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Mechanism of sulfite action on the energy metabolism of *Saccharomyces cerevisiae*

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The pools of ribonucleoside di- and triphosphates decrease within a few min after addition of 5 mM sulfite to a suspension of *Saccharomyces cerevisiae* at pH 3.6. Levels of the corresponding ribonucleoside monophosphates increase in parallel. The strongest effect was observed with the adenosine phosphate pools. Depletion of ATP by sulfite at pH 3.6 occurs both in the presence and absence of glucose. These findings point to at least two different mechanisms for sulfite action on energy metabolism. Glycolysis is effectively impaired by low sulfite concentrations. The enzymes glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase are inhibited by sulfite in vitro. In addition, formation of adducts between sulfite and aldehydes contributes to the inhibition of enzymatic reactions as shown with alcohol dehydrogenase. Sulfite also causes reduction of oxygen consumption of glucose-starved yeast at pH 3.6 which coincides with ATP depletion. In vitro, the oligomycin-sensitive F_1-ATPase of yeast is stimulated 2.8-fold by 1 mM sulfite at pH 5.7. However, this stimulation does not seem to be involved in sulfite-initiated ATP depletion as concluded from experiments with the F_1-ATPase-deficient mutant pet 936. At pH 3.6, the intracellular proton concentration of yeast is increased from $3.2–6.3 \cdot 10^{-8}$ M to $4.0 \cdot 10^{-6}$ M by 1 mM sulfite. In spite of the marked intracellular acidification, stimulation of an ATP-driven proton pump is not the chief cause for the sulfite-initiated ATP decrease. During short exposure of yeast to sulfite the effect on energy metabolism is reversible.

Introduction

Sulfite, known as an antimicrobial agent for a long time, reacts in vitro with a series of biomolecules. Nucleophilic reactions of sulfite are known with disulfides (cystine, proteins) [1], aliphatic aldehydes [2], coenzymes (NAD^+, FAD, FMN) [3–13], vitamins (menadione, thiamine) [14–17], hormones (epinephrine) [18–29] and cytidine [21–25]. Many of these reactions with sulfite are reversible. Concerning NAD^+ [5–8] and FAD [9–13], however, the adducts formed with sulfite are efficiently stabilized in the protein-bound cofactors. The reactions with cytidine [21–25], thiamine [16,17] and epinephrine [18–20] are irreversible. Furthermore, radical reactions of bisulfite with biochemical compounds have been observed, causing chain cleavage of DNA or oxidation of double bonds in unsaturated lipids. An extensive review on this field is given elsewhere [26].

The effect of sulfite pollution on plant metabolism has been reviewed by Ziegler [27]. Sulfite impairs transpiration and photosynthesis in plants [27–33]. The most characteristic action was observed with enzymes involved in CO_2 metabolism. Among them, ribulose diphosphate carboxylase and phospho*enol*pyruvate carboxylase are the most important ones. These enzymes are inhibited

by sulfite in a non-competitive way with respect to the substrates ribulosediphosphate and phospho*enol*pyruvate [34–36]. However, reaction with HCO_3^- is inhibited in a competitive way [34–36]. Damage caused by low concentrations of sulfite have been described for microorganisms [37–40]. Schimz and Holzer [41] observed a rapid decrease of the ATP content in yeast cells after addition of 2 mM sulfite to the medium. Similar results were obtained with bacteria [42]. In addition, Schimz [43] showed that low sulfite concentrations inhibited the colony-forming capacity of yeast cells.

We have been interested in the mechanism of noxious action of sulfite on yeast cells at pH 3.6 at concentrations used in food preservation. The pH used generally occurs in fruit juices and wines. In this paper, a differentiated action of sulfite on the cellular metabolism of yeast is demonstrated.

Materials and Methods

Chemicals. ATP, alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, NAD^+, NADH and CLS reagent (ATP bioluminescence CLS test combination) were purchased from Boehringer GmbH (Mannheim, F.R.G.); bacto peptone and yeast extract were from Difco (Detroit, U.S.A.); carbonyl cyanide *m*-chlorophenylhydrazone and oligomycin were from Sigma (Taufkirchen, F.R.G.). All other chemicals were from Bender und Hobein (Munich, F.R.G.).

Yeast strains. Saccharomyces cerevisiae strain X 2180 B (wild type) and the cytoplasmic petite mutant D 273-10B-1 (α pet ρ^-) [44] were kindly provided by Prof. Dr. D.H. Wolf, Biochemisches Institut der Universität Freiburg. The nuclear F_1-deficient mutant pet 936 (α pet ρ^+) [45,46] was a generous gift of Prof. Dr. G. Schatz, Biozentrum, Basel, Switzerland.

Cultivation of yeast. Cultivation of yeast was performed in Erlenmeyer flasks with 10 g/l yeast extract, 20 g/l bacto peptone and 20 g/l glucose as growth medium at 30°C using a reciprocal shaker. Yeast was grown either to the logarithmic or to the stationary growth phase. The cells were harvested by centrifugation (3000 × *g* for 15 min at 4°C and washed twice with ice cold water. The pellet was stored at 4°C for a maximum of 6 h before use.

