

Perturbation of Growth and Metabolism in *Candida albicans* by 4-Bromobenzyl Isothiocyanate and Iodoacetate

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4-Bromobenzyl isothiocyanate, *Candida albicans* enzymes, inhibition of growth, iodoacetate

The inhibition by PBBI of growing *Candida albicans* was studied; additional information was obtained from the analysis of regeneration. The type of the growth inhibition depended on the cell suspension. At more than 10^7 cells per ml, the inhibition was transient; the inhibitor was taken up rapidly by the cells to a limit of 37 nmoles per mg dry wet. At higher doses, the growth was inhibited permanently, and then, and only then, a lethal effect was observed. At sublethal doses of the inhibitor, the following effects were observed: Inhibition of respiration without appreciable change in R.Q., of incorporation of labelled precursors into proteins and nucleic acids, and of eight of fourteen enzymes studied; the level of total free thiol groups was lowered; and a cross-over between hexose monophosphates and fructose diphosphate was induced. The original activities of the enzymes and metabolic processes inhibited were restored coincidentally with the reappearance of growth of the cells. Inhibition of G-6-PDH, PFK and of an early step in the oxidation of NAD(P)H appeared to be determinative for the growth.

In the cells inhibited transiently by iodoacetate, GAPDH and ADH were the most sensitive enzymes. The activity of the latter but not of the former was readily restored. The R.Q. was lowered, and was restored later than growth. The cells which recovered from the inhibition by iodoacetate appeared to be physiologically different from the control.

Introduction

McKAY *et al.*¹ have drawn attention to benzyl isothiocyanate and its derivatives as the most potent antibacterial agents amongst various types of isothiocyanates. The antibiotic properties of benzyl isothiocyanate were first described by WINTER and WILLEKE². Because of its wide antibacterial spectrum, its activity against genus *Candida*, and the low frequency of resistance to it, benzyl isothiocyanate has recently been recommended for treatment of infections of the urinary and pulmonary tracts^{3,4}. A study of isothiocyanates in our laboratory has shown that PBBI and several other derivatives of benzyl isothiocyanate possess an excellent antifungal activity⁵.

Abbreviations: ADH, alcohol dehydrogenase; ALD, aldolase; GAPDH, NAD-linked glyceraldehyde-3-phosphate dehydrogenase; GDH, NAD-linked glutamate dehydrogenase; GOT, glutamate oxalacetate aminotransferase; GPT, glutamate pyruvate aminotransferase; G-6-PDH, glucose-6-phosphate dehydrogenase; HK, hexokinase; ICDH, NADP-linked isocitrate dehydrogenase; MDH, malate dehydrogenase; NADHox, NADH oxidase; PBBI, 4-bromobenzyl isothiocyanate; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PK, pyruvate kinase.

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The present paper aims at elucidating the mode of action of one of the most potent antifungal isothiocyanates on the occasional parasite *C. albicans*. Proliferating cells and only moderate doses of the inhibitor have been deliberately used in this study. In concentrated *Saccharomyces cerevisiae* suspensions, allyl isothiocyanate and some aromatic isothiocyanates block primarily energy metabolism, the inhibition of GAPDH and perhaps of HK and ADH is what accounts for the inhibition of metabolism and growth observed in this yeast⁶. We expected, therefore, that a comparison of the effects of PBBI and iodoacetate would be useful and include this in the present study.

Materials and Methods

Chemicals

All the enzymes and their substrates, reduced glutathione and triethanolamine · HCl were purchased from Boehringer, Mannheim, except for ATP and glucose-6-phosphate, which were supplied by Reanal, Budapest, and HK, which was obtained from Sigma, St. Louis. 5,5-dithio-bis (nitrobenzoic acid) was supplied by Fluka, Buchs, and cytochrome *c* by Koch-Light, Colnbrook. Iodoacetate, vitamins, dimethyl sulfoxide and all other chemicals were purchased from Lachema,



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Brno, [$8-^{14}\text{C}$] adenine and [$\text{U}-^{14}\text{C}$] L-leucine from ÚVVVR, Praha. PBBI was synthesized at the Dept. Org. Chem, Slovak Techn. Univ., Bratislava. It was applied as a solution in dimethylsulfoxide. Final concentrations of the latter never exceeded 1%. At this concentration no significant change in growth and metabolism of *C. albicans* can be observed⁷.

Organisms and cultivation

The following organisms have been studied: *Candida albicans*, strain Pn 10 of the collection of the Dept. Microbiol. Biochem., Slovak Techn. Univ., Bratislava; *Rhodotorula gracilis*, an organism lacking PFK⁸, kindly provided by Dr. A. KOTYK, Microbiol. Inst., Czechoslov. Acad. Sci., Praha; *Saccharomyces cerevisiae*, a wild strain DT XII and an acriflavine cytoplasmic respiratory mutant DT XIIa, both generously provided by Dr. M. GREKSÁK, Biochem. Inst., Komenský Univ., Bratislava. All were maintained on malt agar slants and cultured in a synthetic medium containing glucose (5%) and ammonium sulfate as the only sources of carbon and nitrogen (if not stated otherwise), and mineral salts and vitamins as described elsewhere⁹. Initial pH of the medium was 6.5, or in the experiments with iodoacetate, 4.5. The cells were cultivated aerobically on a reciprocal shaker at 28 °C. Vigorous aeration on a rotary shaker was necessary for *Rh. gracilis*. Cell concentration was calibrated against the optical density of the culture at 650 nm. No significant changes in cell shape or size were observed in the presence of inhibitors employed.

To study lethality of the inhibitors, 10 ml of the above medium, supplemented with 2% agar, were pipetted into 10 cm Petri dishes. The cells were incubated with the designated dose of inhibitor for 4 hours at 28 °C, washed with saline and, after appropriate dilution, spread on the agar surface. Colonies were counted after 72 hours incubation at 28 °C; three determinations were averaged. All the experiments were performed with the cells from exponential growth phase.

