Arch Microbiol (1986) 145:27–31

Archives of
Microbiology

Analysis of the energy metabolism after incubation of *Saccharomyces cerevisiae* with sulfite or nitrite *

Helga Hinze and Helmut Holzer

Gesellschaft für Strahlen und Umweltforschung (GSF), Abteilung für Enzymchemie, D-8042 Neuherberg bei München, Federal Republic of Germany

Abstract. After addition of 5 mM sulfite or nitrite to glucose-metabolizing cells of *Saccharomyces cerevisiae* a rapid decrease of the ATP content and an inversely proportional increase in the level of inorganic phosphate was observed. The concentration of ADP shows only small and transient changes. Cells of the yeast mutant pet 936, lacking mitochondrial F_1ATPase, after addition of 5 mM sulfite or nitrite exhibit changes in ATP, ADP and inorganic phosphate very similar to those observed in wild type cells. They key enzyme of glucose degradation, glyceraldehyde-3-phosphate dehydrogenase was previously shown to be the most sulfite- or nitrite-sensitive enzyme of the glycolytic pathway. This enzyme shows the same sensitivity to sulfite or nitrite in cells of the mutant pet 936 as in wild type cells. It is concluded that the effects of sulfite or nitrite on ATP, ADP and inorganic phosphate are the result of inhibition of glyceraldehyde-3-phosphate dehydrogenase and not of inhibition of phosphorylation processes in the mitochondria. Levels of GTP, UTP and CTP show parallel changes to ATP. This is explained by the presence of very active nucleoside monophosphate kinases which cause a rapid exchange between the nucleoside phosphates. The effects of the sudden inhibition of glucose degradation by sulfite or nitrite on levels of ATP, ADP and inorganic phosphate are discussed in terms of the theory of Lynen (1942) on compensating phosphorylation and dephosphorylation in steady state glucose metabolizing yeast.

Key words: Nitrite – Sulfite – Saccharomyces cerevisiae – ATP – Energy metabolism – Inorganic phosphate – Glyceraldehyde-3-phosphate dehydrogenase – Glucose-6-phosphate dehydrogenase

Previous work (Schimz and Holzer 1979; Schimz 1980; Hinze and Holzer 1985a) has shown that 0.1 mM sulfite or nitrite causes a rapid decrease in the ATP concentration of yeast cells. Cell death, i.e. the drastic decrease of the cell forming capacity, caused by sulfite or nitrite is probably a consequence of the ATP-depletion (Schimz 1980). Inhibition of the ATP producing degradation of carbohydrate at the step of glyceraldehyde-3-phosphate dehydrogenase has shown to be responsible for the decrease in ATP (Hinze and Holzer 1985b). In the case of sulfite, in addition to the inactivation of glyceraldehyde-3-phosphate dehydrogenase, formation of an aldehyde-bisulfite complex with glyceraldehyde-3-phosphate, which slows down the rate of the dehydrogenase reaction by lowering the concentration of free, uncomplexed glyceraldehyde-3-phosphate, may also contribute to the observed depletion of ATP (Hinze and Holzer 1985b). For further analysis of the effects of sulfite or nitrite on the energy metabolism of yeast, levels of ATP, ADP and inorganic phosphate as well as of non-adenosine nucleoside phosphates were analyzed. Furthermore, the effects of sulfite and nitrite on the yeast mutant pet 936 lacking an intact respiratory chain phosphorylation, were studied to elucidate a possible role of the mitochondrial ATP-synthesizing system in the observed effects on the ATP-levels.

Present address and offprint requests to: H. Holzer, Biochemisches Institut der Universität Freiburg, D-7800 Freiburg im Breisgau, Federal Republic of Germany

Abbreviations. ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; P_i, inorganic orthophosphate

* Dedicated to Prof. Dr. Hans Grisebach on the occasion of his sixtieth birthday

Materials and methods

Chemicals. The reagents for the assays of ATP, ADP and enzyme activities were purchased from Boehringer (Mannheim, FRG) and Sigma (München FRG); bactopeptone and yeast extract were from Difco (Detroit, MI, USA). All other chemicals were obtained from Merck (Darmstadt, FRG).

