Arch Microbiol (1985) 143:220–224

Archives of
Microbiology

Effects of *m*-Cl-peroxy benzoic acid on glycolysis in *Saccharomyces cerevisiae* *

Dhan Prakash [1] **and Helmut Holzer** [1,2]

[1] Gesellschaft für Strahlen und Umweltforschung (GSF), Abteilung für Enzymchemie, D-8042 Neuherberg, Federal Republic of Germany
[2] Biochemisches Institut der Universität Freiburg, D-7800 Freiburg i. Br., Federal Republic of Germany

Abstract. Concentrations of *m*-Cl-peroxy benzoic acid (CPBA) higher than 0.1 mM decrease the ATP-content of *Saccharomyces cerevisiae* in the presence of glucose in 1 min to less than 10% of the initial value. In the absence of glucose, 1.0 mM CPBA is necessary for a similar effect. After the rapid loss of ATP in the first min in the presence of glucose caused by 0.2 mM CPBA, the ATP-content recovers to nearly the initial value after 10 min. Aerobic glucose consumption and ethanol formation from glucose are both completely inhibited by 1.0 mM CPBA. Assays of the activities of nine different enzymes of the glycolytic pathway as well as analysis of steady state concentrations of metabolites suggest that glyceraldehyde-3-phosphate dehydrogenase is the most sensitive enzyme of glucose fermentation. Phosphofructokinase and alcohol dehydrogenase are slightly less sensitive. Incubation for 1 or 10 min with concentrations of 0.05 to 0.5 mM CPBA causes a) inhibition of glyceraldehyde-3-phosphate dehydrogenase, b) decrease of the ATP-content and c) a decrease of the colony forming capacity. From these findings it is concluded that the disturbance of the ATP-producing glycolytic metabolism by inactivation of glyceraldehyde-3-phosphate dehydrogenase may be an explanation for cell death caused by CPBA.

Key words: Peroxy benzoic acid – *Saccharomyces cerevisiae* – ATP – Glycolysis – Glyceraldehyde-3-phosphate dehydrogenase – Colony forming capacity

Organic peroxy acids and their derivatives, such as peracetic acid, diacetylperoxide or peroxyacetyl nitrate (PAN) are known to be strong skin and eye irritants for animals (Patty 1970; Moeschlin 1980) and to be toxic to plants (Heath 1980). Oxidation of cysteine residues to disulfides or cysteic acid and the oxidation of methionine residues have been suggested as possible molecular mechanisms of action of peroxides on enzymes (Knight and Mudd 1984).

To obtain information on the mechanisms of the action of peroxy acids on basic metabolic processes, studies with yeast as a model organism were undertaken. *m*-Chloroperoxy benzoic acid (CPBA) was used as a model peroxide. We observed drastic effects of low concentrations of CPBA on the ATP-content of yeast and therefore studied effects of CPBA on the ATP-producing glycolysis. It is shown in the present paper that glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase are the most CPBA-sensitive enzymes in the glycolytic pathway of yeast. The concentrations of CPBA which inhibit these enzymes are about the same as those which decrease the ATP-content and the colony forming capacity, i.e. the viability of yeast.

Materials and methods

Materials. The *m*-chloro-peroxybenzoic acid of 80–90% purity (10–20% *m*-Cl benzoic acid) was purchased from EGA-Chemie (Steinheim, FRG). Reagents for the assays of ATP, enzymes and metabolites were purchased from Boehringer (Mannheim, FRG). All yeast growth media were from Difco (Schmid, München, FRG) and all other chemicals from E. Merck (Darmstadt, FRG).

Yeast strain and growth condition. Strain X 2180 B (wild type) was kindly provided by Prof. Dr. D. H. Wolf (Biochemisches Institut der Universität Freiburg, FRG). Yeast cells were grown to the stationary phase (24 h) on complete YEPD medium, containing 1% yeast extract, 2% peptone, 2% glucose at 30° C under aerobic conditions. Cells were harvested by centrifugation, washed twice with ice cold distilled water and wet weight was determined on a $4{,}500 \times g$, 15 min pellet.