Manometric experiments

Respiration was measured with aliquots of culture in 20 ml Warburg vessels by standard techniques¹⁰.

Incorporation of ^{14}C -adenine and ^{14}C -leucine

[$8-^{14}\text{C}$] adenine or [$\text{U}-^{14}\text{C}$] L-leucine was added to culture aliquots immediately after inhibitor at doses of 62 μg adenine per ml (0.23 μCi) or 11 μg L-leucine (0.21 μCi) per ml. At suitable intervals 1 ml portions were poured into 1 ml of cold TCA. After 1 hour standing on ice the precipitate was filtered under suction through 4 μ -pore filters ("Synpor", Synthesia, Uhřetěves), and washed with 10 ml each 5% cold TCA and water. The filters were dried at 105 °C for 20 min and radioactivity determined by use of a thin-windowed GM-tube (GMT 30/30 AB, combined

with preamplifier NAZ 615, HV source NBZ 615, and integrator NVZ 612 A, Tesla, Liberec).

Crude extracts

They were prepared from culture aliquots containing 50 to 100 mg wet wt, washed with 5 ml of cold 0.05 M sodium phosphate buffer, pH 7.5, and resuspended in 0.6 ml of the same buffer. The cells were sonicated at 20 KHz 9 times 0.5 min with equal breaks for cooling in an ice bath (either Ultrasonic MSE 100 W, Crawley, or UG 100 TD VÚMA, Nové Mesto n.V.). The period employed sufficed for maximum protein extraction from young cells. Alternatively, the method of ECKSTEIN *et al.*¹¹ was used, providing extracts of similar quality as judged from recovery of enzymatic activity. The homogenates were spun down for 10 min at 3,000 g and the supernates thus obtained were used in subsequent enzyme activity determinations.

Enzyme assays

The activities of the enzymes were determined by the Warburg optical tests in 1 cm vials at room temperature. The absorbance change of nicotinamide dinucleotides was determined at 340 nm, using either spectrometer VSU-1 (Zeiss, Jena) or SP-500, Ser. 2 (Unicam, Cambridge). 3 ml assay solutions were as follows. For HK (EC 2.7.1.1): 6.7 mM glucose, 1.3 mM ATP, 4 μg G-6-PDH, 1 mM NADP, 4 mM MgSO_4 , 50 mM triethanolamine \cdot HCl, pH 7.5. For G-6-PDH (EC 1.1.1.49): 4 mM glucose-6-phosphate, 1 mM NADP, 4 mM MgSO_4 , 50 mM triethanolamine \cdot HCl, pH 7.5. For PFK (EC 2.7.1.11): 3 mM fructose-6-phosphate, 0.3 mM ATP, 3 mM cysteine, 4 mM MgSO_4 , 20 μg glycerolphosphate dehydrogenase/triosephosphateisomerase mixture (Boehringer), 40 μg ALD, 50 mM triethanolamine \cdot HCl, pH 8.0. For GAPDH (EC 1.2.1.12): 4 mM 3-phosphoglycerate, 1.3 mM ATP, 0.16 mM NADH, 40 μg PGK, 2 mM reduced glutathione, 4 mM MgSO_4 , 50 mM triethanolamine \cdot HCl, pH 7.5. For PGK (EC 2.7.2.3): 4 mM 3-phosphoglycerate, 1.3 mM ATP, 0.16 mM NADH, 2 mM reduced glutathione, 40 μg GAPDH, 4 mM MgSO_4 , 50 mM triethanolamine \cdot HCl, pH 7.5. For ADH (EC 1.1.1.1): 0.5 M ethanol, 1 mM NAD, 20 mM glycine, 70 mM semicarbazide \cdot HCl, 75 mM sodium pyrophosphate, pH 8.8. For ICDH (EC 1.1.1.42): 2 mM isocitrate, 0.5 mM NADP, 1.5 mM MnSO_4 , 4 mM MgSO_4 , 50 mM triethanolamine \cdot HCl, pH 7.5. For MDH (EC 1.1.1.37): 1 mM 2-oxoglutarate, 0.16 mM NADH, 40 mM aspartate, 50 μg GOT, 0.1 M sodium phosphate, pH 7.4. For GDH (EC 1.4.1.2): 6.6 mM 2-oxoglutarate, 0.16 mM NADH, 0.1 M ammonium acetate, 1.4 mM EDTA, 0.1 M sodium phosphate, pH 8.0. ALD (EC 4.1.2.13) was assayed with Biochimica Test Combination (Boehringer, Mannheim), GOT (EC 2.6.1.1) and GPT (EC 2.6.1.2) were assayed as in ref. 12. NADH oxidase was determined with 0.16 mM NADH, 0.08 mM cytochrome c, 4 mM MgSO_4 , 50 mM triethanolamine \cdot HCl, pH

7.5. Whenever NADH oxidase was measured, cytochrome *c* was present in the cell suspension during homogenisation.

Metabolite concentrations

The concentrations of intermediates were determined by common spectrometric methods. 50 mM triethanolamine · HCl buffer, pH 7.5, containing 4 mM MgSO_4 was used throughout. HK and G-6-PDH were employed for the determination of ATP and glucose-6-phosphate; glycerolphosphate dehydrogenase, triose phosphate isomerase, ALD and PFK in determination of triosephosphates, fructose-1,6-diphosphate and fructose-6-phosphate. Lactate dehydrogenase in that of pyruvate. Use of diluted cell suspensions necessitated separation of cells: up to 150 ml culture could be filtered on a $4\ \mu$ membrane filter (Synpor 1, 6 cm in diameter, Synthesia, Uhřetíněves) under suction in less than 1 min. During filtration the suspension above the filter was aerated. The filter with the cells was immediately rolled up on a glass rod, submerged into liquid nitrogen, placed into a tube with 0.3 ml 3 N HClO_4 , and kept at -20°C allowing diffusion of the acid. Then the samples were placed on ice and allowed to thaw. The filter was crushed down, mascerated, and once more frozen and thawed. The suspension was centrifuged for 10 min at 5000 g and the sediment washed by 0.4 ml 3 N HClO_4 . The pooled supernates were carefully neutralized by 2 N KHCO_3 . The precipitated perchlorate was spun down twice, when necessary. Between the operations and before the assays the extracts were kept frozen. The intracellular concentration of the metabolites was calculated as in ref. 13.

