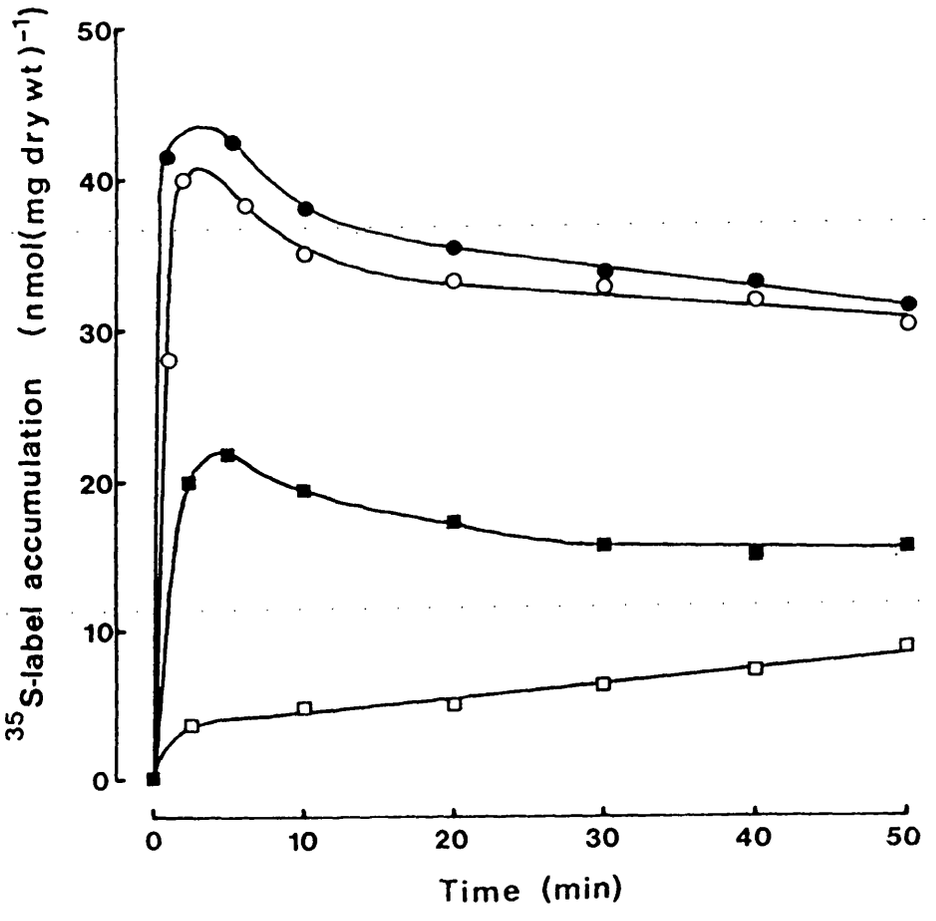



Figure 48. Long-term accumulation of [$^{35}$S] label from sulphite by Saccharomycodes ludwigii TC10 at 30°C and pH 4. Sulphite concentrations were 1.5 mM (□) 6 mM (■), 10 mM (○) and 15 mM (●).

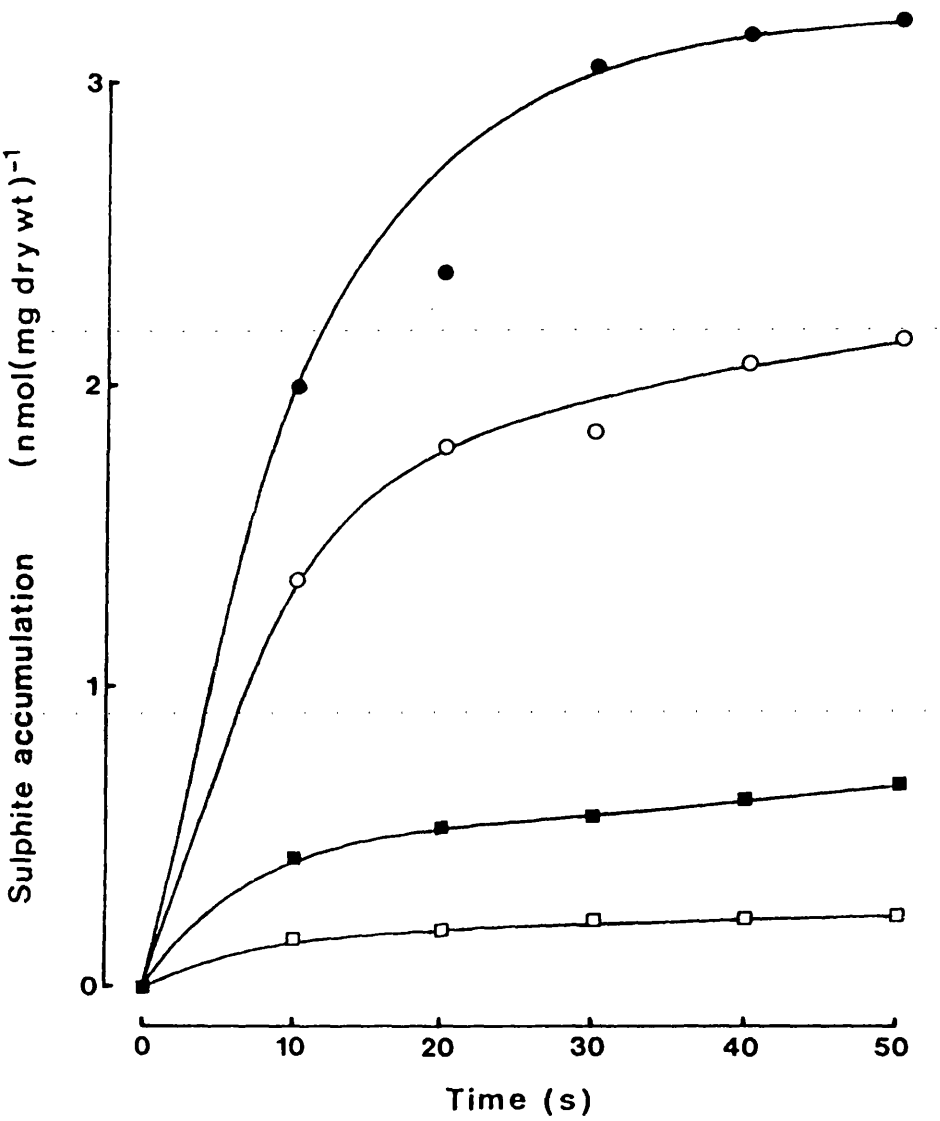



Figure 49. Accumulation of [$^{35}$S] label from sulphite by *Saccharomycodes ludwigii* TC10 at 30°C and pH 4.0. Concentrations of sulphite present were 0.16 mM □, 0.5 mM ■, 1.1 mM ○ and 1.5 mM ●.