Incubation of yeast. Yeast suspensions, given in wet weight/volume (w/v), were prepared in buffer and preincubated at 20°C as described for each experiment. The incubation procedures were started by applying sulfite or another additive to the yeast suspensions.

Determination of ATP. Aliquots of 100 µl of the yeast suspensions were added to 100 µl of ice-cold 35% (w/v) perchloric acid. After 20 min with occasional shaking the extraction procedure was finished by the addition of 550 µl 2 M potassium bicarbonate (final pH value, about 7.3). After further 20 min storage on ice, the potassium perchlorate suspension was centrifuged at 10000 × *g* for 4 min, and the supernatant was used for ATP determination.

ATP was measured with the luciferin-luciferase system by monitoring chemiluminescence with a bioluminescence analyzer (Biolumat LB 9500, Berthold (Wildbad, F.R.G.)). Aliquots of 200 µl of CLS reagent from Boehringer, Mannheim, were dispensed into test tubes and stored for 15 min at room temperature in the dark. After determination of the background chemiluminescence (counts/10 s), 200 µl of the diluted sample were added, followed by immediate measurement of chemiluminescence (counts/10 s). For calibration, an internal standard of $5 \cdot 10^{-9}$ M ATP was present in a parallel assay. The cellular ATP content was calculated in µmol ATP/g yeast cells (wet weight).

Determination of ribonucleoside phosphates in yeast. A 2% (w/v) suspension of stationary cells of wild-type yeast in 0.15 M sodium citrate buffer (pH 3.6) was preincubated at 20°C for 1 h in the presence of 2% (w/v) glucose. After withdrawing a control sample, the cells were incubated for a further 10 min in the presence of 5 mM sulfite. For ribonucleoside phosphate determinations, 1 ml samples were centrifuged at 8000 × *g* for 4 min at 4°C. The sediments, containing 20 µg cells, were treated with 80 µl 20% (w/v) $HClO_4$ and neutralized after 20 min storage on ice by dropwise addition of 140 µl of 2 M potassium bicarbonate (final pH value, about 6.8). After further 10 min the ice-cold extracts were centrifuged and lyophilized. The ribonucleoside phosphates were analyzed by high-performance liquid chromatography as described by Holstege et al. [47].

Glucose consumption and ethanol formation by

yeast (strain X 2180 B). Suspensions of 2% (w/v) in McIlvain buffer (pH 3.6) were preincubated for 1 h at 20°C. After addition of sulfite the incubations were continued for 10 min. Glycolysis was then started by addition of 0.1% (w/v) glucose. At the times indicated glucose was determined [48] after perchloric-acid treatment and neutralization in the supernatant solution (see subsection Determination of ATP). For measuring ethanol formation, the yeast suspensions were pretreated as described above. Glycolysis was started by addition of 2% (w/v) glucose. Ethanol was determined [49] after perchloric acid treatment and neutralization.

Enzyme activities in sulfite-treated yeast cells. From stationary cells of *Saccharomyces cerevisiae* X 2180 B a 2% (w/v) suspension in 0.15 M sodium citrate/HCl buffer (pH 3.6) was prepared and preincubated for 1 h in the presence of 2% (w/v) glucose at 20°C. After applying 5 mM sulfite the incubation was continued for a further 10 min. The cells were collected by centrifugation and washed (1 ×) with ice-cold water. The pellet, containing 4 g yeast, was suspended in 8 ml water and submitted to the French-press procedure. After centrifugation, the supernatant was assayed for the following enzymes: alcohol dehydrogenase [50], glyceraldehyde-3-phosphate dehydrogenase [52], NAD^+- and $NADP^+$-dependent glutamate dehydrogenase and glucose-6-phosphate dehydrogenase [51]. Enzyme activities in the sulfite-treated cells were calculated in % of those in control cells.

Crude membranes from yeast (strain X 2180 B). Crude membranes from stationary yeast were prepared according to Foury et al. [52]. Immediately after harvesting, 5 g yeast was suspended in 5 ml 0.05 M Tris, containing 0.25 M sucrose. The suspension was mixed vigorously with 10 g glass beads (0.5–0.05 mm diameter) on a vortex mixer 10-times for 30 s with 30 s intervals for cooling on ice. The homogenate was centrifuged at $3000 \times g$ for 10 min at 4°C. The pellet was washed with 5 ml buffer and recentrifuged at $3000 \times g$. The combined supernatants were then centrifuged at 25000 $\times g$ for 50 min at 4°C. The pellet was suspended in 1.25 ml of 10 mM Tris/acetate (pH 7.5) containing 0.25 M sucrose and homogenized with a glass-Teflon homogenizer (three strokes). After homogenization 1 mM EDTA and 1 mM ATP were added to the fraction which was kept at 0°C. The extract contained 8–12 mg protein/ml.

Determination of ATPase activity. Assays for ATPase in the crude membrane preparation of yeast (described above) were carried out at pH 5.7 according to Dufour and Goffeau [53]. Inorganic phosphate was determined as described by Ames [54]. One unit of ATPase activity corresponds to the release of 1 μmol P_i per min at 30°C.