Thiol groups

Total SH-groups were determined in crude extracts with Ellman's reagent as described by SEDLÁK and LINDSAY¹⁴, with reduced glutathione as control.

Results

Effects on growth and metabolism

The data in Tab. I show that PBBI inhibits growth of several yeast and yeast-like organisms independently of their fermentative properties. In contrast, sensitivity to iodoacetate varies markedly, being highest for a *petite* mutant and lowest for strictly aerobic *Rhodotorula gracilis*. More detailed studies with *C. albicans* have shown that bromobenzyl isothiocyanate inhibits growth on a variety of carbon sources including glucose, glycerol, glutamate, lactate and ethanol. A relatively higher sensitivity observed in cells grown on the latter two substrates may be due to generally poor growth on these substrates. In this respect PBBI differs from iodoacetate, which inhibits the growth on

glucose or glycerol much more than on any other of the substrates mentioned.

In diluted suspensions of cells growing on glucose PBBI affected primarily the growth rate (Fig. 1), while at cell concentrations exceeding 10^7 cells per ml the addition of a moderate doses of PBBI temporarily

Tab. I. The sensitivity of several yeast and yeast-like organisms to PBBI and iodoacetate. ED_{50} and ED_{100} are extrapolated to those concentrations (μM) at which 50% inhibition of cell number increase (at 6th hour), and full inhibition of the growth (for 24 hours) take place, resp. Generally suspensions of about 0.5 mg dry wt per ml were used.

Strain	ED_{50} (μM)		ED_{100} (μM)	
	PBBI	Iodoacetate	PBBI	Iodoacetate
<i>Rh. glutinis</i> 5 /Fres/Harrison	7	80	16	180
<i>C. albicans</i> Pn 10	6	25	17	80
<i>S. cerevisiae</i> DT XII	6	16	17	40
<i>S. cerevisiae</i> DT XIIa	6	8	17	17

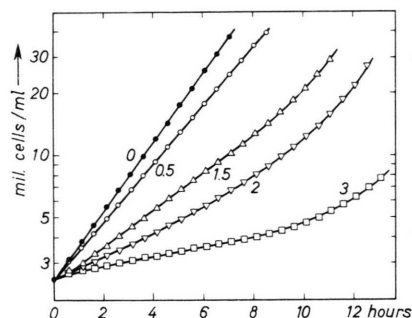


Fig. 1. Inhibition by PBBI of the growth of *C. albicans* in a highly diluted suspension. The inhibitor was added at zero time. The numbers on the curves indicate initial concentrations of PBBI in μmoles .

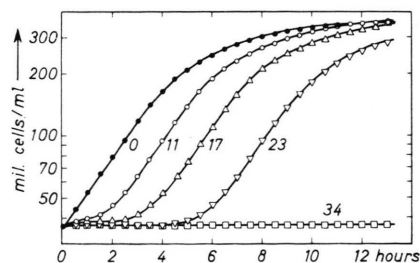


Fig. 2. Inhibition of the growth of *C. albicans* by PBBI. Inhibitor added at zero time at doses (nmoles per mg dry wt) indicated by the numbers on the curves.

arrested multiplication (Fig. 2). Both the growth rate and the cell concentration in stationary phase were identical with those of control, except where the growth delay exceeded 10 hours. When the growth delay (L in min) is plotted vs the dose of PBBI (D in nmoles per mg dry wt) on a double log. scale (Fig. 3) a linear relationship is obtained:

$$L = 2.2 \times D^{1.6} \quad (1)$$

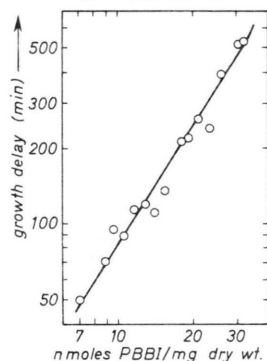


Fig. 3. Dependence of the growth delay on the dose of PBBI applied to exponential cultures of *C. albicans* at initial cell concentrations of 10 to 35 mill. per ml.

The longer the cells were incubated in medium supplemented with PBBI, the more PBBI they removed from the medium and the longer they needed for resumption of the growth when transferred to fresh medium. The dose of PBBI remaining in the medium was determined from the ability of fresh cells to grow in it, and was calculated by means of eqn (1). The results as presented in Fig. 4 show that 1. the cells had

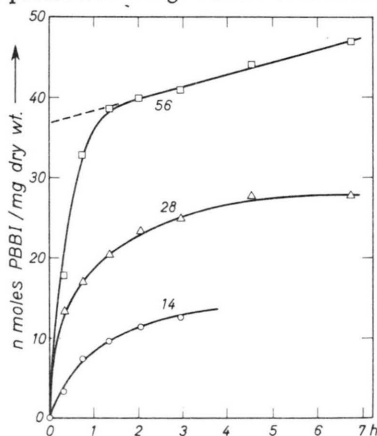


Fig. 4. Kinetics of the disappearance of PBBI from the cultivation medium. *C. albicans* suspensions (0.36 mg dry wt (per ml) were incubated with various doses of PBBI, and the amount of inhibitor remaining in the medium after removal of cells at various intervals was determined as described in the text. The numbers at the curves represent the initial doses in nmoles PBBI per mg dry wt.

a limited capacity for uptake of PBBI, maximally 37 nmoles per mg dry wt, and 2. the rate of uptake depended on the concentration of PBBI. This rate may determine whether PBBI only diminishes the growth rate, or inhibits growth fully (comp. Figs 1 and 2).