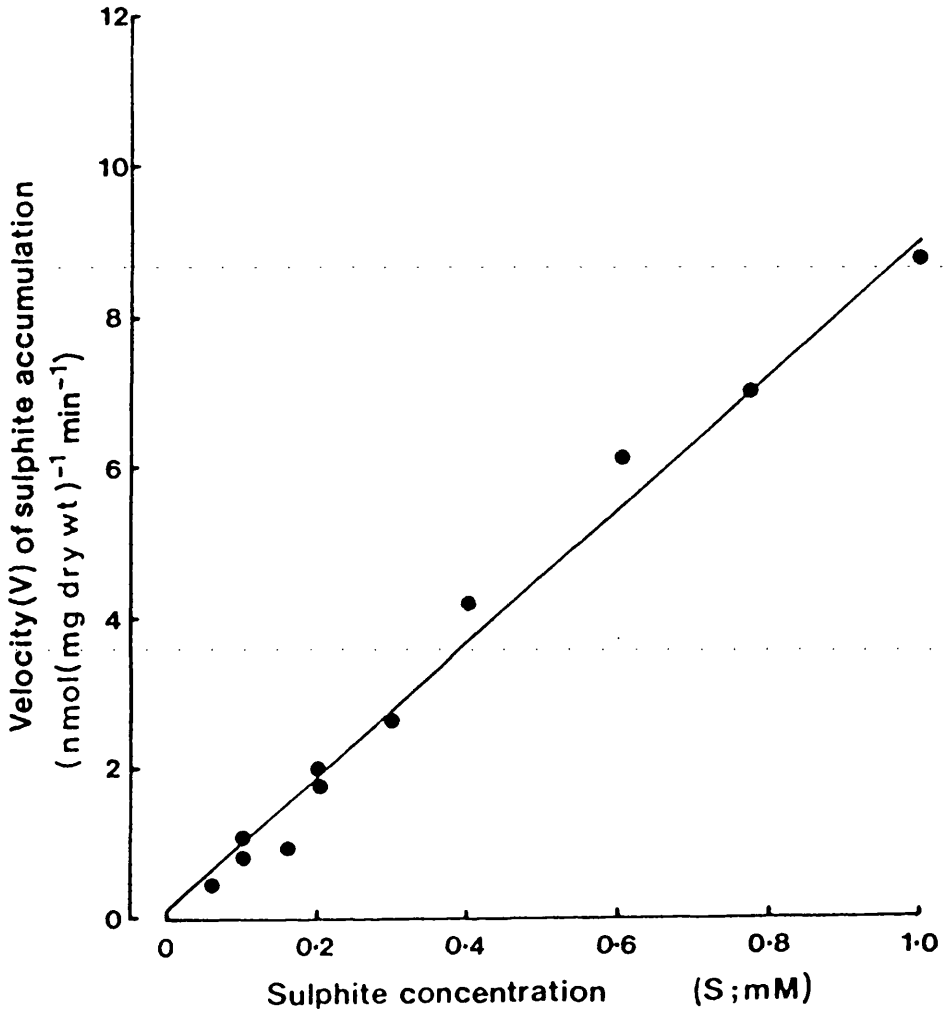



Figure 50. Michaelis-Menten plot of the initial rate of sulphite accumulation against the sulphite concentration for Saccharomycodes ludwigii TC10 at 30°C and pH 4.0. Regression analysis $r^2$ = 0.97.

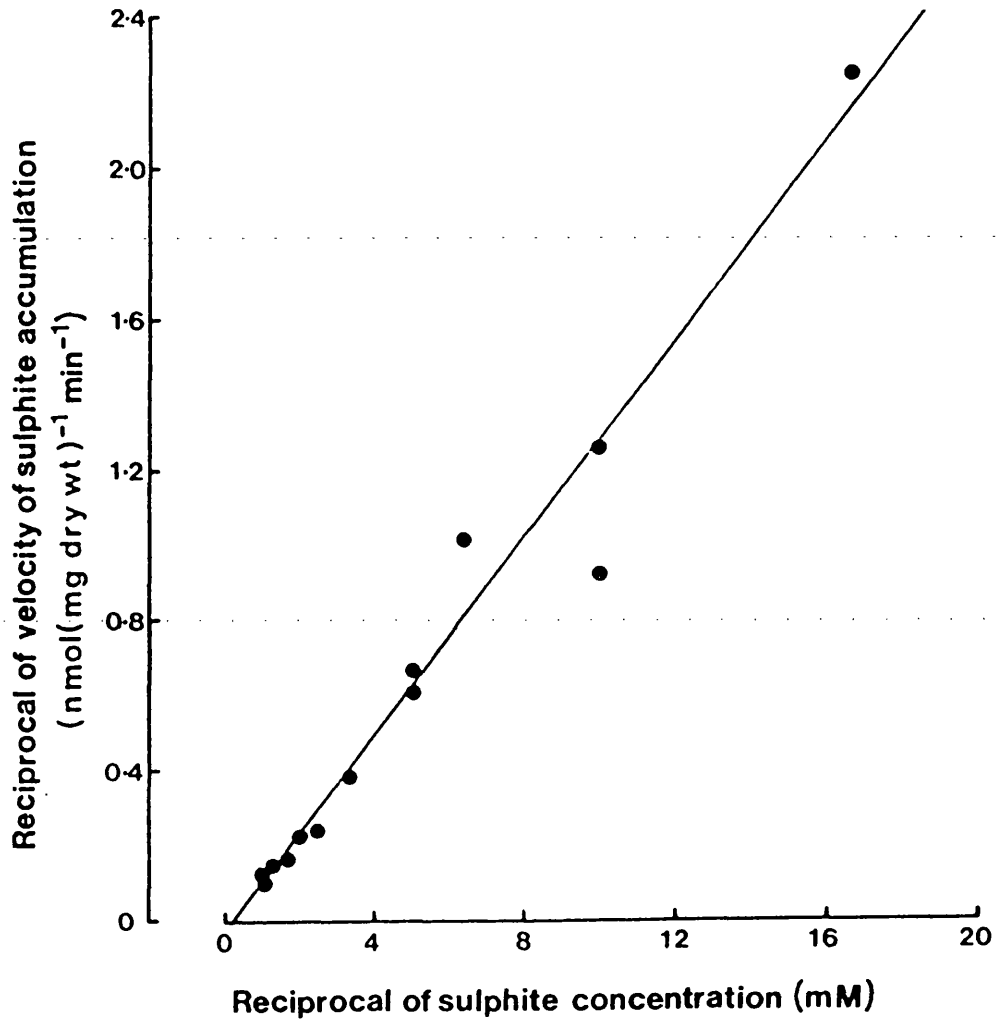



Figure 51. Lineweaver-Burke plot for sulphite accumulation between 0 and 1.07 mM by Saccharomycodes ludwigii TC10 at 30°C and pH 4.0.

Regression analysis $r^2$ = 0.96.

# Discussion

# DISCUSSION

## HYDROGEN SULPHIDE PRODUCTION BY SACCHAROMYCES CEREVISIAE

### Sources of the Sulphur Evolved as Hydrogen Sulphide

Hydrogen sulphide was produced during a discrete period in growth of cultures of Saccharomyces cerevisiae TC8 in enriched apple juice or yeast carbon-base medium. A superficially similar phenomenon has been reported in beer (Nagami et al., 1979; Takahashi et al., 1980). However a more careful examination of the data for beer reveals that this work was completed at relatively low temperatures (9$^{o}$C) in order to produce peaks of hydrogen sulphide production coincident with phases of synchronous cell division. In the present study, hydrogen sulphide was produced as a response by cultures of rapidly growing and synchronously dividing yeast.

Hydrogen sulphide was formed in relatively large quantities, up to 265 μmol $l^{-1}$ of medium. This simplified the question of the origin of the sulphur found emanating as hydrogen sulphide, since only sulphate and sulphite were present in media in concentrations large enough to be responsible for such an amount, unless a major degree of recycling of sulphur sources had occurred prior to hydrogen sulphide production.

Hydrogen sulphide was only produced in media containing appreciable concentrations of sulphite. Two strains of Sacch. cerevisiae, TC16 and 17, which apparently formed small amounts of hydrogen sulphide from sulphate were later discovered to have produced appreciable quantities of sulphite (0.2 mM) from sulphate earlier during exponential yeast growth, and may have utilized this to form

hydrogen sulphide. Of interest to the cider-making industry is the observation that progressively decreasing the sulphite concentrations caused a steady decline in the amount and rate of production of hydrogen sulphide. Lowering the sulphite concentration also caused the lag-phases of yeast growth to be shortened. This will be discussed further in the section concerning sulphite toxicity.