Determination of the intracellular pH of yeast. Yeast suspensions (2% (w/v)) in 0.1 M sodium succinate buffer (pH 3.6) were preincubated for 60 min at 20°C in the absence of glucose. After addition of 10^{-5} M [1-^{14}C]propionic acid, incubation was continued for 10 min in the presence of sulfite or formic acid as acidifying agents. Samples were withdrawn and centrifuged at $10000 \times g$ for 4 min at room temperature. Radioactivity was measured in both pellet and supernatant. The intracellular pH (or the proton concentration) was calculated from partition of [1-^{14}C]propionic acid in the two fractions.

Determination of protein. Protein concentrations were usually determined by the method of Lowry et al. [55] with crystalline bovine albumin as standard.

Determination of oxygen consumption. Oxygen consumption of yeast at 37°C was measured with the Clark oxygen electrode using 0.5% (w/v) yeast suspensions in McIlvain buffer (pH 3.6).

Results

Effect of sulfite on ribonucleoside phosphate pools in yeast cells

The effect of sulfite on the intracellular ATP level was investigated using the following yeast strains from *Saccharomyces cerevisiae*: (1) wild-type strain X 2180 B; (2) mutant pet 936; and (3) mutant D 273 10B-1 ρ^-. The nuclear mutant pet 936 is characterized by mitochondrial ATPase deficiency [46]; the cytoplasmic ρ^--mutant lacks a mitochondrial protein synthesizing system [45]. Both mutants are able to synthesize ATP only via substrate level phosphorylation (for definition of 'substrate level phosphorylation', in contrast to 'respiratory chain phosphorylation' [56]).

After application of 2 mM sulfite a drastic decrease in the intracellular ATP content was

observed at pH 3.6 in both the wild-type and the mutant pet 936 (Table I). Depletion of ATP was much more pronounced in the presence than in the absence of glucose within 4 min of sulfite action. However, after 10 min of incubation the ATP levels in the glucose-starved cells had also dropped below 10% of the control (data not shown). No difference in sensitivity of ATP towards sulfite was observed between yeast cells from the logarithmic and the stationary growth phase.

The sulfite-initiated decrease of ATP was accompanied by a depletion of ADP and by a simultaneous increase of AMP being identical to the sum of loss of ATP and ADP (Table II). Additionally, sulfite caused depletion of other ribonucleoside triphosphates and, to a lesser extent, of the corresponding diphosphates, whereas the ribonucleoside monophosphates (GMP and CMP) increased (Table II).

Effect of sulfite on enzyme activities

The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was found to be most drastically inhibited by sulfite in vivo (98%), followed by alcohol dehydrogenase (80%) and NAD^+-glutamate dehydrogenase (60%). In contrast, the activities of $NADP^+$-dependent enzymes like $NADP^+$-glutamate dehydrogenase and glucose-6-phosphate dehydrogenase were inhibited in vivo to a minor extent (16 and 11%, respectively).

Studies on the in vitro inhibition of the alcohol dehydrogenase catalyzed reduction of acetaldehyde should give some insight into the reactivity of sulfite with metabolites. Reduction of acetalde-

TABLE II

EFFECT OF SULFITE ON THE RIBONUCLEOSIDE PHOSPHATE POOLS OF YEAST AT pH 3.6

A 2% (w/v) suspension of stationary yeast (wild-type strain X 2180 B) in 0.15 M citrate buffer (pH 3.6) was preincubated for 1 h at 20°C in the presence of 2% (w/v) glucose. After taking a control sample incubation was continued for 10 min in the presence of 5 mM sulfite. For ribonucleoside phosphate determinations see 'Materials and Methods'.

Nucleoside phosphate	Control value (nmol/g yeast [a])	After 10 min of incubation	
		without sulfite (nmol/g yeast [a])	with 5 mM sulfite (nmol/g yeast [a])
ATP	1190	1110	15
ADP	227	230	88
AMP	64	41	1440
GTP	190	188	< 3
GDP	74	91	62
GMP	67	55	214
UTP	284	253	53
UDP	71	71	37
CTP	94	91	< 3
CDP	23	24	50
CMP	76	63	118

[a] Wet weight.

TABLE I

EFFECT OF 2 mM SULFITE ON THE ATP CONTENT OF SACCHAROMYCES CEREVISIAE AT pH 3.6

The incubation procedure was carried out with suspensions of 2 g yeast/100 ml McIlvain buffer (pH 3.6) at 20°C using two yeast strains. For ATP determinations see 'Materials and Methods'.

Strain	Growth phase at harvest	Preincubation before addition of sulfite		ATP-content (control value) (μmol ATP/g yeast [a])	ATP-content 4 min after addition of 2 mM sulfite (% of control)
		Time (min)	glucose in the medium (% w/v)		
X 2180 B	stationary	60	–	1.98	33
X 2180 B	stationary	60	2	2.70	9.2
X 2180 B	logarithmic	5	–	1.50	31
X 2180 B	logarithmic	5	2	1.62	4.4
pet 936	logarithmic	10	–	0.93	28
pet 936	logarithmic	60	2	1.50	3.4

[a] Wet weight.