Organisms. Saccharomyces cerevisiae strain X 2180 B (wild type) was kindly provided by Prof. Dr. Dieter H. Wolf, Biochemisches Institut der Universität Freiburg, FRG. The nuclear F_1-deficient mutant pet 936 (pet^+) was a generous gift of Prof. Dr. G. Schatz, Biozentrum, Basel, Switzerland.

Conditions of cultivation. Cells were grown to the stationary phase (24 h wild type; 20 h pet 936) at 30° C under aerobic conditions on complete medium containing 1% yeast extract, 2% bactopeptone and 2% glucose. Details for the growth conditions of the mutant are described by Ebner et al. (1973).

Incubation of yeast and preparation of crude extracts for enzyme activities. Incubation of yeast and crude extract preparation is described by Hinze and Holzer (1985b).

Determination of ATP and ADP. The method used for the assay of ATP is described by Hinze and Holzer (1985a) and for ADP by Schimz et al. (1981).

Assays of enzyme activities. Glyceraldehyde-3-phosphate dehydrogenase was assayed according to Bergmeyer et al.

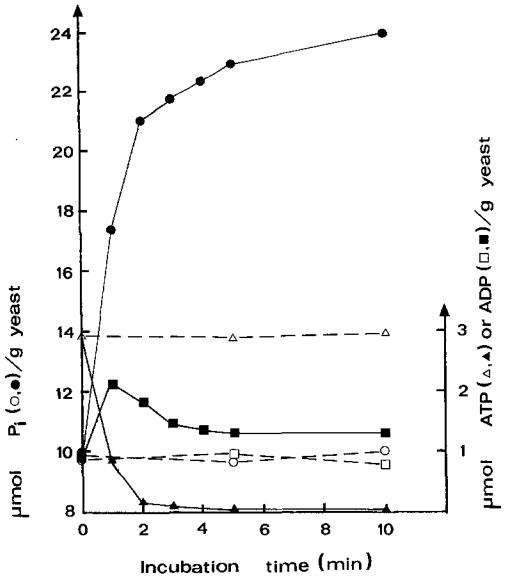

Fig. 1. ATP, ADP and P_i on incubation of *Saccharomyces cerevisiae* X 2180 B with 5 mM sulfite in the presence of glucose. A 2% (wet weight/vol) yeast suspension, preincubated for 1 h with 2% (w/v) glucose at 20° C, was treated with 5 mM sulfite at pH 3.6 and 20° C. At the times indicated, samples were taken and analyzed for ATP-, ADP- and P_i. *Full symbols*: with sulfite; *open symbols*: control without sulfite

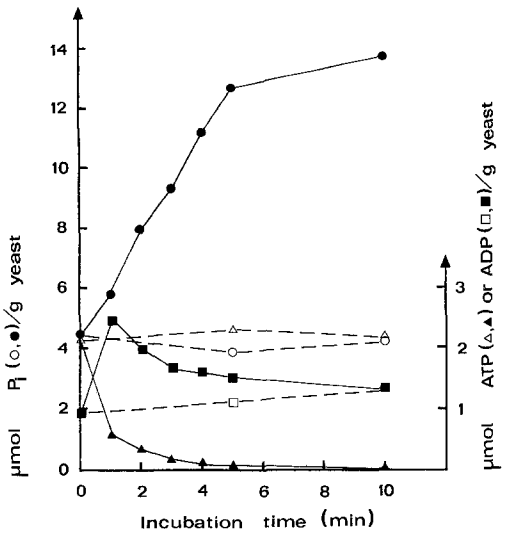

Fig. 2. ATP, ADP and P_i on incubation of *Saccharomyces cerevisiae* X 2180 B with 5 mM nitrite in the presence of glucose. Experimental conditions are described in the legend to Fig. 1. *Full symbols*: with nitrite; *open symbols*: control without nitrite

