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Transport of Sulphur Dioxide by *Saccharomyces cerevisiae*

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Accumulation of label from a suspension (pH 4·0) of *Saccharomyces cerevisiae* containing 100 mM-glucose and 1 mM-[^{35}S]sulphite was initially rapid. Net accumulation ceased after 5 min, but at this time [^{35}S]sulphite was still transported by organisms, and could be washed out to an extent that depended on the wash volume. Pre-incubation in the absence of glucose, and omitting glucose from the reaction mixture, had no effect on initial velocity of sulphite accumulation, although it decreased the total amount accumulated. Initial velocity of accumulation was also unchanged when organisms were pre-incubated in the presence of 2-deoxy-D-glucose and this inhibitor was included in the reaction mixture. Initial velocity of sulphite accumulation decreased logarithmically as the pH value of the suspension was increased from 3·0 to 5·0; the decrease closely paralleled the decline in concentration of molecular SO_2 over this pH range. Woolf–Hofstee plots for accumulation of SO_2, at pH 3·0 or 4·0, gave near-vertical plots. Raising the temperature from 19 to 39 °C increased the initial velocity of SO_2 accumulation. The initial velocity of transport was not affected by pretreatment of organisms with carbonyl cyanide *m*-chlorophenylhydrazone, DNP or iodoacetamide but pretreatment with 20 mM-uranyl nitrate increased the initial velocity almost threefold. It is concluded that SO_2 is transported into *S. cerevisiae* by simple diffusion.

INTRODUCTION

Sulphite in aqueous solution exists as a mixture of three forms, the proportions of which depend on pH value. At low pH values ($<1·77$, the pK value of the equilibrium $SO_2 \rightleftharpoons HSO_3^-$), sulphite exists predominantly as molecular SO_2 and, at higher pH values ($>7·20$, the pK value of the equilibrium $HSO_3^- \rightleftharpoons SO_3^{2-}$), largely as sulphite ions (SO_3^{2-}). At intermediate pH values, it exists in various proportions as the bisulphite ion (HSO_3^-; King *et al.*, 1981). In this paper, the term 'sulphite' is used to denote collectively all three forms.

Very little has been reported on sulphite transport by micro-organisms; in some reports, the pH values used are not stated making it impossible to know the form in which sulphite was transported. In species of *Aspergillus* and *Penicillium*, separate specific transport systems for SO_3^{2-} and SO_4^{2-} were reported by Tweedie & Segel (1970), while Alonso *et al.* (1984), working at pH 6·1, claimed that sulphite is transported into *Candida utilis* on the same system as SO_4^{2-} and $S_2O_3^{2-}$. The first report on sulphite transport into *Saccharomyces cerevisiae* came from Macris (1972) who concluded that, of the three forms of sulphite, only SO_2 was transported. On the basis of the specificity and kinetics of, and the effect of temperature on, SO_2 transport, Macris & Markakis (1974) claimed that it was an active carrier-mediated process. The present paper reports further data on transport of SO_2 into *S. cerevisiae*, and it arrives at a conclusion different from that of Macris & Markakis (1974) concerning the mechanism of the process, and one which was suggested briefly by Stratford & Rose (1985) and Hinze & Holzer (1985).

Abbreviations: DOG, 2-deoxy-D-glucose; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

METHODS

Organism. The strain of *S. cerevisiae* used, TC8, was provided by T. Cowland of the Taunton Cider Co. It was maintained on slopes of malt extract/yeast extract/glucose/peptone/agar medium (Wickerham, 1951).

Experimental cultures. Cultures (1 litre) were grown in 2 litre round flat-bottomed flasks. The medium (pH 4·0 unless otherwise stated) had the following composition (l^{-1} water): glucose 20 g, $(NH_4)_2SO_4$ 3 g, KH_2PO_4 3 g, yeast extract (Lab M) 1 g, $MgSO_4.7H_2O$ 30 mg and $CaCl_2.2H_2O$ 30 mg (Patching & Rose, 1970). Starter cultures (100 ml medium in a 250 ml conical flask) were inoculated with a pinhead of yeast from a slant culture and incubated at 30 °C for 12 h on an orbital shaker (200 r.p.m.). One litre portions of medium were inoculated with a portion of starter culture containing 1 mg dry wt organisms, incubated at 30 °C and stirred magnetically (5 cm stirrer bar; 220 r.p.m.). Growth was followed by measuring optical density at 600 nm, measurements being related to dry wt of yeast by a standard curve.