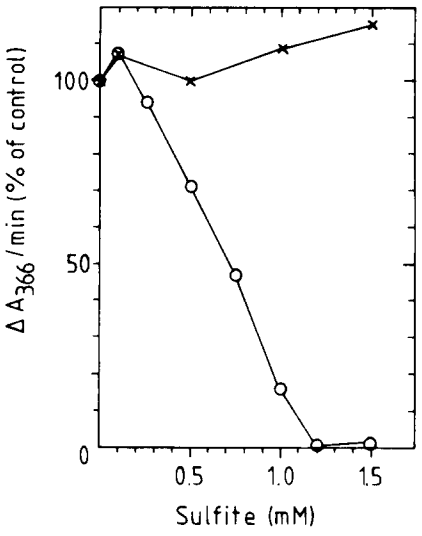

Fig. 1. Effect of sulfite on the alcohol dehydrogenase catalyzed reduction of acetaldehyde. The reaction mixture of 1.5 ml volume contained 0.1 M potassium phoshate (pH 6.5)/0.3 mM NADH and 1 mM acetaldehyde (○———○) or 25 mM acetaldehyde (×———×). The reaction was started with 10 μl of alcohol dehydrogenase obtained by an 1:2500 dilution of the Boehringer enzyme. The reduction rate of acetaldehyde at 30°C was followed by monitoring the decrease of NADH absorbance at 366 nm.

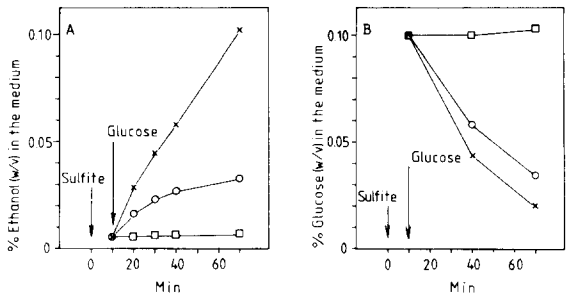

Fig. 2. Influence of sulfite on ethanol formation (A) and on glucose consumption (B) in *Saccharomyces cerevisiae* X 2180 B at pH 3.6. (A) suspensions of stationary yeast cells (2% (w/v)) in 0.1 M sodium succinate buffer (pH 3.6) were preincubated for 60 min in the absence of glucose at 30°C. At zero time sulfite was added to the suspensions: ○———○, 0.2 mM sulfite; □———□, 1 mM sulfite; ×———×, control. After further 10 min of incubation glycolysis was started with 2% (w/v) glucose. Oxidative ethanol consumption was prevented by 0.1 mM sodium azide. At the times indicated 100 μl samples were added to 100 μl of 35% (w/v) $HClO_4$. After neutralization with 2 M $KHCO_3$ and centrifugation ethanol was determined in the supernatant enzymatically [49]. (B) The cells had been pretreated as mentioned under (A). Glycolysis was started 10 min after addition of sulfite with 0.1% (w/v) glucose: ○———○, 0.2 mM sulfite; □———□, 1 mM sulfite; ×———×, control. At the times indicated, 100 μl samples were added to 100 μl of 35% (w/v) $HClO_4$. After neutralization with 2 mM $KHCO_3$ and centrifugation glucose was determined in the supernatant enzymatically [48].

hyde was followed by monitoring disappearance of NADH absorbance given in % of the control value (Fig. 1). In the presence of 1 mM substrate the reaction rate declined with increasing sulfite concentrations. Complete inhibition was obtained with sulfite concentrations above 1 mM. However, at a 25-fold higher substrate concentration (25 mM acetaldehyde), the reaction rate was not diminished even by 1.5 mM sulfite. These findings clearly demonstrate that the formation of an adduct between sulfite and acetaldehyde is decisive for inhibition of the reaction and not a direct interaction of sulfite with the enzyme.

Effect of sulfite on the glycolytic pathway

Inhibition of glyceraldehyde-3-phosphate dehydrogenase by sulfite in vivo initiated further studies on the effect of sulfite on glycolysis, i.e., on glucose degradation and ethanol formation in yeast. The results are summarized in Fig. 2. Ethanol formation was markedly lowered after addition of 0.2 mM sulfite and completely prevented by 1 mM sulfite at pH 3.6 (Fig. 2A). Similarly, aerobic glucose consumption was significantly retarded by 0.2 mM sulfite and came to a halt with 1 mM sulfite (Fig. 2B).

Effect of sulfite on respiratory chain phosphorylation in yeast

Previous results with wild-type yeast implied that sulfite impairs respiratory chain phosphorylation (see Table I). We therefore measured oxygen consumption at pH 3.6 of glucose-starved yeast cells in the presence of different sulfite concentrations. The results are given in Fig. 3. Respiration was found to be markedly reduced by sulfite concentrations between 0.05 and 0.8 mM. Furthermore, the sulfite-initiated decrease of ATP coincided very well with the reduction of oxygen consumption indicating a close relationship between respiration and ATP regeneration in glucose-starved yeast cells.