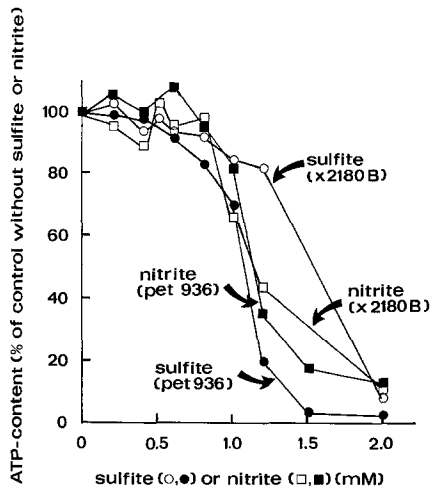

Fig. 3. ATP-content of *Saccharomyces cerevisiae* X 2180 B or mutant pet 936 cells after 10 min incubation with sulfite or nitrite in the presence of glucose. A 2% (wet weight/vol) yeast suspension, preincubated for 1 h with 2% (w/v) (wild type cells) or 3% (w/v) (mutant cells) glucose at 20° C, was treated with 5 mM sulfite or 5 mM nitrite for 10 min at pH 3.6 and 20° C. The measured ATP-content is expressed in % of the values without sulfite or nitrite. The data for X 2180 B are taken from Hinze and Holzer (1985a)

(1974), glucose-6-phosphate dehydrogenase according to Löhr and Waller (1974).

Determination of inorganic phosphate. P_i was measured in the perchloric acid extracts according to Ames (1966) with 0.2 mM disodium hydrogen phosphate as standard.

Determination of ribonucleoside phosphate pools in yeast. A 2% (wet weight/vol) suspension of stationary cells of the 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 nitrite. For ribonucleoside phosphate determinations 1 ml samples were centrifuged at 8,000 × *g* for 4 min at 4° C. The sediments, containing 20 µg cells, were treated with 80 µl ice-cold 20% (w/v) perchloric acid and neutralized after 20 min storage on ice (with occasional mixing) by dropwise additions of 140 µl ice-cold 2 M potassium hydrogen carbonate (final pH about 6.8). The suspension was kept at 0° C for 10 min and precipitated potassium perchlorate was removed by centrifugation for 4 min at 10,000 × *g* at room temperature. The supernatant was then lyophilized. The ribonucleoside phosphate were analyzed by high-performance liquid chromatography as described by Holstege et al. (1982).

Results

It may be seen from Figs. 1 and 2 that sulfite and nitrite show similar kinetics in decreasing the ATP-content and increasing the content of inorganic phosphate in yeast cells. Changes in the contents of ATP and inorganic phosphate are faster in the presence than in the absence of glucose. This may be a consequence of the preferential role of glycolysis, which is inhibited by sulfite or nitrite, in the maintenance of a steady state ATP level. As shown in Fig. 3 the ATP content of the mutant pet 936, defective in the mitochondrial F_1-ATPase (Ebner and Schatz 1973) exhibits a similar sensitivity to sulfite or nitrite as does the wild type X 2180 B: at 1.5 to 2.0 mM sulfite or nitrite almost 100% inhibition is effected. As seen in Table 1 the sensitivity to sulfite or nitrite of the ATP-level and of the glyceraldehyde-3-phosphate dehydrogenase activity is the same in the mutant pet 936 and the wild type cells.

The mutant pet 936 shows a similar time course of disappearance of ATP and appearance of inorganic phosphate after addition of sulfite or nitrite (Figs. 4 and 5) as observed with the wild type (Figs. 1 and 2). It may be concluded that

Table 1. Activities of enzymes in crude extracts from wild type X 2180 B or mutant pet 936 cells, incubated with 1 mM sulfite or 1.5 mM nitrite in the presence of 2% (w/v) (wild type cells) or 3% (w/v) (mutant cells) glucose at pH 3.6 and 20°C. For details of incubation and assays of enzyme activities see "Materials and methods". Enzyme activities are presented in % of controls incubated without addition of sulfite or nitrite

	X 2180 B[a]		pet 936	
	Sulfite 1 mM (%)	Nitrite 1.5 mM (%)	Sulfite 1 mM (%)	Nitrite 1.5 mM (%)
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	17	1	13	9
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	100	100	96	98

[a] Data taken from Hinze and Holzer (1985a)