Stock solution of CPBA. The required quantities of CPBA (for 20 mM or 100 mM) were dissolved in 0.5 ml methanol and then diluted with 0.5 ml distilled water. Stock solutions were prepared 5 min before the start of incubation and were stored at room temperature.

Conditions of incubation with CPBA. Cells were preincubated under air without or with 2 or 4% glucose for 1 h at 30° C in 0.1 M potassium phosphate buffer pH 7.0 in an Erlenmeyer flask on a reciprocal water bath shaker. The incubation procedure was then started by the addition of CPBA stock solution. Shaking was continued during incubation with

* Dedicated to Prof. Dr. Wolfgang Gerok at the occasion of his 60th birthday

Abbreviations. CPBA, *m*-Chloro-peroxy benzoic acid; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P_2, fructose-1,6-bisphosphate; DAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; 2PGA, 2-phosphoglycerate; PEP; phosphoenol pyruvate; Pyr, pyruvate; EtOH, ethanol; PFK, phosphofructokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, ADH, alcohol dehydrogenase

Offprint requests to: H. Holzer, Biochemisches Institut der Universität Freiburg, Hermann-Herder-Str. 7, D-7800 Freiburg i. Br., Federal Republic of Germany

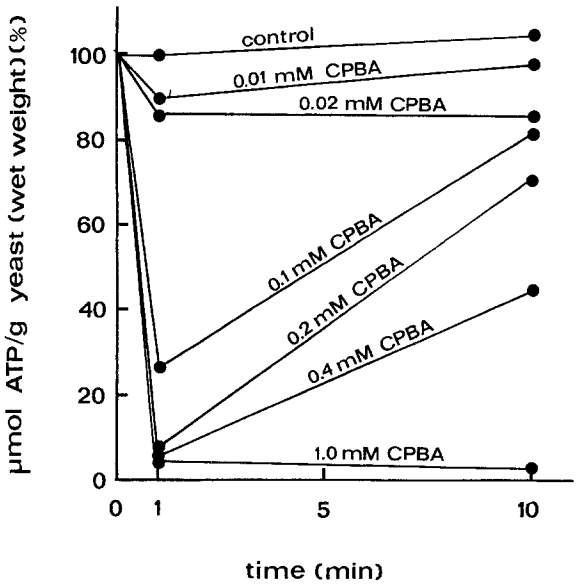

Fig. 1. ATP-content of yeast cells incubated under air with CPBA in the presence of glucose. A 2% yeast suspension (w/v) in 0.1 M phosphate buffer pH 7.0 was preincubated 1 h in the presence of 4% glucose (w/v) at 30° C. At zero time stock solution of CPBA (final concentration as indicated) was added to the yeast suspension. Samples were analyzed for ATP at the times indicated (for details see Materials and methods). The initial concentration of ATP was 2.2 µmol ATP/g yeast wet weight

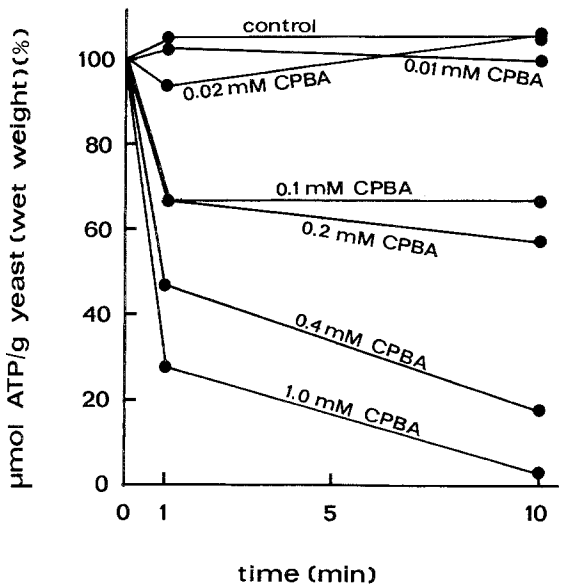

Fig. 2. ATP-content of yeast cells incubated under air with CPBA in the absence of glucose. For details see legend to Fig. 1. The initial concentration of ATP was 1.8 µmol ATP/g yeast wet weight