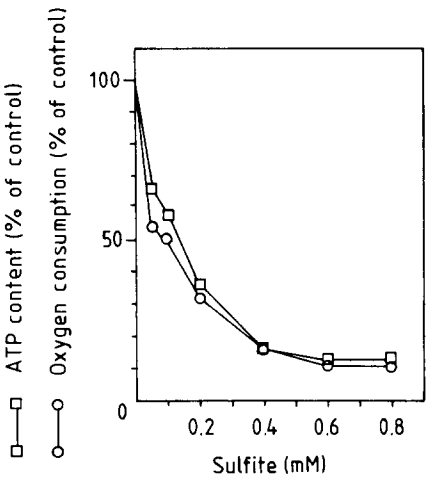

Fig. 3. Effect of sulfite on oxygen consumption and on the ATP content of *Saccharomyces cerevisiae* X 2180 B from stationary growth phase in the absence of glucose. Oxygen consumption of 0.5% (w/v) yeast suspensions in 0.1 M McIlvain buffer (pH 3.6) was measured at 37°C using a Clark electrode. The final suspensions were obtained by addition of 50 μl of a freshly prepared 10% (w/v) stock suspension of yeast to 950 μl of oxygen saturated buffer in the electrode chamber. The control value was taken after 10 min of incubation. Then 50 μl of sulfite from appropriate stock solutions were added (1:21 dilution) and oxygen consumption was measured after 2 min again. Oxygen consumption in the control amounted to 10 nmol/min. In a preceding experiment it was shown that sulfite is not oxidized in the chamber by the oxygen containing buffer during incubation at pH 3.6. ATP depletion was measured in a separate experiment using a 0.1% (w/v) yeast suspension in the same buffer. The cells were preincubated for 30 min before addition of sulfite. After further 10 min of incubation samples were taken for ATP determination (see Materials and Methods). Control: 100% ATP = 2μmol/g yeast (wet weight).

Interaction of sulfite with ATPase activities

We also investigated the effects of sulfite on ATP hydrolyzing systems. The influence of sulfite on ATPase activities was studied using crude membrane preparations from yeast [48] which still contained the mitochondria. In the wild-type about 70% of the ATPase activity at pH 5.7 (pH optimum for plasma membrane ATPase [52]) was derived from the oligomycin-sensitive F_1-ATPase (Table III). We found a marked increase of ATPase activity with 1 mM sulfite. However, in the presence of oligomycin sulfite was totally ineffective, indicating that stimulation occurs only with F_1-ATPase. From the data given in Table III a 2.8-fold activation of F_1-ATPase was calculated. Half-maximal stimulation of the enzyme was observed already with 0.1 mM sulfite (data not shown). The oligomycin-insensitive ATPase which was found to be sensitive to vanadate (data not shown) is identical with the well-characterized plasma membrane ATPase [57–59]. There is no evidence for an interaction of sulfite with the plasma membrane ATPase, as concluded from experiments with the mutant pet 936 (Table III).

Influence of sulfite on the intracellular proton concentration of yeast

Sulfite is highly accumulated in yeast cells during incubation at pH 3.6 as shown by Hinze and Holzer [60]. It has been assumed that the intracellular pH of yeast may be changed as a consequence of sulfite uptake from the acidic medium. Studies

TABLE III

EFFECT OF SULFITE ON THE ATPase ACTIVITY IN YEAST MEMBRANE PREPARATIONS

Crude membranes both from wild-type yeast X 2180 B and mutant pet 936 were prepared according to Foury et al. [52] and assayed for ATPase activity at pH 5.7 [53] (see Materials and Methods). Inorganic phosphate was determined as described by Ames [54]. One unit of ATPase activity corresponds to the release of 1 μmol P_i/min at 30°C. The influence of sulfite was studied with an 1 mM concentration. For inhibition of F_1-ATPase 20 μg/ml oligomycin were present.

Strain	ATPase activity			
	Control	+ Sulfite	+ Oligomycin	+ Sulfite + oligomycin
	(U/mg protein)	(% of control)	(% of control)	(% of control)
X 2180 B	0.756	229	30	28
pet 936	0.302	101	85	85