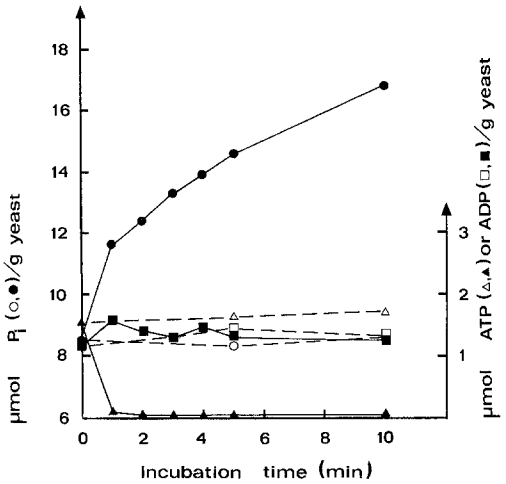

Fig. 4. ATP, ADP and P_i on incubation of mutant cells pet 936 with 5 mM sulfite in the presence of glucose. The experiment was done as described for Fig. 1, except 3% (w/v) instead of 2% (w/v) glucose. *Full symbols*: with sulfite; *open symbols*: control without sulfite

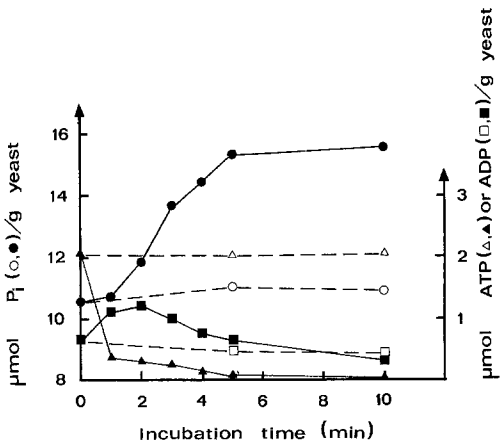

Fig. 5. ATP, ADP and P_i on incubation of mutant pet 936 yeast cells with 5 mM nitrite in the presence of glucose. The experiment was done as described for Fig. 1, except 3% (w/v) instead of 2% (w/v) glucose. *Full symbols*: with nitrite; *open symbols*: control without nitrite

Table 2. Decrease of ATP (−ATP), increase of inorganic phosphate (+P_i), and quotient (+P_i/−ATP) after 10 min incubation of *Saccharomyces cerevisiae* X2180B or mutant pet 936 with 5 mM sulfite or nitrite in the presence of 2% (w/v) (wild type cells) or 3% (w/v) (mutant cells) glucose at pH 3.6 and 20°C

Incubation mixture	+P_i (µmol/g yeast)	−ATP (µmol/g yeast)	(+P_i)/(−ATP)
X 2180 B			
+ 5 mM sulfite	14.04	2.92	4.8
+ 5 mM nitrite	9.30	2.10	4.4
pet 936			
+ 5 mM sulfite	8.39	1.50	5.6
+ 5 mM nitrite	5.05	2.05	2.5

the mitochondrial processes of respiratory chain phosphorylation and ATP-dephosphorylation by ATPase do not participate significantly in the observed changes of ATP and inorganic phosphate. The experiments with the mutant pet 936 are therefore in accordance with the previously formulated idea (Hinze and Holzer 1985b) that an inhibition of glyceral-dehyde-3-phosphate dehydrogenase is responsible for the ATP-depletion caused by sulfite or nitrite.

The steady state levels of ATP and inorganic phosphate are the result of balanced "phosphorylation" (formation of ATP) and "dephosphorylation" (liberation of inorganic phosphate) (Lynen 1942; Lynen and Koenigsberger 1950; 1951). When synthesis of ATP is blocked, dephosphorylation of ATP continues according to equation (1):

$$ATP \rightarrow AMP + 2P_i. \quad (1)$$

Lynen and Koenigsberger (1950; 1951) stopped phosphorylation by addition of 1 mM cyanide. From the rate of P_i formation extrapolated to the time of addition of cyanide, they calculated the steady state rate of "dephosphorylation", which is identical to the steady state rate of "phosphorylation". Similar to the experiments of Lynen and Koenigsberger with cyanide we studied the effects of sulfite or nitrite on "dephosphorylation" as shown in Figs. 1, 2, 4 and 5.