Use of radiolabelled sulphate and sulphite demonstrated that sulphite and not sulphate was being used by yeast as the source of sulphur from which hydrogen sulphide was formed. The rapidity at which radioactive hydrogen sulphide was evolved argues against possible recycling of radiolabelled sulphite prior to reduction to hydrogen sulphide. Indeed, sulphate was not appreciably accumulated under these conditions, possibly because of repression of synthesis of ATP sulphurylase by sulphite as reported by Heinzel and Trüper (1978) or by competitive inhibition of this enzyme by sulphide (Heinzel and Trüper, 1976). Such a loss of enzyme activity would result in a high endogenous sulphate concentration and consequently inhibition of the sulphate permease (Breton and Surdin-Kerjan, 1977). Sulphite accumulation under these conditions was immediate and rapid, but was frequently observed to decelerate around the second minute of the experiment. This was interpreted as an indication of mild sulphite toxicity despite careful pre-incubation of the radiotracer with yeast-free medium taken from previous cultures at this stage of growth. An examination of the disappearance of radiolabel from the medium during such experiments revealed that sulphate concentration did not decline whereas the sulphite concentration did by as much as a third of its original value. These data were supported by chemical analysis of sulphate and sulphite

concentrations. More specifically the sulphite that disappeared was bound sulphite; free sulphite was not detectable in the medium after the time at which yeast growth became exponential. It can be concluded therefore that bound sulphite was the source of sulphur from which hydrogen sulphide was formed.

The source of sulphur used for yeast growth has also to be considered. During growth of cultures, in excess of 2 grams dry weight of yeast were produced. Saccharomyces cerevisiae contains of the order of 0.3% - 0.5% dry weight as sulphur (Maw, 1963a). Using these figures it can be calculated that between 0.18 and 0.31 mmoles sulphur were consumed from the medium during growth of yeast cultures. Only sulphate and sulphite were present in sufficient concentrations to have provided this. Analytical data indicate that the concentration of bound sulphite declined some hours prior to the production of hydrogen sulphide. Furthermore the total drop of bound sulphite concentration was sufficiently large (0.4 mM) to accommodate both the observed hydrogen sulphide production and the estimated sulphur content of the yeast crop. Bound sulphite was probably used both as a source of sulphur for growth and for formation of hydrogen sulphide.

Bound sulphite displays little antimicrobial activity (Rehm, 1964) and moreover it has long been assumed that this form of sulphite plays little part in cellular metabolism or nutrition. Schultz and McManus (1950) claimed the sulphite could be used as a sulphur source, but Macris (1972) cast doubt upon this work. In these studies, relatively large concentrations of bound sulphite were apparently consumed by yeast for growth and reduction to hydrogen

sulphide. Uptake and utilization of bound sulphite could have occurred in one of the following two ways. Firstly the entire bound sulphite complex may be transported, or the bound sulphite complex was cleaved extracellularly and the sulphite moiety transported alone. The kinetics of decomposition of bound sulphite complexes have been examined in order to assess the feasibility of the second explanation. It was discovered that, while the equilibria of free-to-bound sulphite reactions lies very strongly in favour of the bound entity (equilibrium constant $K = 1.42 \times 10^{-6}$ at pH 4.0 and 20°C , for acetaldehyde bisulphite; data supplied by Dr L. F. Burrows). The rate of decomposition and reformation was relatively fast (velocity constant for decomposition of acetaldehyde bisulphite at 20°C and pH 4.0 was $1.01 \times 10^{-3}$). This meant that sulphite could be released from 1.5 mM acetaldehyde bisulphite at a rate of 1.5 μmoles $sec^{-1}$. The maximum rate of hydrogen sulphide formation per litre of medium in the present study was less than 12 nmoles $sec^{-1}$. Thus it would appear entirely feasible that the tiny fraction of free sulphite present with the bound sulphite was efficiently removed by yeast and that bound sulphite was progressively dissociated in order to reform the equilibrium.

In order to investigate whether the entire bound complex was transported or only the sulphite part, uptake of $[^{35}S]$sulphite and $[^{14}C]$pyruvate from sulphite-pyruvate bound complexes was examined. ($[^{14}C]$acetaldehyde was not commercially available). Both sulphite and pyruvate were found to enter the yeast cell although at different rates. However pyruvate accumulation occurred in the absence of sulphite and the rate of accumulation was only marginally diminished if the concentration of free pyruvate was lowered manyfold by the presence of sulphite. This would suggest

that pyruvate was transported alone and that transport was working near the maximum possible rate even at the low concentration of pyruvate in the presence of sulphite. While pyruvate is exported by yeast during growth (Graham, 1979), it has been reported to be re-absorbed at the end of the fermentation (Whiting and Coggins, 1973). As would be expected, the rate of decarboxylation of pyruvate was only marginally affected by the presence or absence of sulphite. Pretreatment of yeast cells with 2-deoxy-D-glucose and antimycin A, to render them deficient in ATP, largely abolished pyruvate transport but left sulphite accumulation barely affected. At pH 6.0 under these conditions sulphite accumulation was lowered These results suggest that pyruvate transport required metabolic energy while sulphite accumulation did not, a system scarcely compatible with the notion that the entire bound sulphite complex was transported. The reduced rate of sulphite transport at pH 6.0 may have been a reflection of the specificity of transport for molecules of $SO_2$. A further piece of evidence concerns the effect of 2,4-dinitrophenol upon uptake of pyruvate and sulphite from the bound complex. The collapse of the membrane proton gradient caused by this compound,caused a cessation of pyruvate accumulation but led to an increase in rate of sulphite accumulation. The arresting of pyruvate accumulation by 2,4-dinitrophenol was consistent with the hypothesis that pyruvate transport was an active process and depended upon a proton gradient for its function. The increase in sulphite accumulation induced by 2,4-dinitrophenol was unexpected and as yet cannot be fully explained. It had been predicted that sulphite accumulation would not have been stopped by 2,4-dinitrophenol if as seemed likely sulphite accumulation from the bound complex was not an active process. The effect of 2,4-dinitrophenol

is discussed at greater length later in this Discussion.

A final piece of evidence indicating that sulphite alone was transported and not the entire bound complex was provided by data on hydrogen sulphide production by yeast cells using several bound complexes other than that of pyruvate-sulphite. Different carbonyl binding compounds at different concentrations caused only slight differences in the rate of hydrogen sulphide formation. It is unlikely that such diverse bound sulphite complexes were taken up and reduced to sulphide by yeast at the same rates. Exposure of yeast cells to certain of the bound complexes undoubtedly led to a degree of sulphite toxicity and after a time loss of viability was observed in some experiments. However in the short term sulphite toxicity did not diminish hydrogen sulphide formation. The use of 2,4-dinitrophenol did not alter the rate of production of hydrogen sulphide despite this reagent's ability to increase sulphite accumulation. A range of free sulphite concentrations had little effect upon the rate of hydrogen sulphide formation and even in the presence of excessive quantities of acetaldehyde (3.0 mM) sufficient free sulphite was available for the normal rate of hydrogen sulphide formation. However very low sulphite concentrations did lead to a smaller amount of hydrogen sulphide being produced thus confirming the earlier results that less hydrogen sulphide was formed in media containing less sulphite. Thus it seems that, at concentrations of free sulphite below a threshold of about 0.5 nM, (calculated from the equilibrium constant) hydrogen sulphide production was limited by the sulphite concentration. Increasing the sulphite concentration above this value up to 1.5 mM free sulphite had little effect, and probably hydrogen sulphide

production was operating at its maximum rate and was thus independent of the sulphite concentration.