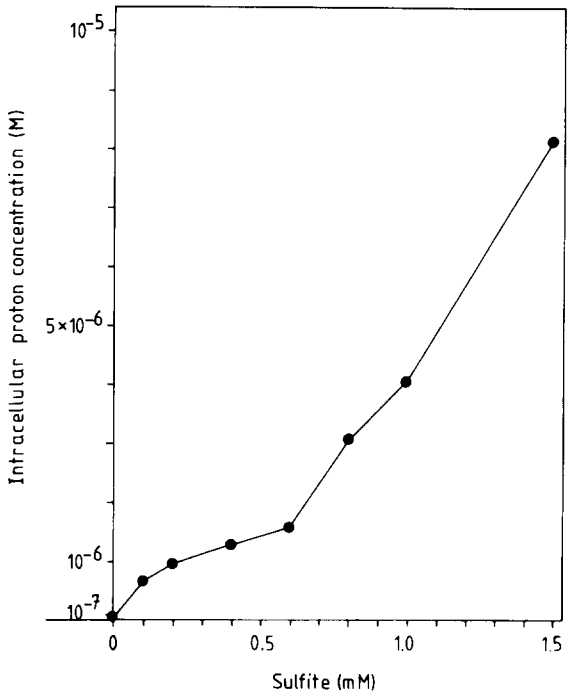

Fig. 4. Effect of sulfite on the intracellular proton concentration in *Saccharomyces cerevisiae* X 2180 B from stationary growth phase in the absence of glucose. Suspensions of 2% (w/v) yeast in 0.1 M sodium succinate (pH 3.6) were preincubated for 60 min. After addition of sulfite incubation was continued for 10 min in the presence of $1 \cdot 10^{-5}$ M [1-^{14}C]propionic acid. Then 1 ml samples were taken and centrifuged at $10000 \times g$ for 4 min. The radioactivity was measured in both supernatant and pellet and the intracellular proton concentration was calculated from partition of [1-^{14}C]propionic acid in the two fractions.

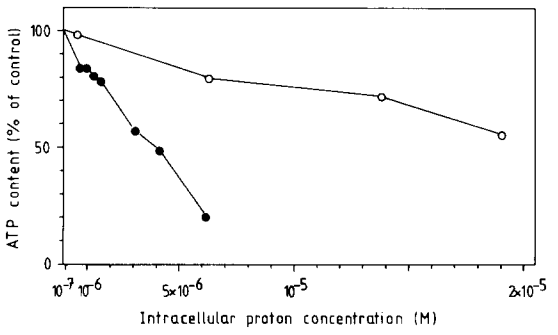

Fig. 5. Relationship between intracellular proton concentration and ATP-content of *Saccharomyces cerevisiae* X 2180 B from stationary growth phase in the absence of glucose. Suspensions of 2% (w/v) yeast in 0.1 M sodium succinate (pH 3.6) were preincubated for 60 min at 20°C. Then, sulfite (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 2 mM (●———●)) or formic acid (2, 4, 6 and 8 mM (○———○)) were added and incubation was continued in the presence of $1 \cdot 10^{-5}$ M [1-^{14}C]propionic acid. After 10 min 1 ml samples were withdrawn and centrifuged at $10000 \times g$ for 4 min. Radioactivity was measured in both supernatant and pellet and the intracellular proton concentration was calculated from partition of [1-^{14}C]propionic acid in the two fractions. Simultaneously, samples were taken for determination of ATP (see 'Materials and Methods'). Control: 100% ATP = 2 μmol/g yeast (wet weight).

on the effect of sulfite on the intracellular pH were performed in the presence of [1-^{14}C]propionic acid. The effect of sulfite on the intracellular proton concentration of glucose-starved yeast cells is shown in Fig. 4. In the absence of sulfite an average intracellular proton concentration of $3.2–6.3 \cdot 10^{-8}$ M was calculated, corresponding to pH 7.2–7.5. With 1 mM sulfite the average intracellular proton concentration of yeast increased to $4.0 \cdot 10^{-6}$ M, corresponding to pH 5.4. This intracellular acidification may stimulate an ATP-driven proton pump [58,59,61] and may thus contribute to ATP depletion.

The influence of intracellular acidification on the ATP level has been investigated by incubation of wild-type yeast at pH 3.6 in the presence of formic acid. At this pH value formic acid easily penetrates the cell membrane similar as propionic acid according to the ion trapping mechanism. With formic acid up to 8 mM a drastic increase in the intracellular proton concentration was observed (Fig. 5). However, compared to sulfite the effect on the ATP content was much less pronounced. These findings suggest that the action of a proton pump during intracellular acidification is of no (or only of secondary) importance for the sulfite-initiated depletion of the ATP level in yeast.

Reversibility of the sulfite initiated ATP decrease in yeast

Reversibility of the ATP decrease in yeast was studied after short-term incubation with 2 mM sulfite both in the presence and absence of glucose. Experiments in the presence of glucose showed that inhibition of substrate chain phosphorylation by sulfite is reversible. Yeast cells from the ρ^--mutant, which do not respire, were incubated with sulfite for 4 min, causing a complete depletion of ATP. After subsequent addition of acetaldehyde as a sulfite trap a recovery of more than 70% of the original ATP level was