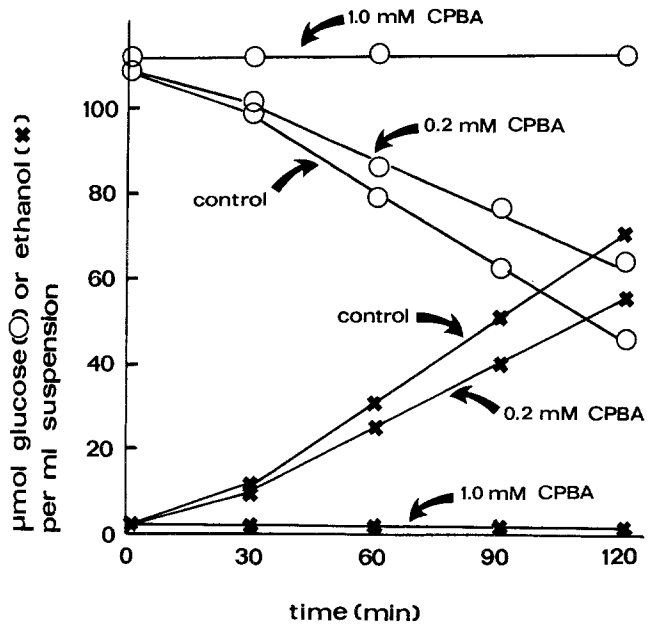

Fig. 3. Effect of CPBA on glucose consumption (○) and ethanol formation from glucose (×). 2% yeast in 100 mM phosphate buffer pH 7.0 was shaken under air in the presence of 2% glucose at 30° C. The indicated concentrations of CPBA were added at zero time. For assays of glucose and ethanol see Materials and methods

Table 1. ATP in sediment and/or supernatant of a yeast suspension incubated with toluene or CPBA. The 5.0 ml of 2% yeast suspensions in phosphate buffer pH 7.0 were preincubated 1 h at 30° C with 4% glucose. A 0.2 ml of 50% toluene/ethanol mixture (v/v, final concentration 2%) or 25 mM CPBA stock solution in 50% ethanol/buffer (v/v, final concentration 1.0 mM) was added at zero time. At the indicated times 0.2 ml aliquots of incubation mixture were centrifuged (10,000 × *g*, 4 min) and in the supernatant ATP was assayed. The sediment was suspended in 0.4 ml 17% perchloric acid. After 20 min on ice the samples were processed and assayed for ATP as described in Materials and methods

	µmol ATP/50 ml yeast suspension (2%)					
	Sediment (1.0 g)		Supernatant (49 ml)		Suspension (50 ml)	
Incubation time (min)	0	10	0	10	0	10
Control	1.6	1.6	0.002	0.003	1.9	1.8
+2% Toluene	1.6	0.5	0.002	0.1	1.9	1.2
+1.0 mM CPBA	1.6	0.02	0.002	0.005	1.9	0.03

CPBA. Samples for the zero time were taken just before the addition of CPBA or similar amount of 50% methanol/water (v/v) to the control experiment.

Assay of ATP. At the times indicated, 0.1 ml aliquots of the incubation mixtures were added to the same volume of ice-cold 35% (v/v) perchloric acid. After 20 min incubation at 0° C with occasional shaking, the extraction procedure was stopped by adding 0.54 ml of 2 M potassium hydrogen carbonate to obtain a final pH of about 7.0. After 10 min of further incubation on ice, the potassium perchlorate formed was centrifuged for 4 min at 10,000 × *g* at 4° C. The supernatants were diluted 1:200 with quartz-distilled water. Aliquots of 0.2 ml of these samples were added to 0.2 ml of luciferin/luciferase reagents solution and the bioluminescence was measured with a Biolumat LB 9500 T (Berthold, Wildbad, FRG). Standard ATP solutions of 5×10^{-9} M concentration were added for calibration in parallel analysis.