Measurement of initial velocities of solute accumulation. Organisms were harvested from late exponential-phase cultures (0·5 mg dry wt ml^{-1}) by filtration through a membrane filter (0·45 μm pore size; 50 mm diam.; Oxoid), washed twice with 10 ml 30 mM-citrate buffer (pH 4·0 unless otherwise stated) containing 100 mM-glucose, resuspended in 5 ml of the same buffer and equilibrated at 30 °C for 3 min. The reaction mixture (15 ml) was citrate buffer (30 mM; pH 4·0 unless otherwise indicated) containing 100 mM-glucose, 5 μM–5 mM-sulphite and [^{35}S]sulphite (0·05–0·20 μCi ml^{-1}; 1 μCi = 37 kBq). This was placed in a 100 ml round-bottomed flask, incubated at 30 °C unless otherwise indicated for 5 min and stirred magnetically (1 cm stirrer bar; 200 r.p.m.). The measurement was started by adding organisms in buffer containing 100 mM-glucose to give a final concentration of 0·5–1·0 mg dry wt ml^{-1}. In certain experiments, organisms were pre-incubated for 1 h at 30 °C in glucose-free buffer, with or without 100 mM-2-deoxy-D-glucose (DOG). Initial velocities of accumulation by these organisms were measured in the same buffer. In other experiments, an examination was made of the effect of potential inhibitors on sulphite accumulation. These were included in the suspension of organisms in buffer which was incubated for 3 min at 30 °C before a portion was taken to start the measurement of initial velocity. To do this, samples (1 ml) were removed at intervals over a 10–35 s period, and rapidly filtered through membrane filters (0·45 μm pore size; 25 mm diam.; Millipore) the filters having been washed with 10 ml 10 mM-sulphite in 30 mM-citrate buffer at the pH value used in the experiment. After filtration, filters with organisms were rapidly washed with 5 ml buffered sulphite solution at the concentration used in the experiment. Filters with organisms were placed in scintillation vials containing 7 ml Optiphase 'Safe' (Fisons). Radioactivity in the vials was measured in a LKB Rackbeta liquid scintillation spectrometer (model 1217).

Initial velocities of accumulation of L-lysine were measured as described by Keenan *et al.* (1982). The suspension (15 ml citrate buffer; pH 4·0) contained organisms (1·0 mg dry wt ml^{-1}), 100 μM-L-lysine (1·0 μCi μmol^{-1}) and unless otherwise stated 100 mM-glucose. The suspension was incubated at 30 °C, the experiment started by adding solute (tracer and carrier) and the velocity of accumulation measured over the first 2 min.

Measurement of equilibrium fluxes. Organisms (1·0 mg dry wt ml^{-1}) in 30 mM-citrate buffer (15 ml; pH 4·0) containing glucose (100 mM) and 1 mM-[^{35}S]sulphite were allowed to accumulate label for 5 min. After this time, efflux of label from organisms was measured by removing five portions (1 ml) of suspension, and filtering each through a membrane filter as already described. Organisms on filters were washed with different volumes (1–100 ml) of 30 mM-citrate buffer containing, except when otherwise stated, 1 mM-sulphite. The times taken to complete the washings were noted, and the radioactivity of organisms recorded for that time. Influx of label into organisms, after net accumulation had ceased (5 min), was measured by adding [^{35}S]sulphite (5 μM; 80 μCi $mmol^{-1}$) to a suspension (1·0 mg dry wt ml^{-1}) in 15 ml buffer containing unlabelled sulphite (1 mM) and glucose (100 mM). Accumulation of label by organisms was measured over the following 2 min.

Chemicals. All chemicals used were Analar or of the highest grade available commercially. Amersham supplied sodium [^{35}S]sulphite which was stored at −20 °C in 5 mM-EDTA to avoid oxidation.