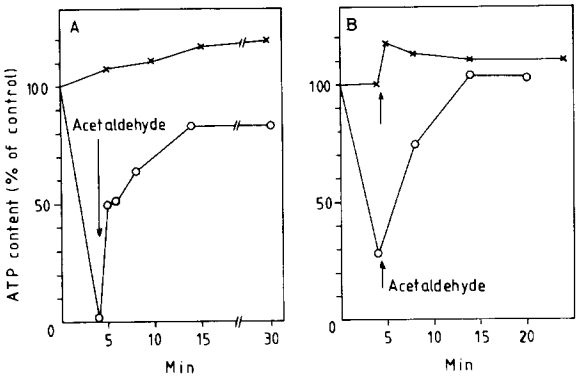

Fig. 6. Reversibility of the sulfite-initiated ATP decrease in yeast. (A) Logarithmic cells of the ρ^--mutant were suspended (0.1% (w/v)) in McIlvain buffer (pH 3.6) and preincubated for 30 min at 20°C in the presence of 2% (w/v) glucose. At zero time, 2 mM sulfite was added to the yeast suspension. After 4 min 25 mM acetaldehyde was added. At the times indicated samples were taken for determination of ATP (see 'Materials and Methods'): O——O, presence of 2 mM sulfite; ×——×, absence of sulfite. Control: 100% ATP = 1.5 μmol/g yeast (wet weight). (B) Stationary cells of the wild-type strain X 2180 B were suspended (0.1% (w/v)) in McIlvain buffer, (pH 3.6) and preincubated for 10 min at 20°C in the absence of glucose. At zero time, 2 mM sulfite was added to the yeast suspension. After 4 min 25 mM acetaldehyde was added. At the times indicated samples were taken for ATP determination (see 'Materials and Methods'). O——O, presence of 2 mM sulfite; ×——×, absence of sulfite. Control: 100% ATP = 2 μmol/g yeast (wet weight).

obtained (Fig. 6A). Studies in the absence of glucose indicated that respiratory chain phosphorylation is reversibly blocked by sulfite. In glucose-starved yeast cells pretreated with sulfite for 4 min the ATP content was strongly reduced. However, within a few min after addition of acetaldehyde, more than 90% of the original ATP was regenerated (Fig. 6B). Regeneration of ATP was observed also after filtration of the cells and resuspension in a sulfite-free buffer at pH 7.5 (data not shown).

Discussion

Sulfite lowers the levels of ATP and ADP as well as of other ribonucleoside di- and triphosphates in yeast cells. However, the most dramatic effect was observed with ATP and ADP (see Table II). Independent of the mechanism of ATP depletion, the decrease of ADP and increase of AMP may be effected by the enzyme adenylate kinase, which reversibly catalyzes the reaction of ATP + AMP $\rightleftharpoons$ 2 ADP. Lack of ATP may shift the equilibrium to the left-hand side. In the presence of glucose, sulfite changes the energy charge, as defined by Atkinson [62], from 0.89 to 0.05. In such a situation presumably most of the energy-dependent reactions come to a halt. The present paper shows that sulfite acts both on glycolysis and on respiratory chain phosphorylation in yeast causing ATP depletion.

Yeast cells of the mutant pet 936 which are not able to respire respond with a complete decrease of the ATP content within a few min after addition of sulfite in the presence of glucose (cf. Table I). This effect can therefore be directly correlated to inhibition of glycolysis by sulfite (cf. Fig. 2), which is the consequence of the following different mechanisms.

(a) Formation of sulfite adducts. Sulfite reacts very easily with acetaldehyde and thus blocks formation of ethanol. During this last step in alcoholic fermentation, NAD^+ is regenerated from NADH which is consumed by the glyceraldehyde-3-phosphate dehydrogenase catalyzed oxidation step in glycolysis. Trapping of acetaldehyde by sulfite effectively stops regeneration of NAD^+ as shown in vitro (cf. Fig. 1). According to Holzer et al. [63] the quotient of ethanol/acetaldehyde is proportional to the quotient of $NADH_{free}/NAD^+_{free}$ in different metabolic stages of yeast. Depletion of acetaldehyde by formation of the sulfite adduct would be expected to increase the quotient of ethanol/acetaldehyde and consequently to change the ratio of $NADH_{free}:NAD^+_{free}$ in favour of $NADH_{free}$. Regeneration of NAD^+ may also occur by reduction of dihydroxyacetone phosphate to glycerol-3-phosphate [64,65]. However, since yeast contains only low activities of enzymes to reduce triose phosphates [65], insufficient NAD^+ would be regenerated to support glycolysis effectively.

(b) Interaction of sulfite with the NAD^+-dependent dehydrogenases in the glycolytic pathway. It has been demonstrated that sulfite very efficiently inhibits glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase in intact cells. Inhibition of the two enzymes by sulfite was observed also in a yeast extract (data not shown). Meyerhof et al. [3] found that sulfite inhibits lactate dehydrogenase by forming an adduct with NAD^+.

Pfleiderer et al. [5,6] studied interaction of sulfite with NAD^+-dependent enzymes and titrated NAD^+ binding sites by monitoring formation of the NAD^+ · sulfite adduct which exhibits maximal absorbance at 320 nm. The authors recognized that enzyme-bound NAD^+ reacts much more readily with sulfite than does unbound NAD^+. This has been confirmed with lactate dehydrogenase and malate dehydrogenase from pig heart by Parker et al. [8]. It is supposed that yeast glyceraldehyde-3-phosphate dehydrogenase is also inhibited by binding the NAD^+-sulfite complex.