Only at doses of PBBI exceeding 40 nmoles per mg dry wt did a significant decrease in the viable count take place (Fig. 5). The dose response curve is very steep and depends to some extent on the duration of the contact of the cells with PBBI. The break in the

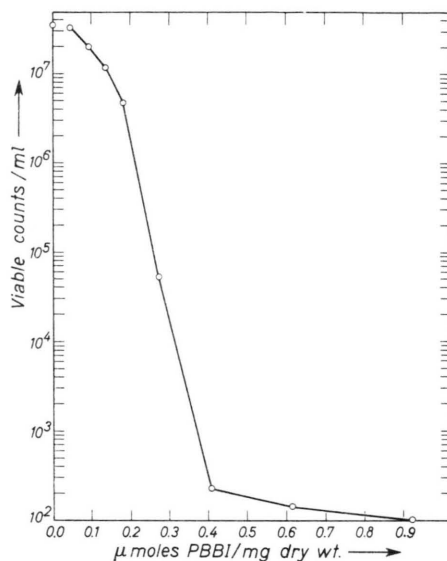


Fig. 5. Loss of the colony forming ability in cultures of *C. albicans* incubated with various doses of PBBI for 4 hours at 28 °C.

curve corresponds roughly to the solubility limit of PBBI (ca. 100 μM).

Figure 6 illustrates changes occurring in the level of free thiols in crude extracts obtained from growing *C. albicans* supplemented with various doses of PBBI. Measured in the same cultures growth delays of about 2 and 7 hours were observed at the two lower doses of PBBI shown. At the lowest dosage at least, resumption of growth coincided with the restoration of the free thiol level.

PBBI inhibited respiration and aerobic fermentation of glucose in both growing and resting *C. albicans* cells without significant change in R.Q. Correlated with this an inhibition of incorporation of ^{14}C -leucine and ^{14}C -adenine into TCA insoluble cell fraction was determined (Fig. 7). Fig. 8 shows the temporal correlation of the reappearance of the growth (vertical dashed lines) with restoration of normal respiration,

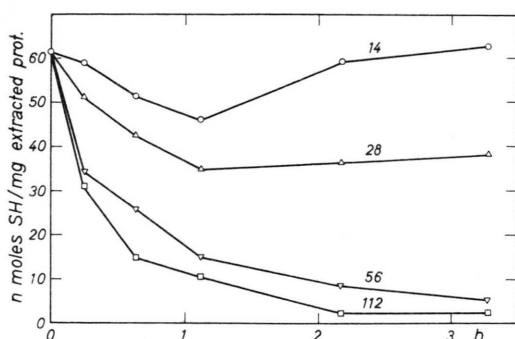


Fig. 6. Thiol content of crude extracts prepared from suspensions of *C. albicans* inhibited by PBB1. Numbers on the curves indicate initial doses in nmoles PBB1 per mg dry wt. Initial cell concentration: 0.36 mg dry wt per ml.

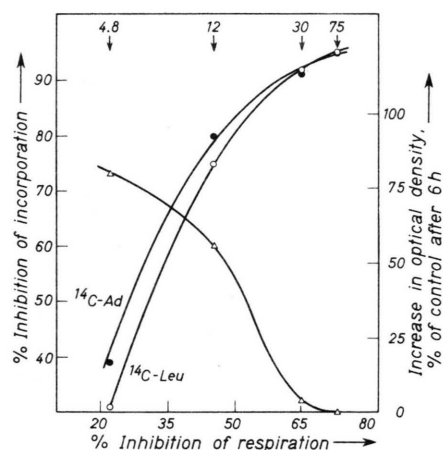


Fig. 7. Relationship between the incorporation of leucine and adenine and the inhibition of the respiration in *C. albicans*. The numbers at the top of the figure indicate the doses in nmoles PBB1 per mg dry wt. Growth of the culture is given by relative optical density changes.

fermentation and macromolecular synthesis (horizontal dashed lines).

Like PBB1, iodoacetate at suitable conditions caused cessation of growth of *C. albicans* only temporarily. The growth rate, however, was not fully restored (Fig. 9) except at very low concentrations. The fungicidal effect of iodoacetate was weak: as much as 17% of the cells retained their colony-forming ability after 4 hours incubation in presence of 20 mM iodoacetate. The count was doubled when the cells were plated on

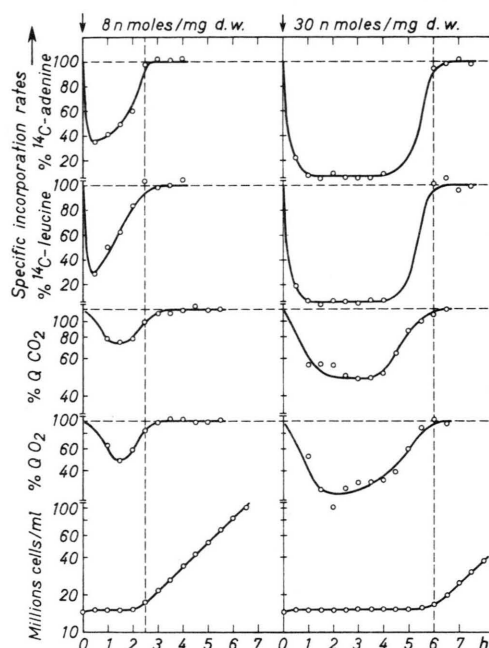


Fig. 8. Transient inhibition of the growth and metabolism in *C. albicans* by PBB1 at two different doses: 12 and 30 nmoles per mg dry wt. Immediately after adding PBB1 the culture (0.14 mg dry wt per ml) was divided into aliquots for determination of the incorporation of ^{14}C -adenine and ^{14}C -leucine, and for manometric determinations.

glucose agar supplemented with 0.5% glutamate. The growth of cells adapted to glutamate, lactate or ethanol was first inhibited at concentrations of iodoacetate nearly 10-times higher than necessary to inhibit growth of cells on glucose or glycerol. The inhibition of the cell growth on glucose medium may be substantially overcome by glutamate even when added much later than iodoacetate. In an experiment shown in Fig. 9, the influence of iodoacetate on respiration and aerobic fermentation was studied in parallel to its effect on growth. The inhibition of the growth appears to be closely related to that of carbon dioxide production and/or oxygen uptake. At lower concentration of iodoacetate a recovery to normal respiration and fermentation was observed. The latter was subsequent to the former as judged by a reduced R. Q., which persisted for an appreciable period after the onset of growth. At higher iodoacetate concentrations R. Q. decreased well below 1, as in *S. cerevisiae*¹⁵. At 25 μM iodoacetate, concomitant with the resumption of multiplication, a rather high specific respiration rate exceeding that of control was observed (Fig. 9).