## Mechanism of Hydrogen Sulphide Formation

Hydrogen sulphide may be produced by yeast from a variety of sources and by several methods. In this study, several lines of evidence indicate that hydrogen sulphide was being produced as a consequence of sulphite reductase activity. These pieces of evidence are as follows:

i. Production of hydrogen sulphide at different temperatures indicated that the rate of production was increased by a factor of three to four by raising the temperature by $10^{o}$ Centigrade. Such a value of $Q_{10}$ indicates involvement of an enzymic rate-limiting step in formation of hydrogen sulphide.

ii. Hydrogen sulphide was formed from sulphite. It was possible that sulphite was converted into another sulphur source prior to its reduction to hydrogen sulphide but $[^{35}S]$sulphite was reduced to hydrogen sulphide sufficiently rapidly as to make the possibility of sulphite recycling very remote. If sulphite were directly reduced by yeast to hydrøgen sulphide, the only enzyme reported to catalyse this reaction is sulphite reductase.

iii. Hydrogen sulphide was formed after uptake and accumulation of sulphite. Were the hydrogen sulphide formed by external contact of the yeast with the sulphur source, as in production of hydrogen sulphide from elemental sulphur (Schutz and Kunkee, 1977), it is unlikely for there to have been an eleven minute delay in its formation.

iv. Hydrogen sulphide production was suppressable by addition of methionine (0.2 mM), an amino acid which has been implicated in repression of a number of enzymes involved in methionine biosynthesis including sulphite reductase (Cherest et al., 1971; Dott and Truper, 1978). Methionine has also been found to repress formation of hydrogen sulphide (Wainright, 1970b, 1971a) which was assumed to be derived from the activity of sulphite reductase.

v. An assay for NADPH-sulphite reductase revealed that this enzyme was present, and its activity throughout the growth of cultures of Saccharomyces cerevisiae TC8 was proportional to the potential hydrogen sulphide production throughout growth of these cultures.

It is concluded that formation of hydrogen sulphide by Saccharomyces cerevisiae TC8 under the conditions examined almost certainly involved the activity of NADPH-sulphite reductase.

## Causes of Hydrogen Sulphide Production

Ammonium ion concentration invariably reached undetectable levels on or about the time during the growth of cultures that hydrogen sulphide was formed. The final depletion of ammonium ion concentration was, probably a sudden and traumatic event for the yeast. In enriched apple juice medium, after depletion of the α-aminonitrogen content, a certain amount of total nitrogen was still detectable. Presumably this residue was unusable by the yeast. The relevance of ammonium ion depletion to hydrogen sulphide production and yeast

growth was confirmed by addition of ammonium ions to sulphide-forming cultures. Hydrogen sulphide formation was abruptly terminated and yeast growth was resumed. Further depletion of ammonium ion concentration resulted in resumption of hydrogen sulphide formation and cessation of yeast growth. It seems that hydrogen sulphide production was a readily reversible process and the rapidity at which the switch could be made argues against protein synthesis being required for these events. Furthermore a need for protein synthesis was unlikely in view of the fact that such cultures had ceased growing due to nitrogen starvation.

The use of alternative nitrogen sources, namely L-asparagine and L-proline, in media and the consequent production of hydrogen sulphide upon depletion of these nitrogen sources, confirmed the hypothesis that hydrogen sulphide was formed in response to acute nitrogen starvation and not specifically to a deficit in ammonium ions. Production of hydrogen sulphide in enriched apple juice was also caused by nitrogen starvation and this is probably the cause of most hydrogen sulphide production encountered during cider making. There have been suggestions that this also occurs during winemaking. Vos and Gray (1979) found a correlation between the free amino nitrogen content of grape musts and their hydrogen sulphide-producing potential, although Eschenbruch (1974b) could not find any such correlation. This confusion was only to be expected since the situation was complicated by peaking of sulphite reductase activity, and hydrogen sulphide potential during yeast growth. An alteration of available nitrogen concentration could conceivably increase or decrease the rate of hydrogen sulphide

production depending on where in relation to the peak of activity that growth is terminated and hydrogen sulphide is produced. Furthermore, the cidermaker or winemaker is concerned with the rate and amount of hydrogen sulphide produced per litre of beverage, unlike here in relation to the dry weight of yeast cells.

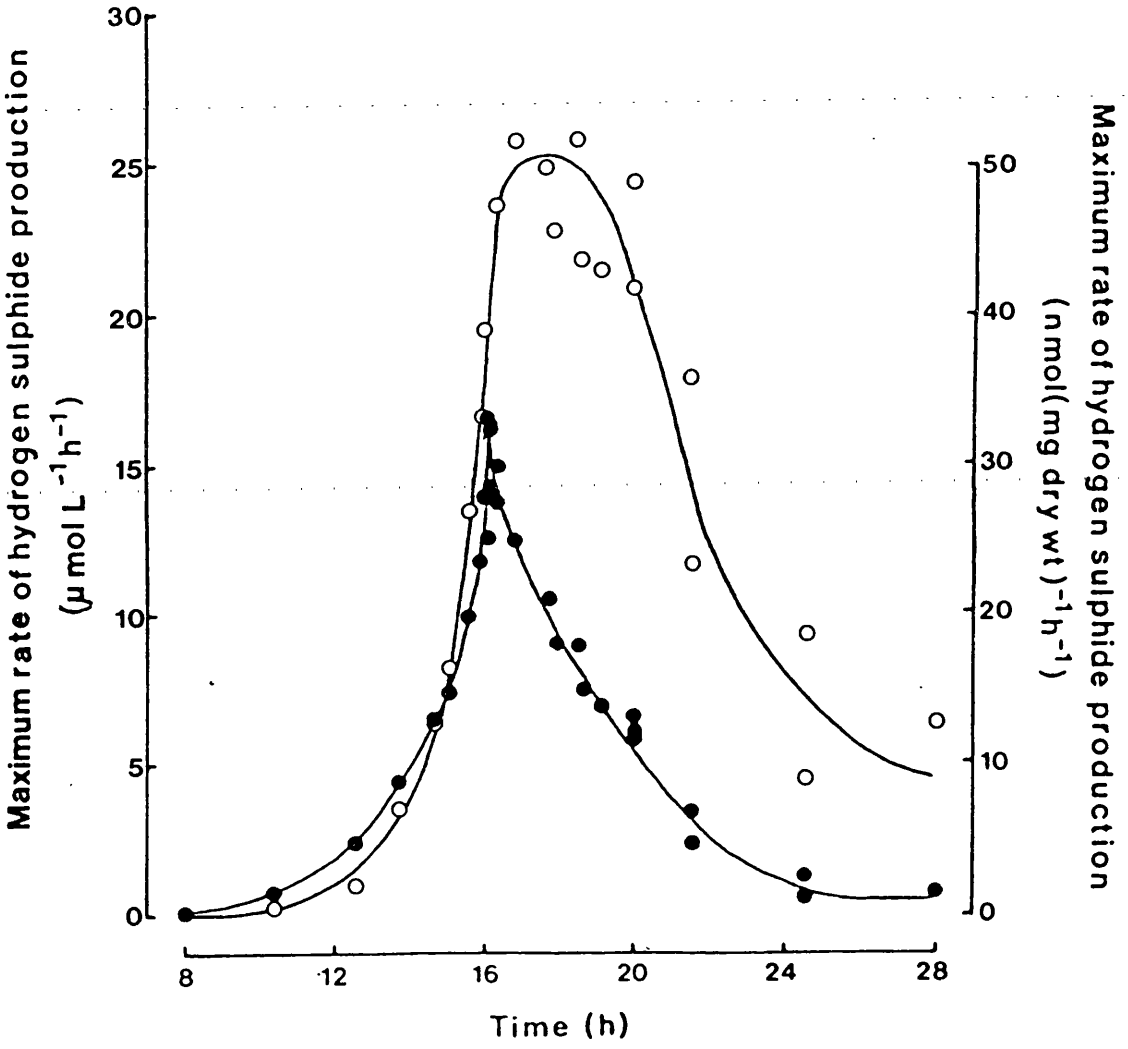



Figure 52.

Maximum rates of $H_2S$ production in relation to the dry weight of yeast (●) (n moles (mg dry wt)$^{-1}h^{-1}$) and to the volume of medium (O) (µmol $L^{-1}h^{-1}$) at different times throughout the growth of Saccharomyces cerevisiae TC8 in enriched apple-juice at 25°C.

Thus it is possible by increasing the free amino-nitrogen concentration to decrease the rate of hydrogen sulphide production per unit of yeast mass but to increase hydrogen sulphide production per litre of medium. It is not surprising therefore that no consistent correlation between the free amino-nitrogen content of grape musts and hydrogen sulphide production has been found.