Sulfite strongly impairs respiratory chain phosphorylation. Both oxygen consumption and the ATP content of glucose-starved yeast are drastically lowered by sulfite during incubation at pH 3.6 (cf. Fig. 3). Under the conditions used for glucose starvation (10–60 min preincubation in absence of glucose) at least 60% of the cellular ATP content are regenerated by respiratory chain phosphorylation. This has been found by uncoupling of respiration with carbonyl cyanide *m*-chlorophenylhydrazone or by inhibition with sodium azide, both causing an ATP depletion of about 60%. The remaining ATP level turned out to be most sensitive to iodoacetic acid, a potent inhibitor of glycolysis [66], indicating ATP generation by fermentation (unpublished results). It is known that in the absence of external nutrients intact yeast cells can both respire and ferment their endogenous carbohydrate reserves, like glycogen or trehalose, [67] to maintain energy metabolism. Sulfite reacts easily with FAD containing proteins [9–13]. Cytochrome b_2 (l-lactate dehydrogenase) from bakers yeast, a typical flavoprotein, is competitively inhibited by sulfite which reacts with the FMN moiety of the enzyme. The K_m value for lactate is 0.4 mM, whereas the K_i value for sulfite is 1.4 μM [13]. It is therefore assumed that impairment of respiration by sulfite might be caused by an effective inhibition of flavoproteins involved in respiration. This should be proved by further detailed investigations.

The decline of ATP effected by sulfite is closely related to inhibition of the ATP regenerating systems. However, beside these mechanisms stimulation of an ATP-hydrolyzing system by sulfite should be discussed as a reason to disturb energy-balance. A 2.8-fold stimulation of the oligomycin-sensitive F_1-ATPase by sulfite at pH 5.7 was detected in a crude membrane fraction of yeast (cf. Table III). Ebel and Lardy [68] reported activation of bovine heart F_1-ATPase by several anions. Among these sulfite was the most potent activator. With homogeneous F_1-ATPase the authors observed an 8.6-fold stimulation by sulfite. However, in a submitochondrial fraction they found only a 2-fold stimulation of the enzyme which agrees with our results obtained from yeast (cf. Table III). In spite of this marked effect of sulfite on the F_1-ATPase in vitro we do not believe it is of importance for ATP depletion, since F_1-ATPase functions as a coupling factor as long as the electrochemical potential is maintained by intact respiration. This argument is supported by the results obtained with the mutant pet 936 which is deficient in a functional F_1-ATPase [46]. We found that in the absence of glucose ATP is depleted in this mutant as fast as in the wild type after addition of sulfite (cf. Table I). If stimulation of F_1-ATPase by sulfite would be involved in ATP decrease the depletion rate in the pet mutant should be distinctly lower. Furthermore, the results shown in Table III clearly demonstrate that the oligomycin-insensitive ATPase, which is identical with the plasma membrane ATPase [57–59], is not stimulated by sulfite in vitro.

Hinze and Holzer [60] observed a drastic accumulation of sulfite in yeast cells during incubation at pH 3.6. This effect has been explained by the ion trapping mechanism which functions with sulfite in an acidic medium [43,60]. The authors found a 50-fold accumulation in yeast cells when applying 0.5 mM sulfite into the incubation medium [60]. On the basis of these findings it should be realized that high intracellular concentrations of sulfite may arise even at low extracellular concentrations, sufficient to promote reactions with enzymes, cofactors and metabolites.

Sulfite strongly increases the intracellular proton concentration in yeast during incubation at pH 3.6 (cf. Fig. 4). We therefore proved whether stimulation of an ATP-driven proton pump [58,59,61] is involved in the sulfite-initiated ATP depletion. Formic acid also causes a strong intracellular acidification in yeast when added to the incubation medium at pH 3.6. However, the effect on the cellular ATP content is much less pro-

nounced than observed with sulfite (cf. Fig. 5). It is therefore concluded that the sulfite-initiated ATP depletion is predominantly based on sulfite specific actions as discussed above and not, or to a much lesser extend on the action of an ATP-driven proton pump responsible for extrusion of protons out of the cells. The proton pump of yeast is thought to be identical with the plasma membrane ATPase [58,59,61], which is not stimulated by sulfite (cf. Table III).

The effect of sulfite on energy metbolism in yeast is reversible during short-time incubations as indicated by a high recovery of ATP after removal of sulfite (cf. Fig. 6). During incubations of yeast in the presence of sulfite at pH 3.6 for more than 60 min the effect becomes increasingly irreversible leading to cell death as shown by Schimz and Holzer [41].

Beck-Speier et al. [69] showed that bakers yeast and *Saccharomyces cerevisiae* exhibit very low activities of sulfite oxidase, the detoxifying enzyme for sulfite. In contrast, liver tissues and hepatocytes are endowed with high activities of sulfite oxidase. The presence of this enzyme totally prevents ATP depletion in liver slices and hepatocytes. The authors concluded that a strong reciprocal correlation exists between the sensitivity of ATP to sulfite and the activity of sulfite oxidase in tissues and cells [69].

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