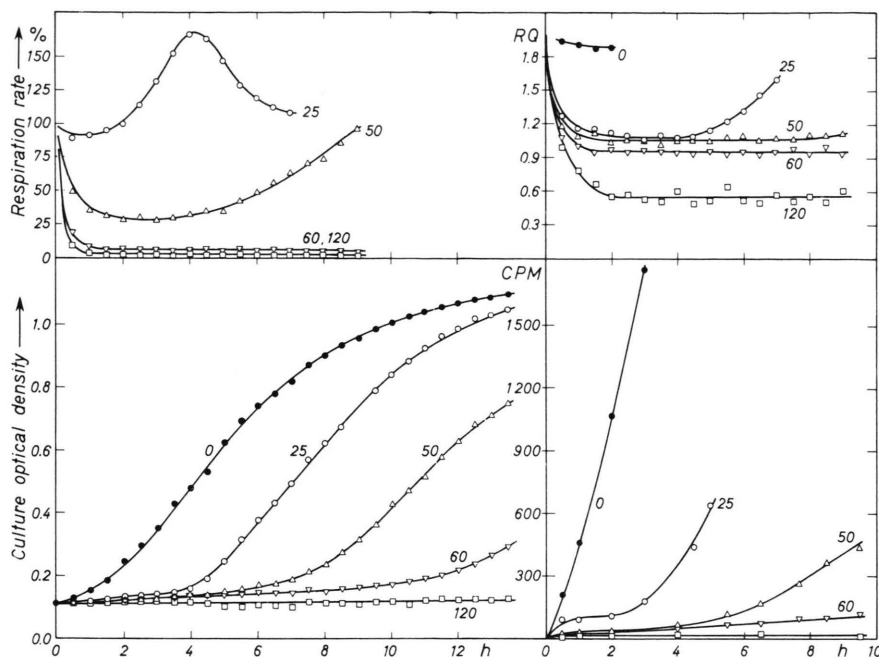


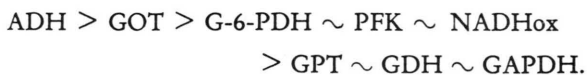
Fig. 9. Effect of iodoacetate on growing cells of *C. albicans*. Growth (bottom left), incorporation of ^{14}C -adenine (bottom right), respiration (top left), and respiratory quotient (top right) are plotted against time after addition of iodoacetate. The various parameters were measured in parallel in separate aliquots of the same culture. Numbers on the curves give initial concentration of iodoacetate in μM .

Iodoacetate inhibited incorporation of ^{14}C -adenine into nucleic acids, and, generally parallel but somewhat less, that of ^{14}C -leucine into proteins (not shown). The dependence of isotope incorporation on respiration is quite obvious. In this respect the effects of both inhibitors studied are very similar. It is concluded that PBBI, like iodoacetate, interferes with carbon-energy metabolism in *C. albicans*.

Effects on enzyme activities

In regard to its inhibition of GAPDH in *C. albicans*, PBBI resembles some other isothiocyanates in *S. cerevisiae*⁶. As can be seen in Fig. 10, the rapid loss of GAPDH activity following administration of PBBI (100 nmoles/mg dry wt) was essentially recovered before growth resumed. The inhibition of GAPDH, however, could not be observed at lower doses of PBBI (see for example Fig. 11), unless reduced glutathione was omitted from the assay mixture. The data presented in Tab. II illustrate the dose and time dependence of the inhibition of several enzymes *in vivo*

by PBBI. The inhibition of GAPDH lags behind that of other sensitive enzymes. Its actual activity *in vivo* may reflect changes in the concentration of thiol groups. The effect of the inhibitor on another set of the enzymes is shown in Tab. II. Analogous experiments indicated HK, ALDO, PGK, PK, ICDH and MDH were not inhibited *in vivo*, after application of moderate doses of PBBI (*i. e.* when the growth inhibited only temporarily) while G-6-PDH was inhibited to a similar extent as PFK. Inhibition of some sensitive enzymes could also be demonstrated when PBBI was added to crude extracts (Fig. 12). The relative sensitivity to PBBI under these conditions was the same as *in vivo*. The doses of PBBI (based on protein content) needed to cause appreciable inhibition of the enzymes were comparable to those used *in vivo*. Remarkably, no turbidity was observed in the crude extracts, even at highest PBBI concentrations where its solubility in water was far exceeded. The enzymes ranked according to their sensitivity as follows:



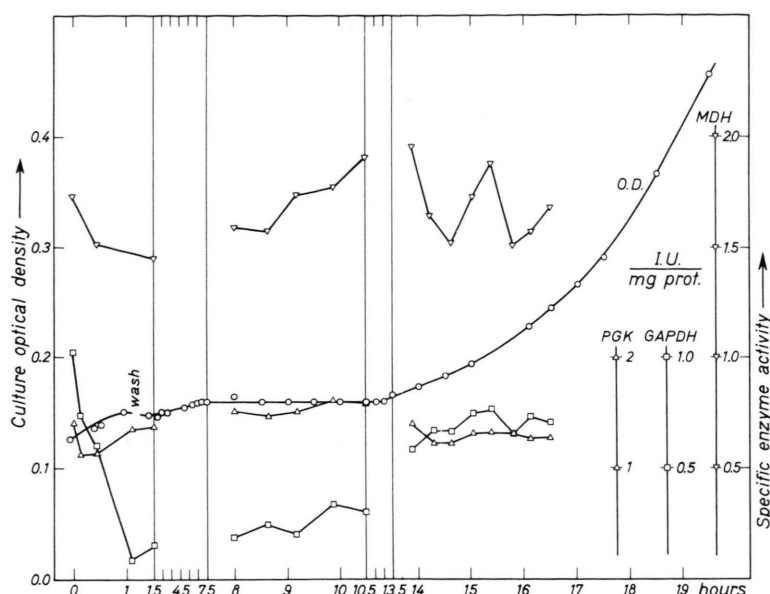


Fig. 10. Activities of GAPDH (\square), PGK (\triangle), and MDH (∇) in *C. albicans* after addition of a high dose of PBBI, and during the period of renewed growth. The cells were incubated for 1 hour after addition of 100 nmoles PBBI per mg dry wt, then washed and resuspended in equal volume of fresh medium.

Tab. II. Inhibition of enzymes in *C. albicans* by PBBI. Various doses of the inhibitor were added to aliquots of a culture of exponentially growing cells. The initial dose is given in nmoles PBBI per mg dry wt.

Enzyme	Dose of PBBI	% activity remaining after:			
		10	40	80	170 min
ADH	8	33	20	32	79
	20	22	5	9	94
	50	3	1	< 1	< 1
	125	< 1	< 1	< 1	< 1
PFK	8	82	67	71	67
	20	77	53	65	50
	50	52	39	33	27
	125	28	13	11	9
GDH	8	82	80	77	100
	20	99	87	79	139
	50	80	36	47	35
	125	41	1	1	3
GOT	8	48	40	48	71
	20	54	36	38	63
	50	34	9	7	10
	125	8	4	6	7
GPT	8	70	69	87	98
	20	70	67	65	107
	50	53	26	25	36
	125	39	16	18	12
GAPDH	8	109	90	106	89
	20	90	105	108	130
	50	85	95	67	9
	125	87	2	1	< 1

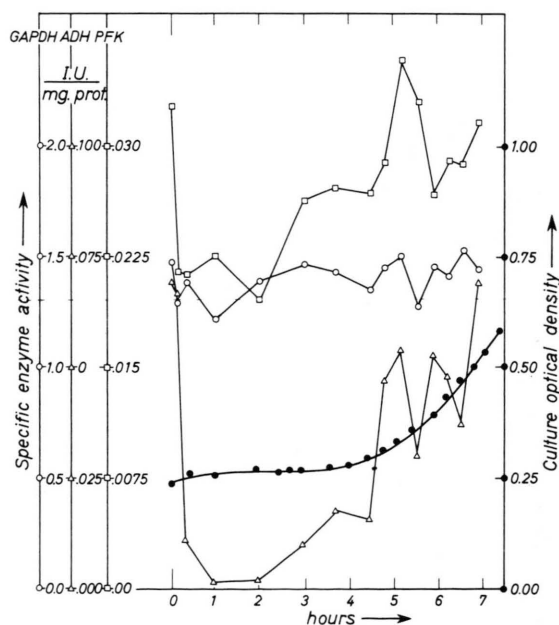


Fig. 11. Activities of ADH (\triangle), PFK (\square), and GAPDH (\circ) in temporarily inhibited *C. albicans*. 20 nmoles PBBI per mg dry wt were added at zero time.

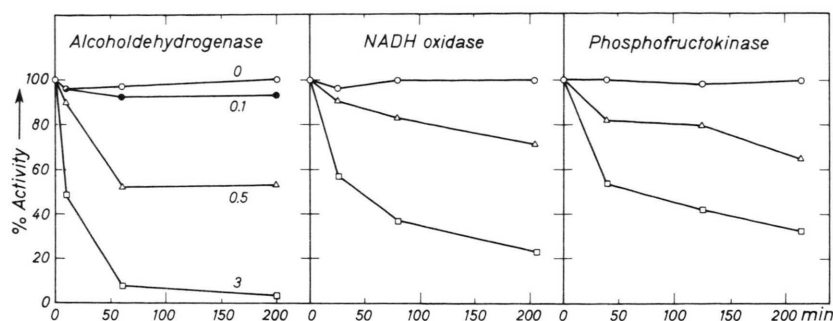


Fig. 12. Inhibition of ADH, PFK and NADH oxidase in crude extracts from *C. albicans*. PBBI was added to the crude extract (15 mg protein per ml) at zero time at final concentrations as indicated by the numbers on the curves. The extracts were incubated at 20 °C. The activities of NADH oxidase at the last interval were corrected for activity loss in the control.

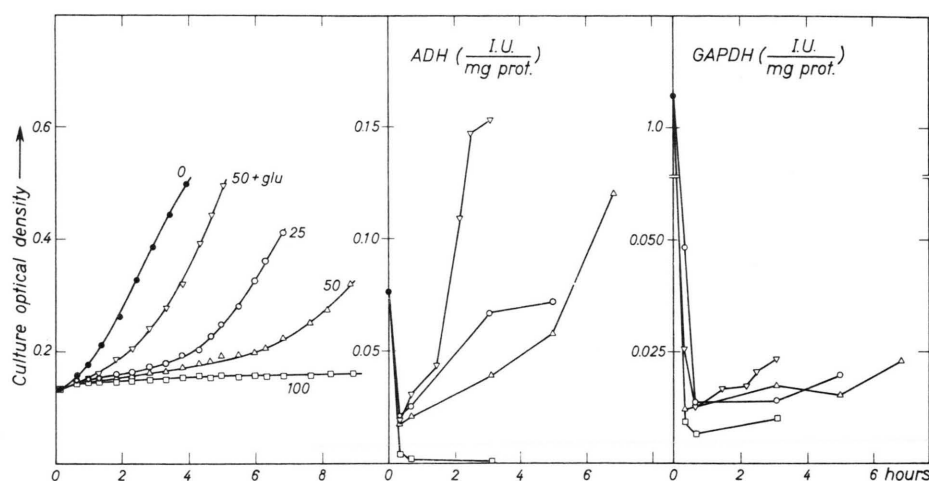


Fig. 13. Inhibition by iodoacetate of growth and activities of ADH and GAPDH in *C. albicans*, and partial protection by glutamate. Glutamate (0.5 % w/v) was added simultaneously with iodoacetate at zero time. The final concentrations of the inhibitor are given by the numbers on the curves.