In Table 2 the ratios of increase in inorganic phosphate and of decrease in ATP after 10 min incubation with sulfite or nitrite are depicted. The ratios observed in the absence of glucose are somewhat higher than expected from equation (1): 2.4 instead of 2.0. In the presence of glucose ratios of up to 4−5 have been observed. The higher quotients of phosphate/ATP are explained by the presence of non-adenosine nucleotides which are in enzymatic equilibrium with the adenosine nucleotides. Therefore, the formation of inorganic phosphate is not only the results of degradation of ATP according to Eq. (1) but also to the degradation of GTP, CTP and UTP as summarized in Eq. (2):

$$XTP \rightarrow XMP + 2P_i. \quad (2)$$

When the concentration of GTP, CTP and UTP which almost completely disappear under the influence of nitrite (Table 3) or sulfite (Maier et al. 1985) are included for calculation of the appearance of inorganic phosphate in relation to the disappearance of ATP, ratios in the range of 3−5 are obtained. The higher quotient (+P_i)/(−ATP) in the presence than absence of glucose may be the result of higher concentrations of phosphorylated metabolites in the

Table 3. Effect of 5 mM nitrite on the nucleoside phosphate pools in wild type X 2180 B yeast cells. Incubation of yeast and determination of ribonucleoside phosphates were performed as described in "Materials and methods"

Nucleoside-phosphate	Initial value (nmol/g)	After 10 min of incubation	
		without nitrite (nmol/g)	with 5 mM nitrite (nmol/g)
ATP	1440	1470	16
ADP	290	240	61
AMP	34	31	1360
GTP	256	251	<8
GDP	n.d.	n.d.	197
GMP	<10	<10	147
UTP	223	207	<9
UDP	n.d.	n.d.	n.d.
UMP	n.d.	n.d.	191
CTP	96	101	<13
CDP	<10	<10	<10
CMP	n.d.	n.d.	n.d.

presence of glucose which are in rapid enzymatic equilibrium with ATP.

Discussion

Lynen (1942) and Lynen and Koenigsberger (1950; 1951) have shown that addition of cyanide to glucose-metabolizing yeast cells initiates a rapid liberation of inorganic phosphate. They postulated that the formation of inorganic phosphate by "dephosphorylation" is in the steady state compensated by "phosphorylation", i.e. disappearance of inorganic phosphate as a consequence of ATP synthesis. If this assumption is correct rate of "dephosphorylation", extrapolated to the time of inhibition of "phosphorylation" by addition of cyanide, is a measure for the steady state rate of "phosphorylation". In the present work, this technique of Lynen et al. has been used to get information about the effects of sulfite or nitrite on "phosphorylation" and "dephosphorylation" in yeast. In addition to measurements of the rate of appearance and disappearance of inorganic phosphate as done by Lynen and Koenigsberger (1950, 1951) the rates of appearance and disappearance of ATP and ADP were also measured. Our observations lead to the following conclusions: the synthesis of ATP from inorganic phosphate (and ADP) is stopped immediately on addition of sulfite and nitrite by inhibition of glyceraldehyde-3-phosphate dehydrogenase. The ATP-degrading and inorganic phosphate liberating processes of "dephosphorylation" continue after addition of sulfite or nitrite. They have been measured and tabullated as the ratio $(+ P_i)/(-ATP)$. When equilibration of the adenosine nucleotides with non-adenosine nucleotides of the purine and pyrimidine nucleotide series is taken into consideration, quotients of $(+ P_i)/(-ATP) = 2.5-5$ are expected. This is in accordance with the observations summarized in Table 2. Synthesis of ATP in the course of "phosphorylation" may be the result of "substrate phosphorylation", i.e. phosphorylation at the level of glyceraldehyde-3-phosphate dehydrogenase, and/or "respiratory chain phosphorylation", i.e. "oxidative phosphorylation" (Holzer 1951). As shown in the present work, the yeast mutant pet 936, which is characterized by a defect in the F_1-ATPase and is therefore deficient in respiratory chain phosphorylation, shows the same rate of decrease of ATP after addition of sulfite or nitrite as does the wild type X 2180 B. This is strong evidence that an inhibition of the "substrate phosphorylation" at the level of glyceraldehyde-3-phosphate dehydrogenase causes the observed changes in the concentrations of ATP, ADP and inorganic phosphate after addition of sulfite or nitrite. This is also in agreement with our previous work (Hinze and Holzer 1985b) showing that the concentrations of sulfite or nitrite which cause a rapid decrease in the ATP-level also inhibit glyceraldehyde-3-phosphate dehydrogenase. It may, however, be that in yeast cells, containing intact mitochondria in addition to the inhibition of substrate phosphorylation at the level of glyceraldehyde-3-phosphate dehydrogenase, the respiratory chain phosphorylation is also damaged by sulfite or nitrite. In vitro experiments with purified ATPase from yeast mitochondria demonstrate sensitivity of this system to sulfite (Maier et al. 1985). In summary, it is concluded that the drastic decrease in the ATP-level of yeast cells observed after incubation with sulfite or nitrite is a consequence of the inhibition of ATP synthesis linked to the glycolytic degradation of glucose.