RESULTS

Kinetics of sulphite accumulation

Accumulation of ^{35}S-label from a suspension containing [^{35}S]sulphite was rapid over the first minute, after which the velocity declined (Fig. 1). Net accumulation ceased after approximately 5 min. Organisms incubated in buffer containing unlabelled sulphite for 5 min were able to incorporate added [^{35}S]sulphite at a velocity similar to that observed over the first minute in suspensions containing [^{35}S]sulphite. It was also possible to wash label out of organisms after 5 min incubation, to an extent which depended on the volume of buffer used for washing (Fig. 1). The velocity of efflux did not change when organisms were washed with sulphite-free buffer. Woolf–Hofstee plots (Hofstee, 1959) for accumulation of SO_2 by organisms, at pH 3·0 or 4·0,

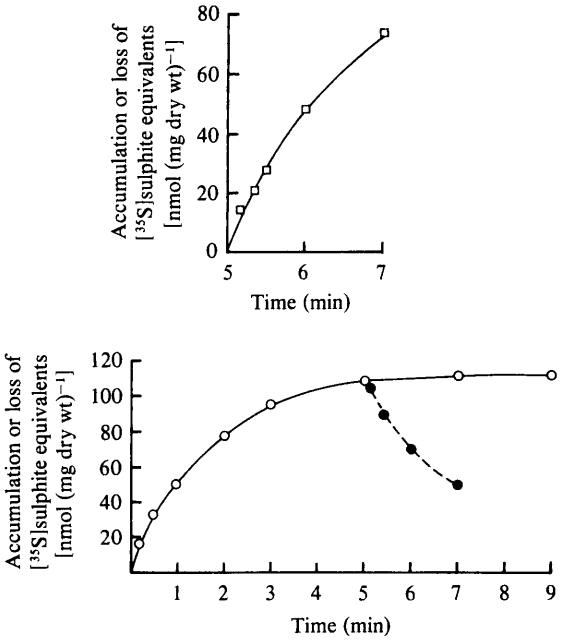

Fig. 1

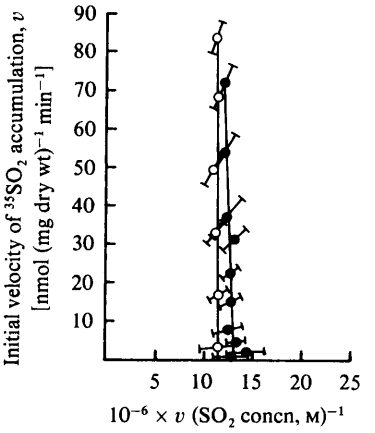

Fig. 2

Fig. 1. ○, Time-course of accumulation of sulphite equivalents by *S. cerevisiae* TC8 (1·0 mg dry wt ml^{-1}) from 30 mM-citrate buffer (pH 4·0) containing glucose (100 mM) and [^{35}S]sulphite (1 mM); ●, time-course of efflux of sulphite equivalents from organisms harvested after 5 min and washed on filters with different volumes of buffer, as described in Methods; □, accumulation of sulphite equivalents by organisms initially incubated in a suspension containing unlabelled sulphite for 5 min and then supplemented with [^{35}S]sulphite, as described in Methods. Data shown are representative of those obtained in three separate experiments.

Fig. 2. Woolf–Hofstee plots for accumulation of molecular SO_2 by *S. cerevisiae* TC8 suspended in buffer at pH 3·0 (○) or pH 4·0 (●). Concentrations of molecular SO_2 at the different pH values were calculated from the data of King *et al.* (1981). Bars indicate SD.

Table 1. *Effect of glucose and DOG on initial velocities of accumulation of SO_2 and L-lysine by Saccharomyces cerevisiae TC8*

Organisms were suspended in supplemented or unsupplemented citrate buffer (pH 4·0) for 1 h before initial velocities were measured over the first 10 s. The reaction mixture also contained the supplement at the concentration stated. Values quoted are means of at least three separate measurements ± SE.

	Initial velocity (percentage of initial velocity in a glucose-containing suspension)	
Supplement	SO_2 (5·8 μM)	L-Lysine (0·1 mM)
Glucose (100 mM)	100·0 ± 7·9	100·0 ± 3·6
None	95·5 ± 12·1	9·8 ± 0·7
DOG (100 mM)	100·2 ± 13·7	5·3 ± 0·8

gave near vertical plots (Fig. 2). Pre-incubating organisms in glucose-free buffer and omitting glucose from the reaction mixture had no effect on initial velocities of SO_2 accumulation, measured at 2 s intervals over the first 10 s. It did, however, approximately halve the amount accumulated after 5 min. Pre-incubating organisms in the presence of DOG (100 mM), and including this inhibitor in the reaction mixture at the same concentration, also had no effect on initial velocities of SO_2 accumulation (Table 1).