Assay of enzyme activities. A 2% yeast suspension (50 ml) in 0.1 M phosphate buffer pH 7.0 was preincubated by shaking

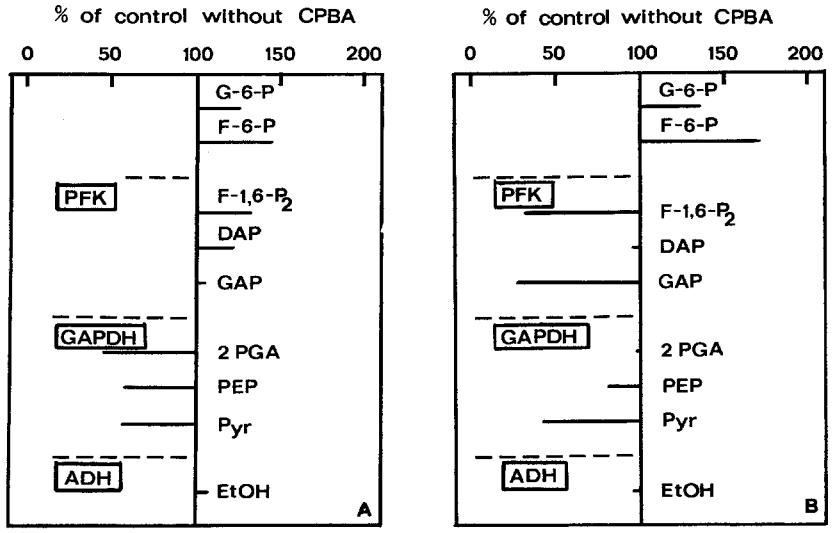

Fig. 4A, B
Effect of 30 min incubation with CPBA on metabolite concentrations and enzyme activities in yeast. **A** Yeast (2%) was shaken under air in 100 mM phosphate buffer pH 7.0 at 30° C in the presence of 2% glucose and 0.2 mM CPBA or **B** 1.0 mM CPBA added at zero time. All values are presented in % of the values at zero time without CPBA. Metabolites are indicated with full lines, enzymes with dotted lines

Table 2. Effect of CPBA on enzyme activity in yeast cells. Incubation of 2% yeast was in the presence of 2% glucose. For other details see Materials and methods

Enzyme	Concentration of CPBA (mM)	Enzyme activity in percent of a control without CPBA after incubation with CPBA for	
		1 min	30 min
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	0.2	8	15
	1.0	<1	<1
Alcohol dehydrogenase (EC 1.1.1.1)	0.2	10	16
	1.0	<1	<1
6-Phosphofructokinase-1 (EC 2.7.1.11)	0.2	55	59
	1.0	<1	<1
Glutamate dehydrogenase (NAD^+) (EC 1.4.1.2)	0.2	94	100
	1.0	8	7
Pyruvate decarboxylase (EC 4.1.1.1)	0.2	87	88
	1.0	21	15
Glutamate oxalacetate transaminase (EC 2.6.1.1)	0.2	97	106
	1.0	67	91
Hexokinase (EC 2.7.1.1)	0.2	97	101
	1.0	83	80
Glutamate dehydrogenase ($NADP^+$) (EC 1.4.1.4)	0.2	95	95
	1.0	92	100
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	0.2	94	94
	1.0	85	82

for 1 h in the presence of 2% glucose at 30° C. After addition of CPBA, 10 ml samples were withdrawn at the times indicated and poured into ice cold tubes containing ice, followed by immediate centrifugation (6,000 × *g*, 5 min). After washing with ice cold buffer (2.0 ml) the pellet was suspended in 2.0 ml buffer. Crude extract was prepared by passing this suspension twice through a French pressure cell (Aminco Silver Spring) at a pressure of 137,895.2 kN/m^2. The supernatant obtained after centrifugation of the homogenate at 10,000 × *g* for 4 min was used for determination of enzyme activities. Activities of glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase, glutamate dehydrogenase (NAD), glutamate dehydrogenase (NADP), pyruvate decarboxylase, glutamate oxalacetate transaminase and hexokinase were assayed as described by Bergmeyer et al. (1974a). Phosphofructokinase and glucose-6-phosphate dehydrogenase were assayed according to Afting et al. (1971) and Löhr and Waller (1974), respectively.