Acknowledgements. This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 206 and the Verband der Chemischen Industrie e.V. (Fonds der Chemischen Industrie). We are greatly obliged to Prof. Dr. Gottfried Schatz for supplying the yeast mutant. We are grateful to Prof. Dr. Dietrich Keppler for determination of ribonucleosidephosphates by HPLC. The authors also thank Dr. Henryk Kalisz for critical reading of the manuscript and Ulrike Eitel for help with the figures and for typing the manuscript.

References

Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. In: Neufeld EF, Ginsburg V (eds) Methods in enzymology, vol 8. Academic Press Inc., New York, pp 115–118

Bergmeyer HU, Gawehn K, Graßl M (1974) Glycerinaldehyde-3-phosphat-Dehydrogenase. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, vol 1, 3. edn. Verlag Chemie, Weinheim, pp 495–496

Ebner E, Schatz G (1973) Mitochondrial assembly in respiration-deficient mutants of *Saccharomyces cerevisiae*. III. J Biol Chem 248:5379–5384

Ebner E, Menucci L, Schatz G (1973) Mitochondrial assembly in respiration-deficient mutants of *Saccharomyces cerevisiae*. I. J. Biol Chem 248:5360–5368

Hinze H, Holzer H (1985a) Accumulation of nitrite and sulfite in yeast cells and synergistic depletion of the intracellular ATP-content. Z Lebensm Unters Forsch 180:117–120

Hinze H, Holzer H, (1985b) Effect of sulfite or nitrite on the ATP content and the carbohydrate metabolism in yeast. Z Lebensm Unters Forsch 181:87–91

Holstege A, Schulz-Holstege C, Henninger H, Reiffen KA, Schneider F, Keppler D (1982) Uridylate trapping induced by the C-2-modified D-glucose analop glucosone, fluoroglucose and glucosamine. Eur J Biochem 121:469–474

Holzer H (1951) Photosynthese und Atmungskettenphosphorylierung. Z Naturf 6b:424–430

Löhr GW, Waller HD (1974) Glucose-6-phosphat-dehydrogenase. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, vol 1, 3. edn. Verlag Chemie, Weinheim, pp 673–681

Lynen F (1942) Die Rolle der Phosphorsäure bei Dehydrierungsvorgängen und ihre biologische Bedeutung. Naturwissenschaften 30:398–406

Lynen F, Koenigsberger R (1950) Nachweis der Phosphorylierung bei der alkoholischen Gärung durch lebende Hefe. Liebigs Ann Chem 569:129–138

Lynen F, Koenigsberger R (1951) Zum Mechanismus der Pasteur'schen Reaktion: Der Phosphat-Kreislauf in der Hefe und seine Beeinflussung durch 2,4-Dinitrophenol. Liebigs Ann Chem 573:60–84

Maier K, Hinze H, Leuschel L (1985) Mechanism of sulfite on the energy metabolism of *Saccharomyces cerevisiae*. Biochim Biophys Acta (in press)

Schimz KL (1980) The effect of sulfite on the yeast *Saccharomyces cerevisiae*. Arch Microbiol 125:89–95

Schimz KL, Holzer H (1979) Rapid decrease of ATP-content in intact cells of *Saccharomyces cerevisiae* after incubation with low concentrations of sulfite. Arch Microbiol 121:225–229

Schimz KL, Rütten B, Tretter M (1981) Determination of adenosine nucleotides with luciferine/luciferase from crude firefly lantern extract on a bioluminescence analyzer. In: Moo-Young M, Robinson CW, Vezina C (eds) Advances in biotechnology, vol 1. Pergamon-Press, Toronto, pp 457–462

Received November 14, 1985/Accepted February 20, 1986