In contrast to PBBI iodoacetate, at concentrations insufficient for permanent inhibition of growth, caused a severe inhibition of GAPDH *in vivo*; ADH was less severely inhibited (Fig. 13). Glutamate, which ameliorates the growth inhibition, permits an earlier recovery of ADH activity.

Figure 14 shows that renewed growth is possible in spite of the sluggish reappearance of GAPDH activity. A partial inhibition of GDH was also observed (Fig. 14), while under the same conditions no inhibition of PFK, ALDO, PGK and MDH could be observed. GOT was found to be as sensitive as GDH, and GPT slightly more. ICDH was insensitive to low concentrations of iodoacetate and a partial inhibition of HK was observed at concentrations permanently inhibiting the growth. The *in vivo* sensitivities of the enzymes

studied rank as follows: GAPDH > ADH > GOT > GDH ~ GPT > HK.

Changes in metabolic pools

Growth inhibition by PBBI would appear to involve more complex mechanisms than that by iodoacetate. An attempt to assess a possible perturbation by PBBI of the early steps of glycolysis was made by measuring the influence of PBBI on the concentrations of several intermediates. The results presented in Fig. 15 show, that within few minutes after adding a moderate dose of PBBI to exponentially growing culture an abrupt increase in the intracellular level of hexosemonophosphates takes place. Concomitantly the level of fructose-1,6-diphosphate decreases. Essentially the same crossover was observed also at a higher dose of PBBI.

Its appearance may be explained by the inhibition of PFK and G-6-PDH.

Discussion

The pattern of enzyme activities in *C. albicans* as determined in this study (summarized in Fig. 16) resembles that known for related organisms^{27, 28}. At variance with *S. cerevisiae*, the activity of MDH in *C. albicans* is high even when growing at high glucose concentration. In *C. albicans* as in *S. cerevisiae* NAD-linked GAPDH is one of the most active enzymes Rao *et al.*²⁹ interpreted the reduction of NADP by crude extracts from *C. albicans* in presence of fructose-6-phosphate and arsenate as a demonstration of PFK activity and of NADP-dependence of GAPDH. Most probably G-6-PDH, shown here to be quite abundant in *C. albicans*, interfered with their assay. NADH oxidase activity may be underestimated in the present study, due to preparation of crude extracts by sonication.

That the type of growth inhibition by PBBI is dependent on the cell concentration urges the exercise of caution in attempts to classify antimicrobial agents according to their influence on the shape of the growth curves, as presented *e.g.* in ref. 18. Without any doubt the characteristic nature of the growth inhibition by PBBI is related to both physical and chemical properties of this substance: the lipophilic character seems to be a prerequisite to the high antimicrobial activity of many isothiocyanates. Within the group of substituted benzyl isothiocyanates, at least, a high correla-

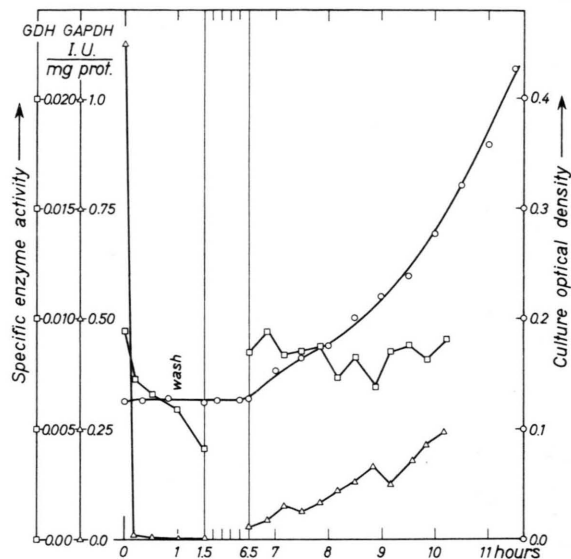


Fig. 14. Inhibition of growth (○) and of GAPDH (△) and GDH (□) activities by iodoacetate in *C. albicans* and their restoration. The inhibitor, 1 mM initial concentration, was washed out after 1 hour.

tion exists between their solubility in water or their partition coefficient in water-n-octanol and their effectiveness in inhibiting the growth of *E. coli* and *A. niger*, as well as of *C. albicans* (L. Drobnica, personal comm.). The high affinity of PBBI for the yeast cell, as evidenced in this report, explains the fact that its inhibitory effect depends on the dose of the inhibitor rather than on its initial concentration in the medium (Fig. 3). The dose-response observed here is described

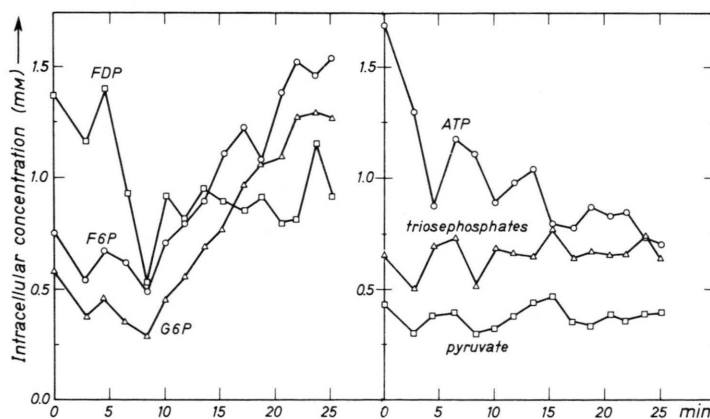


Fig. 15. Effect of PBBI on the intracellular concentration of some metabolites in *C. albicans* *in vivo*. The inhibitor was added at zero time at a dose of 18 nmoles per mg dry wt.