Glucose consumption and ethanol formation. Solutions of 2% glucose in 0.1 M phosphate buffer pH 7.0 were preincubated for 20 min at 30° C. After addition of CPBA glycolysis was started by addition of yeast suspension (2% final concentration). At times indicated, 0.1 ml samples were mixed with 0.1 ml ice cold 35% perchloric acid. The extraction was performed as described for ATP-determination. Glucose and ethanol were determined enzymatically in the supernatant

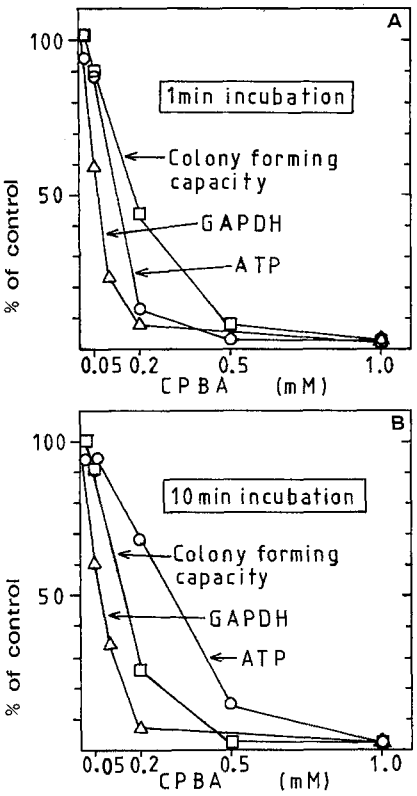

Fig. 5A, B. Effect of CPBA on ATP-content, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity and colony forming capacity of yeast cells. **A** Yeast (2%) with the indicated concentrations of CPBA was shaken under air at pH 7.0 and 30° C for 1 min or **B** 10 min. For other details see Materials and methods. Results are expressed as percent of the control without CPBA

according to Bergmeyer et al. (1974b) and Bernt and Gutmann (1974), respectively.

Determination of colony forming capacity. A 2% yeast suspension in 0.1 M phosphate buffer pH 7.0 containing 4% glucose was preincubated for 1 h at 30° C. At the indicated times after addition of CPBA, 0.5 ml samples were diluted $1:10^5$ and $1:5\times10^5$ with sterilised 0.9% NaCl solution. Aliquots of 0.1 ml from both dilutions were plated in duplicate on sterilised YEPD agar-medium. The colonies were counted after 48 h at 30° C.

Assay of metabolites. Yeast was preincubated and incubated with CPBA as described for the assay of enzyme activities. At the times indicated 100 ml samples were transferred to ice cold tubes containing ice and were immediately centrifuged ($6{,}000\times g$, 5 min). The pellet was suspended in 2.0 ml of 35% perchloric acid, incubated for 20 min on ice and centrifuged ($4{,}500\times g$, 5 min). The supernatant was neutralized with 2 M potassium hydrogen carbonate solution. After 10 min of further incubation at 0° C the potassium perchlorate formed was separated by centrifugation ($10{,}000\times g$, 4 min) and the supernatant assayed for metabolites as described by Lang and Michal (1974), Michal and Beutler (1974) and Czok and Lamprecht (1974).

Assay of protein. Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard.

Results and discussion

The time-dependence of the effect of *m*-chloro peroxy benzoic acid (CPBA) on the ATP-content of yeast incubated with glucose is shown in Fig. 1. A significant effect was seen at concentrations ≥ 0.1 mM CPBA. With 0.1 to 0.4 mM CPBA 70 – 90% of the ATP disappeared in less than 1 min. After 10 min, however, a partial recovery of the ATP-level was observed. With 1.0 mM CPBA the ATP-content decreased in the first minute to about 5% of the initial value, with no subsequent recovery. In contrast, in the absence of glucose, CPBA effected smaller changes in the ATP-level with no subsequent increase in the levels of ATP (Fig. 2). *m*-Chlorobenzoic acid (15 mM), an impurity in the preparation of CPBA used, had no effect on the ATP-level (data not shown). The more pronounced effects of 1 min incubation with CPBA in the presence of glucose as compared to incubation without glucose, is evident from Figs. 1 and 2. The reason might be a different conformation and therefore a different sensitivity of protein attacked by CPBA caused for example by different intracellular pH-values (Nicolay et al. 1982; Gillies et al. 1981) under the different metabolic situations.