Artificial Induction of Hydrogen Sulphide

Several inhibitors of yeast metabolism were used to induce hydrogen sulphide formation. Treatment of cultures with iodoacetamide or cycloheximide terminated exponential yeast growth and induced formation of hydrogen sulphide. Hydrogen sulphide was detectable within 15 minutes following addition of inhibitor. Rates of hydrogen sulphide production in both yeast carbon-base medium and in enriched apple juice when adjusted for the concentration of yeast present were found to differ at various stages of growth. A pronounced peak in activity was evident during exponential growth. The peak of activity in yeast carbon-base medium occurred at an earlier stage of growth than that observed in enriched apple juice. In both media, the maximum rate of hydrogen sulphide formation was of the order of 30 - 35 nmol (mg dry weight)$^{-1}$ h$^{-1}$. Rates of hydrogen sulphide formation did not differ whether induced by nitrogen starvation or by addition of inhibitors.

It was very unlikely that synthesis of sulphite reductase was induced in response to factors causing induction of hydrogen sulphide because (i) there was insufficient time and (ii) cycloheximide prohibits protein synthesis. It is possible that inhibition of protein synthesis led to removal of an inactivator

of sulphite reductase, thereby enabling hydrogen sulphide to be formed. However, it is much more probable that sulphite reductase was present in an active form throughout, and that presence of the inhibitors caused a diversion of reduced sulphide from its normal route in methionine biosynthesis thereby leading to liberation of hydrogen sulphide. The second possibility is supported by the discovery of sulphite reductase activity during the growth of Saccharomyces cerevisiae TC8. Sulphite reductase, in the normal course of events, reduces sulphite to sulphide, which passes on to the enzyme homocysteine synthetase to be combined with a carbon-nitrogen skeleton, homoserine or O-acetylhomoserine to form cysteine or homocysteine en route to methionine. Any interference in production and availability of homoserine or O-acetylhomoserine would lead to accumulation of sulphide.

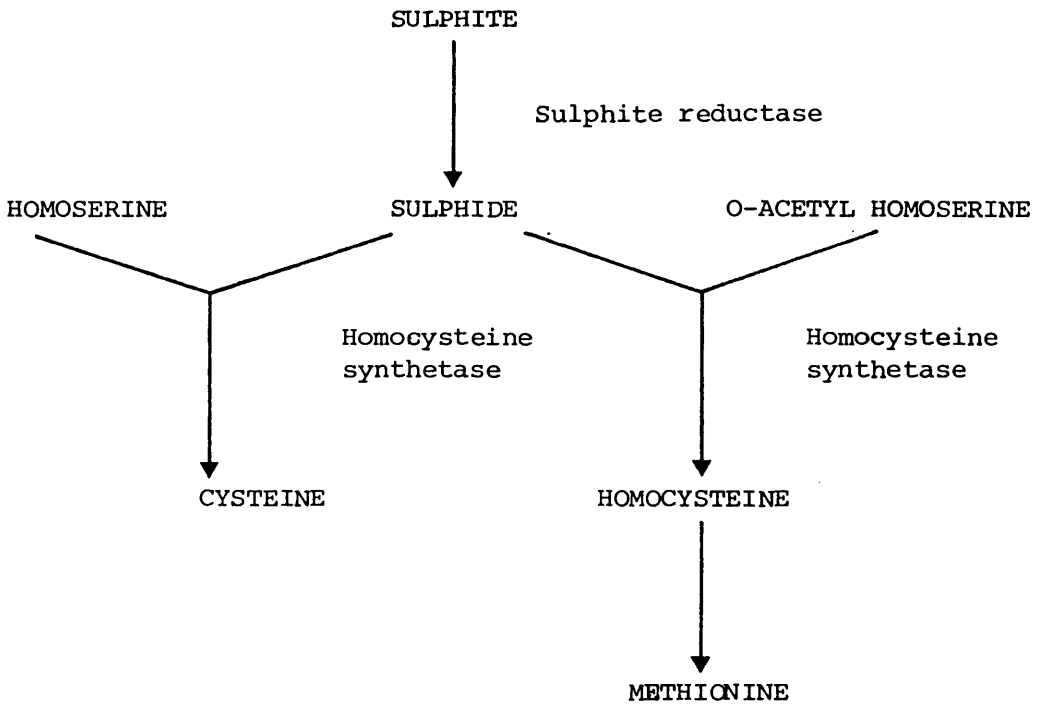



Figure 53.

Later stages on pathways leading to synthesis of cysteine and methionine in Saccharomyces cerevisiae.

Sulphite reductase synthesis is known to be repressed by methionine (Dott and Trüper, 1978), but such a repression would not seem to operate in Saccharomyces cerevisiae in this study, since there could be no build up in concentration of methionine. Such a repression could not have a rapid effect and influence hydrogen sulphide formation after it had started. Cycloheximide treatment demonstrated that protein synthesis was not required for hydrogen sulphide formation and thus no repression of sulphite reductase could have any effect after hydrogen sulphide was produced.

Inhibition of sulphite reductase activity by hydrogen sulphide would seem to be the only possible feedback control available to yeast after hydrogen sulphide formation had been induced. Some slight inhibition of this kind was noted by Yoshimoto and Sato (1968a, b). The yeasts Saccharomyces cerevisiae TC8 and NCYC 366 seem to lack any inhibition and indeed Saccharomyces cerevisiae NCYC 366 produces hydrogen sulphide throughout its growth. The yeasts Saccharomyces cerevisiae TC16 and TC17 however do possibly display a measure of such control since hydrogen sulphide was formed at a low rate and this ceased relatively quickly. It is easy to understand why little hydrogen sulphide was formed when yeast strains were grown using sulphate as the sulphur source, since there exists a barrage of repressive and allosteric inhibition aimed at the first stages of the sulphate reduction sequence (Heinzel and Trüper, 1976; 1978).

It can be deduced that hydrogen sulphide production from sulphite would be relatively difficult to stop. Sulphite entry, as discussed earlier, appears to be by diffusion and is thereby uncontrollable. Only

upon the sulphite reductase enzyme may controls be imposed. A negative feedback by sulphide would be an obvious control on this enzyme and possibly a positive control by O-acetylhomoserine such that sulphite reductase could only operate in its presence. In Saccharomyces cerevisiae TC8 any such controls appear to be lacking or operating very inefficiently.

If hydrogen sulphide were produced from that reduced sulphide that was destined for incorporation into sulphur-containing cell constituents, it might be expected that the rate of hydrogen sulphide formation would be related to the expected sulphur content of the yeast which would otherwise have been formed in this period. In the exponential growth phase 0.68 mg dry weight of yeast would be expected to increase to 1 mg cells during an hour. Using the value of 0.4% sulphur in yeast cells (Maw, 1965) this increase represents 1.28 µg sulphur which is a rate of accumulation of 40 nmol hydrogen sulphide (mg dry wt)$^{-1}h^{-1}$. This bears reasonable comparison with the observed rates of hydrogen sulphide formation (30 - 35 nmol (mg dry wt)$h^{-1}$)

Nitrogen starvation, it is easy to envisage, would cause depletion of intracellular concentrations of homoserine and O-acetyl-homoserine, thereby causing sulphide to be liberated. Cycloheximide causes inhibition of protein synthesis by preventing chain initiation and elongation (Cooper and Bossinger, 1976). A consequence of this would be a build up of amino acids within the cell leading to a retardation of further amino-acid synthesis (Messenguy, 1979). This would in turn prevent further synthesis of homoserine and O-acetyl-homoserine. Actinomycin D had surprisingly little effect either

upon yeast growth or hydrogen sulphide formation. It was discovered however that Saccharomyces cerevisiae is largely immune to the action of this inhibitor of RNA synthesis (Gorenstein et al., 1978) due to poor permeation of the cells (Schindler and Davis, 1975). An explanation of iodoacetamide induction of hydrogen sulphide formation is not immediately apparent. Blockage of glycolysis caused by this inhibitor may prevent assembly of carbon skeletons for formation of homoserine and O-acetylhomoserine, or it may act by depleting the cell of energy, thereby preventing metabolism of aspartate to O-acetylhomoserine which requires ATP. The lack of effect of ethanol or acid shock in the induction of hydrogen sulphide was predictable and only served to confirm that the high concentration of ethanol and low pH value in existence at the end of fermentations were unlikely to cause evolution of hydrogen sulphide.