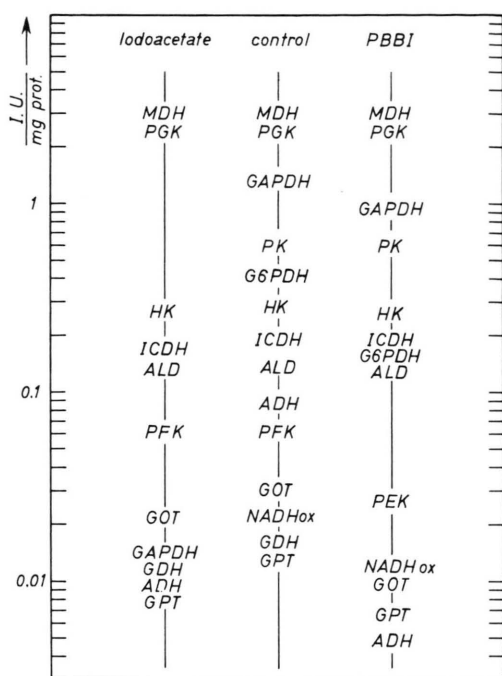


Fig. 16. Enzyme pattern of *C. albicans* cells in log. growth phase (at 0.2 — 0.6 mg dry wt. per ml), and of such cells moderately inhibited by iodoacetate (left) and PBBI (right).

by the general eqn suggested by TAMIYA *et al.*¹⁸ ($H = G^n/\phi^n + G^n$, where H is measure of inhibition, ϕ concentration of the substance, which causes 50% inhibition, and G actual concentration), for analysis of inhibitory effects, with one important modification: dose of inhibitor must be substituted for concentration, and correspondingly the ϕ value may not be treated as a constant.

AUGUSTÍN *et al.*¹⁹ have demonstrated the accumulation of some isothiocyanates by yeast and other cells and their reaction with macromolecular cellular components by use of ³⁵S-labelled derivatives. They recovered the bound radioactivity chiefly in protein, partly in some other cell fractions. In the present paper a limit to the uptake capacity of the cells for PBBI is demonstrated. When this limit is exceeded, growth inhibition becomes permanent and lethality sets in. At lesser doses, there is no decrease in the viable count during the temporary cessation of multiplication, and in these cases at the moment growth resumes, there is no more free, extracellular PBBI.

As concerns the chemical features of the interaction of PBBI with the cell, there is now ample evidence

that the high antimicrobial activity of many isothiocyanates lies in their reaction with sulfhydryl components of the cell: 1. high reactivity of isothiocyanates towards low molecular weight thiol compounds²⁰, 2. inactivation *in vivo* of many enzymes which are regarded as sulfhydryl enzymes, 3. depression of free intracellular thiol groups demonstrated in present paper, 4. long known antagonism of thiols towards isothiocyanates and closely related dithiocarbamates^{21,22}, and 5. direct spectral evidence of the formation of dithiocarbamate derivatives of isothiocyanates *in vivo*²³.

Having in mind the reactivity towards thiol groups as well as the suggestion that the effect of some isothiocyanates on yeast resides in inhibition of GAPDH⁶, it was originally expected iodoacetate might mimic the effect of PBBI on *C. albicans*. The lower sensitivity of *C. albicans* to iodoacetate as compared with fermenting *S. cerevisiae* may be related to the more aerobic nature of the former organism. The initial depression of R. Q. and the coincidence of its restoration with the slow reappearance of GAPDH activity (see Fig. 14), support the view that in *C. albicans*, as in *S. cerevisiae*^{16, 17}, GAPDH is the focal point of inhibition by iodoacetate. It is interesting that ADH activity is regenerated much more readily than GAPDH. Those cells which grow after transient inhibition by iodoacetate appear to be physiologically different from control cells as indicated by lower R. Q. A transient inhibition, however, is limited to very narrow range of iodoacetate concentrations. Only within this range can a stimulation of the respiration and a full restoration of the growth rate be observed. Stimulatory effects of iodoacetate on respiration in frog muscle³⁰ and *S. cerevisiae*³¹ have been reported by others.

The comparison of the effects of PBBI and iodoacetate makes it clear that the target of the former must be sought elsewhere than in GAPDH.

Aliphatic isothiocyanates, but not aromatic ones, have been observed to cause changes in the R. Q. of yeast²². In concentrated suspensions of *S. cerevisiae* GAPDH seemed to be the most sensitive enzyme towards phenyl- and various other isothiocyanates⁶. Aromatic isothiocyanates also inhibit G-6-PDH in Ehrlich ascites carcinoma cells and NADH oxidation by mitochondria from these cells²⁴.

In spite of interference with several dehydrogenases and with oxidation of NAD(P)H (as evidenced in the present paper, Fig. 12, and ref. 25) PBBI apparently does not change R. Q. in *C. albicans* but the end effect

may result from a coincidental canceling of opposing effects. The failure to deplete intracellular triose phosphates or pyruvate, while the levels of hexose phosphates severely change points to the involvement of multiple inhibition sites. This concept is compatible also with the obvious unspecificity of PBBI. Yet the simple fact of spontaneous resumption of growth suggests that only a few of these inhibition sites are determinative for growth. These crucial sites would seem to be the enzymes PFK and G-6-PDH, the early steps in NAD(P)H oxidation (see also ref. 25), and perhaps some enzymes of tricarboxylic acid cycle.

In *E. coli*, 4-bromophenyl- and some other aromatic isothiocyanates inhibit glutamate metabolism as judged from the fact that addition of glutamate to synthetic media decreases markedly the sensitivity of the cells²⁶. In *C. albicans*, however, glutamate does not ameliorate the effect of PBBI even though the latter inhibits GDH and transaminases somewhat.

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