After 1 min incubation of 0.2 or 1.0 mM CPBA with intact yeast in the presence or absence of glucose as described in the legends to Figs. 1 and 2, the supernatant exhibits no more ATP-decreasing activity when incubated with fresh yeast (data not shown). In contrast, in the absence of yeast, 0.2 mM CPBA retained 97% of its ATP-decreasing activity after 1 min. Metabolic intactness of yeast was not necessary to cause disappearance of the ATP-decreasing activity of CPBA. Yeast heated for 10 min at 90° C had the same effect as intact yeast.

The data shown in Fig. 1 demonstrate a recovery of the ATP-content between 1 and 10 min after incubation with CPBA. The recovery phenomenon is not observed in the absence of glucose (cf. Figs. 1 and 2). A functioning respiratory system was not necessary for ATP recovery after treatment of yeast cells with 0.2 mM CPBA because addition of 1.0 mM cyanide had no decreasing effect on the ATP recovery in the presence of glucose (data not shown). Ethanol was considerably less effective than glucose for the recovery of ATP (data not shown). Therefore, we assume that the ATP-recovery is the consequence of a glucose dependent repair of the ATP-generating system damaged by CPBA in the first min of its influence. The glucose-dependence of the repair system concerns the metabolism of glucose insensitive against 1 mM cyanide, i.e. anaerobic fermentation.

Effects of peroxides on the permeability properties of membranes are well known (Frew et al. 1983). We have studied if the decrease of intracellular ATP may be the consequence of increased permeability of the yeast cell membrane for ATP.

This would lead to the appearance of ATP in the suspension medium. As demonstrated in Table 1, toluene, a yeast permeabilizing agent (Murakami et al. 1980) decreased intracellular ATP to about 30% and caused a significant increase of the ATP-concentration in the supernatant. In contrast, with 1.0 mM CPBA, which decreased the ATP-content in the cells more than 95%, ATP in the supernatant was similar to the control without toluene or CPBA. It is evident that the decrease in the ATP-content in yeast cells observed after incubation with CPBA is not a consequence of permeabilization of the cell membrane for ATP.

As illustrated in Fig. 3, 1.0 mM CPBA which decreased the ATP-content of yeast (cf. Fig. 1) inhibited completely glucose consumption as well as ethanol formation from glucose. To localize the effect of CPBA on the carbohydrate metabolism, activities of different enzymes in extracts from yeast cells treated with 0.2 or 1.0 mM CPBA were assayed. As shown in Table 2, glyceraldehyde-3-phosphate dehydrogenase, NAD-alcohol dehydrogenase and 6-phosphofructokinase-1 were most sensitive. *m*-Chloro benzoic acid (10 mM) showed no effect on these three enzymes (data not shown). The results of assays of intracellular concentrations of glycolytic metabolites and of the activities of the three enzymes most sensitive to CPBA, after incubation of yeast cells with 0.2 or 1.0 mM CPBA are shown in Fig. 4. Incubation of yeast in the presence of glucose with CPBA, under the conditions which resulted in a drastic decrease of the ATP-content led to an inhibition of carbohydrate degradation at the metabolic steps catalyzed by 6-phosphofructokinase-1, glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase.

To find if the observed effects of CPBA on yeast cell metabolism may be related to cell death the colony forming capacity was measured. Figure 5 summarizes the observed effects of different concentrations of CPBA after 1 or 10 min incubation with yeast. From comparison with the corresponding effects of CPBA on the ATP content and on glyceraldehyde-3-phosphate dehydrogenase activity, it becomes evident that for all three parameters depicted in Fig. 5 distinct effects are seen at 0.05 mM CPBA and are almost maximal at 0.5 mM CPBA. Considering also the results summarized in Fig. 4, it may be postulated that under conditions where the energy metabolism is dependent on glycolysis, cell death (measured as colony forming capacity) caused by CPBA may be a consequence of disturbance of the ATP producing glycolytic metabolism by inactivation of glyceraldehyde-3-phosphate dehydrogenase. The respiratory ATP production (all experiments were done under aerobic conditions) is not able to prevent or to overcome the CPBA-caused ATP depletion. This suggests that an inhibitory attack of CPBA on the mitochondrial oxidative system, i.e. on respiratory chain phosphorylation (Lynen and Holzer 1949), takes place at concentrations of CPBA similar or less than those inhibiting glyceraldehyde-3-phosphate dehydrogenase. Studies on interactions of CPBA with the yeast respiratory system are in progress in our laboratory.