It is probable that a great many other potent causes of hydrogen sulphide formation have yet to be discovered. Wainwright (1970 a,b; 1971 a,b) cited deficiencies of the vitamins pantothenic acid and pyridoxine as being causes of hydrogen sulphide formation. His explanation for this rather needlessly involved methionine as an intermediary. Probably any inhibitor or treatment abiding by the following conditions would cause hydrogen sulphide formation. These conditions are that (i) NADPH formation is unimpaired, (ii) homoserine and O-acetylhomoserine production is stopped, and (iii) sulphite reductase activity is not impaired.

## Factors Influencing Hydrogen Sulphide Formation

### (i) Yeast Strain

Rankine (1963, 1968) reported considerable variation among yeast strains in ability to produce hydrogen sulphide. Strains examined in the present study varied by as much as a factor of 10 both in amount and in the rate of production of hydrogen sulphide. Saccharomyces cerevisiae TC16 and TC17 produced little sulphide while TC8 and NCYC 366 produced vast amounts. The decline in hydrogen sulphide production by these yeasts resembled the decay of radioactive isotopes, with half lives of about 2.5 to 3 hours. This may be a reflection of rates of decay of the sulphite reductase activity in yeast populations. In strains TC16 and TC17 the decline was very much faster and may indicate inhibition or degradation of sulphite reductase.

### (ii) Rate of Yeast Growth

Factors influencing the rate of yeast growth, such as temperature, degree of aerobiosis, and rate of stirring, were all found to alter the rate of hydrogen sulphide formation. This confirms reports of a number of other workers that fast fermentations and aerobic conditions favour production of hydrogen sulphide (Ricketts and Coutts, 1951a; 1951b; Rankine, 1963; Wainwright, 1971a; Dott and Trüper, 1976). Since rate of hydrogen sulphide production in Saccharomyces cerevisiae TC8 was of the same order of magnitude as the calculated rate of accumulation of sulphur in yeast, sulphite reductase activity was probably adjusted to keep pace with yeast growth and availability of NADPH was not a rate governing issue.

(iii) Methionine

Methionine suppression of hydrogen sulphide formation via repression of sulphite reductase has for several years been known. In the present study, a 50% dimunution of the rate of hydrogen sulphide production was achieved by the inclusion of methionine (0.2 mM) in the medium. It was considered possible that methionine concentration could be partly responsible for time-course of changes in sulphite reductase activity and formation of hydrogen sulphide throughout growth of yeast Saccharomyces cerevisiae TC8. However over the range examined, methionine caused a similar reduction in rate of hydrogen sulphide formation at several stages in growth of the yeast, and there is no evidence of a greater effect earlier on in the fermentation when methionine would be expected to be present at a higher concentration. Possibly sequestration of methionine in the form of S-adenosyl methionine is involved (Sumrada and Cooper, 1978). It is known that the vacuolar contents, including S-adenosyl methionine, are released into the cytoplasm under conditions of nutrient deprivation (Law and Ferro, 1980). Such a release might be partly responsible for the gradual decline in activity after this had peaked. This aside, there is probably still much to be researched concerning factors governing the activity of the sulphite reductase in different stages of yeast growth.

## Production of Thiols

Only very low concentrations of volatile thiols were detected in cultures examined in the present study. Greatest amounts were produced by cultures producing large amounts of hydrogen sulphide. It is probable that thiols were formed by reaction of sulphide with medium components (Rankine, 1963) and the small concentrations

found were probably a reflection of efficient removal of hydrogen sulphide by sparging of gas through the medium.

## SULPHITE TOXICITY AND TRANSPORT IN SACCHAROMYCES CEREVISIAE AND SACCHAROMYCODES LUDWIGII

### Effects of Sulphite on Growth and Viability

Saccharomyces cerevisiae TC8 was more resistant to sulphite than strains X2180 Aα and NCYC 366. This may have been a reflection of the fact that Saccharomyces cerevisiae TC8 is a strain used in cidermaking during which sulphite treatment occurs and some selection for a sulphite-tolerant strain is likely.

Sulphite at low concentrations (0.1 mM total sulphite) barely affected fermentations; viability was unaltered while the duration of the lag-phase was marginally increased in duration. Further increases in sulphite concentration progressively increased the duration of the lag-phase, to such an extent that on one occasion a lag-phase duration of 150 hours was recorded before exponential yeast growth occurred. The shapes of growth curves obtained from sulphited cultures were altered by the presence of sulphite. Growth, after prolonged lag-phases, was slow for several hours before exponential growth began. The eventual rate of exponential yeast growth was identical in sulphited and control cultures.

It should be recalled that similar prolongation of lag-phase and subsequent slow growth occurred both in yeast carbon-base medium and in enriched apple juice. During the long lag-phases there was a gradual decline in free sulphite concentration. This happened

faster than the rate of oxidation of sulphite to sulphate, as measured by the rate of sulphite oxidation in yeast-free controls. Since no such rapid change was observed in total sulphite concentrations, it was assumed that the yeast was lowering the free sulphite concentration by secretion of sulphite-binding agents. Slow growth of yeast began while traces of free sulphite were still present, and exponential yeast growth was only initiated after free sulphite had completely disappeared from the medium.

Loss of viability of yeast was observed when sulphite concentrations exceeded certain threshold values which were dependent upon the strains of yeast. The effects of sulphite concentrations close to these threshold concentrations were examined. Thus phenomena which require high concentrations of sulphite could be distinguished from those reactions which led to death of yeast cells.

## Effect of Sulphite on Transport of Solutes

Addition of sulphite to yeast cell suspensions during measurement of accumulation of L-lysine or L-arginine caused an inhibition of transport. An interval of several minutes elapsed between addition of sulphite to suspensions and a decline in rate of accumulation. Increasing the concentration of sulphite caused this interval of time to become slightly shorter and the degree of inhibition to increase. Even at the highest concentrations of sulphite examined (1.5 mM total sulphite) there was no evidence of loss of solute from cells, making it unlikely that the integrity of the plasma-membrane had been altered by addition of sulphite.

Transport of L-lysine and L-arginine is an active process involving

membrane-bound proteins (Eddy, 1982) and it is possible that the observed inhibition was due to conformational distortion of these proteins by sulphite in the manner suggested by Clarke (1932). Alternatively the inhibition could have been caused by interference of sulphite with the energy metabolism of the cell and thereby denying active transport processes a source of energy. As a third possibility, sulphite may have caused an inhibition of transport of L-lysine and L-arginine by directly collapsing the membrane proton gradient necessary for active transport to occur.

Sulphite was not observed to affect significantly the net rate of accumulation of radiolabels from D-glucose, implying that sulphite does not influence the rate-limiting step in glucose transport and accumulation. Uptake of glucose by Saccharomyces cerevisiae has been reported to be a facilitated diffusion process (Eddy, 1982). Were sulphite to be causing conformational distortion of those proteins involved in the transport of L-lysine and of L-arginine, it would be unlikely that similar effects would not also occur in those proteins concerned with transport of D-glucose. In this light, it seems more likely that sulphite inhibits transport of L-lysine and L-arginine by affecting the energy source rather than by directly acting on the proteins .

Sulphite Action on Glycolysis

Sulphite lowered the rate of carbon dioxide evolution by anaerobically-grown yeast cells. Shortly after addition of sulphite the rate of carbon dioxide evolution declined to some 40% of its original value. In complete contrast, evolution of carbon dioxide by aerobically-grown cells was not impaired by such an addition of sulphite. However, the rate of oxygen utilization, was diminished by sulphite.

This last point is in agreement with Rehm (1964).