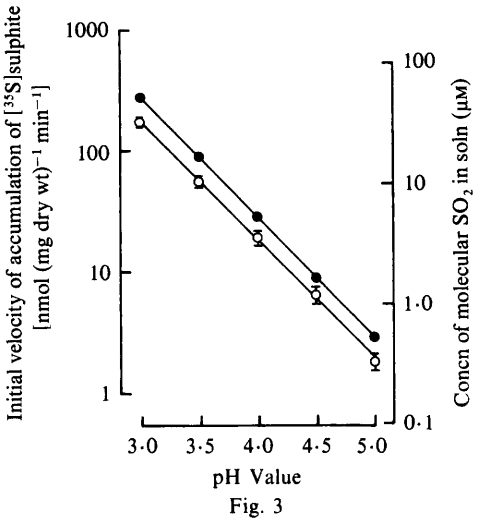

Fig. 3

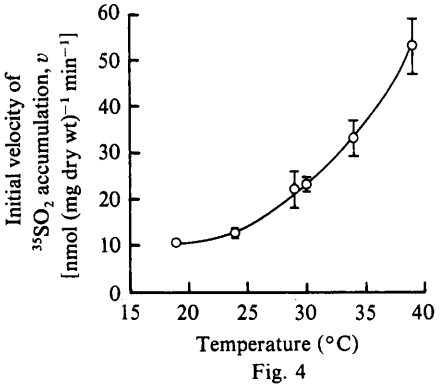

Fig. 4

Fig. 3. Effect of pH value on initial velocities of accumulation of [^{35}S]sulphite equivalents by *S. cerevisiae* TC8 suspended in buffer containing 100 mM-glucose and 0·5 mM-[^{35}S]sulphite (○) and on the concentration of molecular SO_2 in the suspensions (●) calculated from the data of King *et al.* (1981). Bars indicate SD.

Fig. 4. Effect of temperature on initial velocities of accumulation of SO_2 by *S. cerevisiae* TC8 suspended in buffer containing 100 mM-glucose and 1·77 μM-SO_2. Bars indicate SD.

Table 2. *Effect of inhibitors on initial velocities of accumulation of SO_2 and L-lysine by Saccharomyces cerevisiae TC8*

Initial velocities were measured over the first 10 s. Values quoted are means of at least three separate measurements ± SE.

Inhibitor	Concn (mM)	Initial velocity (percentage of initial velocity in a suspension lacking inhibitor) SO_2 (5·8 μM)	L-Lysine (0·1 mM)
None	–	100 ± 9·7	100 ± 4·0
CCCP	0·5	104·4 ± 11·5	6·2 ± 1·8
DNP	0·5	111·8 ± 8·6	13·3 ± 3·2
Iodoacetamide	5·0	94·4 ± 12·1	31·0 ± 2·5
p-Chloromercuribenzoate	2·0	95·0 ± 13·2	42·3 ± 5·1
Uranyl nitrate	10·0	115·3 ± 12·4	22·9 ± 2·0
	20·0	282·3 ± 35·6	20·1 ± 3·1

Factors affecting accumulation of sulphite

The initial velocity of sulphite accumulation decreased logarithmically as the pH value of the suspension was increased from 3·0 to 5·0 (Fig. 3). This decrease closely paralleled the decline in concentration of molecular SO_2 as the pH value was increased over this range as calculated from the data of King *et al.* (1981). There was no evidence for loss of SO_2 from solutions in this pH range over the time scale of these experiments. Raising the temperature of the suspension from 19 °C to 39 °C increased the initial velocity of SO_2 accumulation by organisms (Fig. 4). Over the range 19–39 °C, the Q_{10} value was 2·5. When potential inhibitors were included in cell suspensions, none except uranyl nitrate at 20 mM had a significant effect on the initial velocity of transport of SO_2 present at 5·8 μM (Table 2). When the inhibitors were included in suspensions

of organisms at the same concentrations, DNP and CCCP almost completely prevented accumulation of L-lysine, while accumulation of the amino acid was considerably retarded by iodoacetamide, *p*-chloromercuribenzoate and uranyl nitrate (Table 2).

DISCUSSION

The close correlation between the rate of accumulation of label from [^{35}S]sulphite by organisms, over the pH range 3·0–5·0, and the concentration of SO_2 in solution calculated from the data of King *et al.* (1981), strongly suggests that, over this pH range, the only form that passes into organisms is SO_2. This conclusion, and the inference that *S. cerevisiae* does not transport HSO_3^-, are in agreement with Macris & Markakis (1974) and Hinze & Holzer (1985).