Acknowledgements. Thanks are due to Drs. A. G. Lenz, W. Göggelmann and H. Hinze for valuable discussions, to Dr. Henryk Kalisz for critical reading of the manuscript, to Mrs. Ulrike Eitel for her excellent help to prepare the figures and typing the manuscript. The financial assistance from Gesellschaft für Strahlen- und Umweltforschung mbH, München, Neuherberg, FRG, with a postdoctoral fellowship to Dr. Dhan Prakash (National Botanical Research Institute, Lucknow, India) is gratefully acknowledged.

References

Afting EG, Ruppert D, Hagmeier V, Holzer H (1971) Yeast phosphofructokinase: Effector-controlled conversion of an ATP-sensitive to an ATP-desensitized form. Arch Biochem Biophys 143:587–592

Bergmeyer HU, Gawehn K, Graßl M (1974a) Enzyme als biochemische Reagenzien. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, vol 1. Verlag Chemie, Weinheim, pp 454–558

Bergmeyer HU, Bernt E, Schmidt F, Storck H (1974b) Bestimmung mit Hexokinase und Glucose-6-phosphat-Dehydrogenase. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, vol 2. Verlag Chemie, Weinheim, pp 1241–1246

Bernt E, Gutmann I (1974) Äthanol: Bestimmung mit Alkohol-Dehydrogenase und NAD. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, vol 2, Verlag Chemie, Weinheim, pp 1545–1548

Czok R, Lamprecht W (1974) Pyruvat, Phosphoenolpyruvat und D-Glycerat-2-phosphat. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, vol 2. Verlag Chemie, Weinheim, pp 1491–1496

Frew JE, Jones P, Scholes G (1983) Spectrophotometric determination of hydrogen peroxide and organic hydroperoxides at low concentrations in aqueous solution. Anal Chim Acta 155:139–150

Gillies RJ, Ugurbill K, Den Hollander JA, Shulman RG (1981) ^{31}P NMR studies of intracellular pH and phosphate metabolism during cell division cycle of *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 78:2125–2129

Heath RL (1980) Initial events in injury to plants by air pollutants. Ann Rev Plant Physiol 31:395–431

Knight KL, Mudd JB (1984) The reaction of ozone with glyceraldehyde-3-phosphate dehydrogenase. Arch Biochem Biophys 229:259–269

Lang G, Michal G (1974) D-Glucose-6-Phosphat und D-Fructose-6-Phosphat. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, vol 2. Verlag Chemie, Weinheim, pp 1283–1287

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

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Lynen F, Holzer H (1949) Über den aeroben Phosphatbedarf der Hefe. II. Liebigs Annalen 563:213

Michal G, Beutler HO (1974) D-Fructose-1,6-diphosphat, Dihydroxyaceton-phosphat und D-Glycerinaldehyd-3-phosphat. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse, vol 2, Verlag Chemie, Weinheim, pp 1359–1364

Moeschlin S (1980) Maximale Arbeitsplatzkonzentrationen gesundheitsschädlicher Arbeitsstoffe. In: Moeschlin S (ed) Klinik und Therapie der Vergiftungen, Thieme, Stuttgart New York, p 77

Murakami K, Nagura H, Yoshino M (1980) Permeabilization of yeast cells: Application to study on the regulation of AMP deaminase activity in situ. Anal Biochem 105:407–413

Nicolay K, Scheffers WA, Bruinenberg PM, Kaptein R (1982) Phosphorus-31 nuclear magnetic resonance studies of intracellular pH, phosphate compartmentation and phosphate transport in yeasts. Arch Microbiol 133:83–89

Patty FA (ed) (1970) Industrial hygiene and toxicology, vol 2. Interscience Publishers, New York London, p 1805

Received August 12, 1985/Accepted September 24, 1985