The difference in inhibition of carbon dioxide evolution between anaerobically and aerobically grown cells, was explicable in several ways. Most of the carbon dioxide produced by Saccharomyces cerevisiae in the presence of glucose arises through the activity of pyruvate decarboxylase. The carbon dioxide is formed from the carbon atoms originally in positions 3 and 4 in the glucose molecule. Inhibition of sulphite in anaerobically-grown cells obviously operated at a point between uptake of glucose and pyruvate decarboxylase. Either aerobically-grown cells avoided such an inhibition or increased carbon dioxide was evolved by an alternative decarboxylation reaction, such as that found in the reductive pentose phosphate pathway. Operation of such a reaction would provide an increased proportion of carbon dioxide, originally derived from position 1 in the glucose molecules.

These possibilities were investigated by use of glucose, radio-labelled at specific positions, and radioactivity present in the carbon dioxide evolved from the different sources was measured. Rates of carbon dioxide evolution from [U- $^{14}$C]glucose confirmed those results obtained by Warburg manometry, both in rates of production and in effects of sulphite. Aerobic formation of carbon dioxide was barely altered while formation of carbon dioxide by anaerobically-grown cells was inhibited . It was discovered that evolution of carbon dioxide from [1- $^{14}$C]glucose was not affected by sulphite, either aerobically or anaerobically, while evolution from [6- $^{14}$C]glucose was inhibited under both conditions. The pentose-phosphate pathway activity is customarily estimated by

subtracting the rate from position 6 from that rate derived from position 1.

Decarboxylation rates using the pentose phosphate pathway were marginally increased by treatment with sulphite. Obviously any blockage of glycolysis by sulphite must occur at a stage later than formation of glucose-6-phosphate. Sulphite affects sugar catabolism subsequent to formation of glucose-6-phosphate anaerobically, but not aerobically, although an explanation of this is not immediately apparent. The low rates of decarboxylation of [6- $^{14}$C]glucose confirmed that little TCA cycle decarboxylation was occurring, probably due to the presence of glucose.

A hypothesis that fits all of the known facts is as follows: In anaerobic cells, sulphite binds intracellular acetaldehyde. This prevents regeneration of oxidized $NAD^+$ by ethanol dehydrogenase. It has been stated that 'the main physiological function of ethanol dehydrogenase in yeast is the regeneration of $NAD^+$ during the fermentative breakdown of sugars' (Ciriacy and Breitenbach, 1979). Shortage of $NAD^+$ would prevent the activity of glyceraldehyde-3-phosphate dehydrogenase and thus prevent flow of intermediates through glycolysis and further decarboxylation of pyruvate. Accumulation of glyceraldehyde-3-phosphate would promote formation of glycerol by sulphited yeast (Freeman and Donald, 1957) and would regenerate some $NAD^+$. However no net energy, as ATP, could be formed by this means. Schimz and Holzer (1974) reported a rapid decline in the ATP content of sulphited cells. In aerobic cells, the rate of decarboxylation by pyruvate decarboxylase was maintained in the presence of sulphite, in spite of binding of sulphite to

acetaldehyde. Probably, $NAD^+$ was regenerated using oxygen as the terminal electron acceptor. Little damage could thus arise to aerobic cells from sulphite binding to acetaldehyde.

Many theories have been advanced to explain sulphite toxicity yet the issue is still a matter for speculation. Were a limited quantity of sulphite to be mixed with yeast cell contents it would be logical to expect to find sulphite bound to those cellular constituents with which sulphite has greatest affinity. Acetaldehyde probably ranks top of these. Work by Macris,(1972) revealed that sulphite was associated with the small molecule fraction of the cell constituents, a fraction undoubtedly containing acetaldehyde. Sulphite also causes a rapid depletion of cellular ATP content (Schimz and Holzer, 1979) in a manner identical to that observed when cultures of yeast mutants blocked at different points in glycolysis, were placed in glucose-containing media (Ciriacy and Breitenbach, 1979). The sulphite-activated ATPase postulated by Schimz (1980) seems unnecessary to account for the observed phenomena of ATP depletion. Inhibition of anaerobically-grown yeast by sulphite may be explained in terms of binding of sulphite to acetaldehyde thereby depriving glycolysis of the $NAD^+$ required for further activity. However in aerobically grown yeast glycolysis has been demonstrated to be unaffected by sulphite treatment, and another mechanism is required to explain the observed yeast inhibition and energy depletion in aerobic cells.

Sulphite may act by preventing ATP generation from glycolysis or by increasing the rate of utilization of ATP by the yeast. In the first instance, sulphite would be acting as an uncoupler

of glycolysis, enabling intermediate flow to be maintained while preventing generation of ATP from this flow. A possible mechanism for this could arise by binding of sulphite to glyceraldehyde-3-phosphate thereby impairing formation of ATP from this molecule while not preventing its further metabolism.

Schimz and Holzer (1979) speculated that sulphite could enhance utilization of ATP by yeast by activating ATPase. However, no evidence exists for such activation. A more likely alternative method of enhancing ATP utilization was recently suggested by Dr. A.D. Warth (personal communication). He suggested that acid antiseptics, including sulphite, exert their toxic effects by lowering the internal pH value of cells. When uncharged molecules of antiseptic enter the interior of cells they became ionized due to the higher pH value within yeast cells, and thereby liberating protons which lower the internal pH value. Such a production of protons within the yeast would cause the membrane proton gradient to disappear and the yeast to expend ATP in a vain attempt to expel the protons and re-establish the gradient. This would continue until the concentration of molecular antiseptic on either side of the membrane was equal, by which time irreversible damage may have been caused by ATP depletion. Binding of sulphite to carbonyl-containing compounds would also liberate protons and could not act to detoxify the antiseptic.

Whether either or both of energy uncoupling or of enhanced energy utilization are occurring in aerobic yeast treated with sulphite, is impossible to determine from the existing data. The decline of ATP content of yeast cells is similar in those treated

with sulphite (Schimz and Holzer, 1979) to those mutants blocked in glycolysis (Ciriacy and Breitenbach, 1979). This suggests that glycolytic uncoupling may be occurring. However to test this suggestion, yeast cells would have to be treated with several acid antiseptics as well as sulphite. If equal toxic effects were found in all experiments when equal antiseptic molecules were transported, sulphite would be acting only by lowering the internal pH value. If unequal toxic effects were found, this would demonstrate the existence of additional toxic effects by some of the antiseptics.

## Transport of Sulphite by Saccharomyces cerevisiae

A solution of sulphite, in the range of pH values examined, contains a mixture of sulphite and bisulphite ions and molecular sulphur dioxide. Transport was measured by accumulation of radioactive atoms and it is not possible to state which of the readily interchangeable sulphur dioxide, sulphite or bisulphite ions is being transported. To overcome this difficulty in terminology, the word 'sulphite' will be used to indicate collectively sulphur dioxide, bisulphite and sulphite ions, and 'sulphite ion' to mean the form of sulphite present at higher pH values, $SO_3^{2-}$.

To date, sulphite transport has been described by workers in one laboratory (Macris and Markakis, 1974). It was concluded as a result of this examination that sulphite transport was an active saturable process which probably involved a sulphite permease. These conclusions are called into question by the results obtained in the present study and an explanation of how such misleading conclusions arose will be forthcoming.

Sulphite (1.5 mM) entered cells of Saccharomyces cerevisiae TC8 at a fast rate for up to three minutes after which time sulphite was lost from the cells progressively. An examination of the initial rates of transport at low concentrations of sulphite revealed that initial rates of accumulation were proportional to the concentrations of sulphite. Also the plateau heights of accumulation, levels at which accumulation stopped, were also proportional to sulphite concentration. This evidence is explicable only in terms of a diffusion process. Furthermore the Lineweaver-Burke plot of these data is linear, and passes very close to the origin. This is strong evidence that sulphite transport is a diffusive process.

Sulphite was accumulated up to a level of 45 nmol (mg dry wt)$^{-1}$ from 1.5 mM total sulphite. This represents a concentration of sulphite inside the yeast of the order of thirtyfold. How then can sulphite be concentrated by diffusion? Sulphite can be drawn into yeast cells by a combination of two methods.