However, we do not agree with Macris & Markakis (1974) with regard to the manner in which SO_2 passes into organisms, and we suggest that our data lead to the conclusion that SO_2 is transported into *S. cerevisiae* by simple diffusion. Strong evidence in favour of a protein not being involved in SO_2 transport came from the near-vertical Woolf–Hofstee plots for transport at pH 3·0 and 4·0. Neither plot is perfectly vertical, so that kinetic constants could be derived from them [pH 4·0; K_T 0·11 mM, V_{max} 1·4 μmol (mg dry wt)$^{-1}$ min^{-1}: pH 3·0; K_T 3·2 mM, V_{max} 1·4 μmol (mg dry wt)$^{-1}$ min^{-1}]. These K_T values are considerably greater than the threshold concentration of SO_2 required to kill *S. cerevisiae* TC8 under these conditions (Stratford, 1983). Deviation from the vertical for the Woolf–Hofstee plot for accumulation at pH 4·0 is caused very largely by data points at low concentrations of SO_2. This might suggest that, as the concentration of HSO_3^- increased when the pH value was raised, a slow transport system for this ion which was probably saturated may have been functioning, activity of which was masked in the presence of higher concentrations of SO_2. Additional evidence for lack of involvement of proteins came from the inability of iodoacetamide and *p*-chloromercuribenzoate to affect the initial velocity of transport. The absence of active transport during entry of SO_2 into organisms is suggested by the inability of CCCP and DNP (Borst-Pauwels, 1981) to affect the initial velocity. The effect of CCCP and DNP on accumulation of L-lysine indicated that they were used at concentrations that almost completely inhibit active transport of the amino acid. The ability of uranyl nitrate, at 20 mM but not 10 mM, to increase the initial velocity of SO_2 accumulation is probably explained by its causing an increase in the permeability of the yeast plasma membrane after the large uranyl ions react with phospholipid polar headgroups. A concentration-dependent inhibition of yeast transport systems by uranyl nitrate has been reported by Maxwell *et al.* (1970). Further evidence for lack of active transport of SO_2 came from the finding that, although glucose was included in the reaction mixture because Macris & Markakis (1974) claimed that SO_2 accumulation is an active process, excluding the sugar from reaction mixtures had no effect on initial velocities of accumulation. The ability of glucose to increase the extent of accumulation is probably explained by the maintenance of a relatively high internal pH value as a result of glycolysis (Conway & Downey, 1950). The inability of DOG to affect initial velocities of SO_2 accumulation also argues against the process being an active one. A Q_{10} value of 2·5 over the temperature range 19–39 °C is somewhat higher than values usually encountered with diffusion processes (Stein, 1967), although the absence of an overall decline in the rate of SO_2 transport up to a temperature of 39 °C might be taken as evidence for no protein involvement. Unlike Macris & Markakis (1974) it was considered injudicious to examine transport at temperatures higher than 39 °C because of the possibility of causing cell death. Additional evidence for a lack of protein involvement in SO_2 transport is provided by the absence of an effect of pH value on the process other than that predicted by changes in substrate concentration.

The time-course of accumulation of label from $^{35}SO_2$ requires a more detailed explanation. Accumulation of label in organisms was initially very rapid, but levelled off after about 5 min when the concentration of label in organisms, assuming a water content of 2 μl (mg dry wt)$^{-1}$ (Beavan *et al.*, 1982), was approximately 60 times that in the suspension. Accumulation of label from SO_2 under these conditions can be explained by combination of sulphite with intracellular carbonyl-containing compounds to produce α-hydroxysulphonates (Burroughs & Sparks, 1964)

and by the greater intracellular (about pH 6·5), compared with extracellular, pH value (Borst-Pauwels, 1981). From the latter consideration, it is inferred that free SO_2 after diffusion into organisms is largely converted into HSO_3^- ions. This in turn lowers the intracellular concentration of SO_2 thus allowing further diffusion into organisms until the concentration of SO_2 is equal on both sides of the plasma membrane. This explanation is supported by the equilibrium fluxes that were demonstrated in suspensions of organisms that had accumulated SO_2 for up to 5 min.

Our disagreement with the conclusion by Macris & Markakis (1974) as to the manner of transport of SO_2 requires justification. Many of the findings reported by these workers, especially the time-course of SO_2 accumulation and the results of double-reciprocal plots, are similar to those reported herein. However, additional evidence reported in the present paper leads us to conclude that SO_2 transport into *S. cerevisiae* is not an active process (Macris & Markakis, 1974) but is by simple diffusion.

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