(i) The pH value inside the yeast cells is fairly close to neutrality. Macris (1972) has demonstrated that only molecular sulphur dioxide is transported. Thus sulphite will be accumulated by diffusion until the concentration of molecular sulphur dioxide inside the cell equals that outside, which due to the pH difference means a concentration of total sulphite inside the cell of at least 20 times.

(ii) Binding of sulphite inside yeast cells will lower the internal molecular sulphur dioxide concentration and further accumulation of sulphite would occur until the molecular sulphur dioxide concentration gradient across the plasma membrane is zero. The contribution that sulphite-binding makes to accumulation of

sulphite is difficult to estimate but would be dependent upon concentration of binding agents within the yeast.

Macris (1972; Macris and Markakis, 1974) reported that the sulphite permease in Saccharomyces cerevisiae (var. ellipsoides had a Km value for sulphite of $2.65 \times 10^{-2}$ M and a $V_{max}$ value of $41.7 \times 10^{-3}$ mol $min^{-1}$. This Km value indicates an incredibly low affinity for the permease while the enormous $V_{max}$ value is unusual to say the least. Macris and Markakis (1974), while demonstrating the specificity of transport for molecular sulphur dioxide, showed that the transport process does not display an optimum pH value. The irreversible inactivation of sulphite uptake by temperature occurs at a temperature ($60^{o}C$) more consistent with thermal inactivation of central metabolism and yeast cells as a whole than the thermal inactivation of transport processes which are inhibited at lower temperatures ($40^{o}C$). Such high temperatures could prevent sulphite concentration by diffusion through loss of the pH gradient across the membrane and inactivation of the metabolism producing sulphite-binding compounds. The specificity of the transport process cited by Macris (1972) as evidence for a mediated process is equally valid as evidence for diffusion since only molecular sulphur dioxide would diffuse readily through the hydrophobic interior of the plasma membrane (Jacobs, 1940; Ingram et al., 1956). The use of metabolic inhibitors to prevent sulphite transport (Macris, 1972) was inconclusive, with only slight inhibition of transport by 2,4-dinitrophenol being found. This inhibition may have been due to collapse of the membrane proton gradient caused by this inhibitor.

Sulphite transport in Saccharomyces cerevisiae appears to occur as passive diffusion. There is no real evidence of active transport or of a mediated process. However, the diffusion is dependent indirectly upon active cell metabolism for maintenance of a pH gradient and for formation of sulphite-binding compounds. In view of this sulphite transport is dubbed a pseudoactive process.

The effect of 2,4-dinitrophenol upon sulphite accumulation was most unexpected. A similar occurrence was noted during sulphite uptake from bound sulphite-pyruvate complexes. This inhibitor is reputed to enable free passage of protons across the plasma membrane thereby collapsing the pH gradient across the membrane. It would have been expected that such a collapse would precipitate a rapid loss of sulphite from the yeast cells. Perhaps the drop in internal pH value causes a tighter binding of sulphite thereby lowering the free sulphur dioxide concentration and causing further sulphite to diffuse in.

## Resistance of Saccharomycodes ludwigii to sulphite

The resistance of Saccharomycodes ludwigii to sulphite toxicity is legendary in the cider industry. In this study, Saccharomycodes ludwigii strain TC10 was inhibited by sulphite concentrations ten-fold greater than those required to have a similar effect upon most strains of Saccharomyces cerevisiae. But, apart from the far greater sulphite concentrations required, Saccharomycodes ludwigii responded to sulphite treatment in a very similar fashion to that recorded in Saccharomyces cerevisiae. Increased concentration of sulphite caused progressively longer duration of lag-phase in Saccharomycodes ludwigii. Through the duration of lag-phase, the

concentration of free sulphite in the medium declined, reaching undetectable concentrations shortly before exponential growth began. This decline in free sulphite concentration in the medium, was achieved by secretion of sulphite-binding compounds by Saccharomycodes ludwigii. Analysis of the binding compounds revealed that they consisted almost entirely of acetaldehyde, (P. Morgan, personal communication), the residue being pyruvic acid. The quantity of acetaldehyde produced during lag-phase growth of Saccharomycodes ludwigii was so great as to preclude the possibility that it arose from cell reserves. It was almost certainly formed from break-down of glucose, despite the lack of growth of these cultures. After the free sulphite concentration present in the medium had reached zero and exponential growth had begun, production of acetaldehyde ceased. No such production of binding compounds was observed in cultures of Saccharomycodes ludwigii that had not been treated with sulphite. This implies that the production of acetaldehyde is a direct response to the presence of sulphite.

An examination of sulphite accumulation by Saccharomycodes ludwigii showed that rates of uptake were very much slower than those observed in Saccharomyces cerevisiae but the general manner of accumulation was similar with an initial high rate of uptake reaching a peak followed by a subsequent progressive loss of radio-label from the yeast cells. An examination of the initial rates of transport at low concentrations of sulphite revealed that sulphite concentrations were proportional to the initial rate of uptake observed. The Lineweaver-Burke plot of these data was also linear and passed very close to the origin. Thus it appears that sulphite transport in Saccharomycodes ludwigii is by diffusion.

An interesting observation was that, at the sulphite concentrations that begin to cause loss of cell viability (10 mM for Saccharomycodes ludwigii and 1.5 mM for Saccharomyces cerevisiae) , very similar amounts of sulphite were found accumulated in the cells, 40 - 45 nmol (mg dry wt)$^{-1}$. In other words when this concentration of sulphite is achieved internally in cells of either yeast, loss of viability occurs. This implies equal sensitivity to sulphite of the internal cell metabolism of both yeasts and, therefore, that the mechanism of sulphite resistance in Saccharomycodes ludwigii lies only in the uptake of sulphite by this yeast.

Having already established that the mechanism of sulphite transport in both Saccharomyces cerevisiae and Saccharomycodes ludwigii is probably simple diffusion, how then may diffusion be altered in Saccharomycodes ludwigii to enable it to be 10-fold more resistant to sulphite? Two factors may be involved in this: firstly the rate of diffusion, and secondly the equilibrium point the eventual concentration of sulphite within the cell after all net transport has ceased.

The rate of diffusion may be altered by the composition of the plasma membrane. The lipids present in Saccharomycodes ludwigii are unusual, in that there is a high proportion of $C_{18:1}$ (oleyl) residues in the membrane (Kaneko et al., 1976),as compared with Saccharomyces cerevisiae which contains a higher proportion of the shorter chain fatty acids. This could conceivably have an influence upon the rate of diffusion of sulphur dioxide through the membrane.

However just how much influence will the rate of diffusion have upon the sulphite toxicity? Sulphite transport is exceptionally rapid and ceases within minutes of addition of sulphite. It seems much more likely that the total amount of sulphite accumulated determines the degree of sulphite toxicity and that, if the rate of diffusion were altered, only the time taken to transport this amount would be changed, not the amount itself.

The equilibrium point is reached when the concentration of molecular sulphur dioxide is equal on both sides of the plasma-membrane. In order for a change in the internal sulphite concentration to occur, a change must occur in the concentration of carbonyl-containing compounds within the cell or of the internal pH value of the cell. The concentration of sulphite-binding compounds would influence the total sulphite concentration within the cell but not the free sulphite concentration. This latter is most likely to cause toxic effects to the cell. Experimentally little difference was found between Saccharomycodes ludwigii and Saccharomyces cerevisiae in their carbonyl-compound content.

Alternatively if the internal pH value of Saccharomycodes ludwigii were to be a little lower than that of Saccharomyces cerevisiae less sulphite would be accumulated before equilibrium was reached. In order for the observed 10 fold resistance of Saccharomycodes ludwigii to sulphite to be explained in this manner, its internal pH value would have to be lowered by the order of one pH unit below that of Saccharomyces cerevisiae. To date the internal pH value of Saccharomycodes ludwigii has not been reported.

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