ORIGIN OF THE INCREASE IN ADAPTABILITY TO OXYGEN OF YEAST PRETREATED WITH BENZIMIDAZOLE

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Benzimidazole (BZI : 1,3 benzodiazole) inhibits the induced biosynthesis of a functional respiratory system by non-proliferating yeast cells as well as yeast growth (Slonimski, 1954[a]; 1954[b]; 1956[a]). The mode of action of BZI remains unknown and for yeast at least, the capacity to act as an antimetabolite of purines (Woolley, 1944) has been invalidated by Slonimski (1954[a]).

The biological activity of BZI has been shown to depend on the entry of the non-ionized form of the molecule into living cells. The impermeability of the cell membrane to the BZI^+ ion and a bidirectional diffusion of the non-ionized molecule explain the rapid interruption of the inhibition of respiratory adaptation when the extracellular medium is acidified. At pH above the pKA_1 (5.3-5.4) the partition coefficient of BZI between the intracellular fluid and the external medium may considerably exceed unity (Slonimski, 1956[a]).

Futher insight into the mode of action of BZI on the mechanism of induction of the respiratory enzymes could be gained by reanalyzing the increased acceleration of the respiration rate which is observed when the capacity of the yeast cells to adapt to oxygen is restored i.e. after the exclusion of BZI from the aeration buffer (Slonimski, 1956[b]). Two hypotheses are available to explain such a result : (i) precursors of the respiratory enzymes, catalytically inactive, accumulate; these precursors, quickly transformed into holoenzymes, unite to form a functional respiratory system when BZI is excluded (ii) a regulating factor is exhausted, subsequently allowing a transitory acceleration of the de novo synthesis of respiratory enzymes.

To decide which of these alternatives is the right one, two different antibiotics were used in combination with BZI : (i) cycloheximide (actidione, abbreviated CYCLO), a powerful inhibitor of the de novo syn-

thesis of proteins,principally cytoplasmic in their origin with however secondary inhibition of the mitochondrial system (ii) chloramphenicol (abbreviated CAP),effective in inhibiting more specifically the synthesis of the particulate respiratory enzymes,resulting in a "petite" phenotype (Huang et al.,1966;Clark-Walker and Linnane,1967). These criteria of specificity will be considered however with care since recent results have revealed complex inter-relations in vivo between the cytoplasmic and the mitochondrial protein synthesizing systems (Mahler et al.,1968; Ashwell and Work,1968).

Results -

For all experiments we used the yeast strain "Yeast Foam" 237 (Saccharomyces cerevisiae, "grande" ρ^+,prototrophic,primitively diploïd). Cells were grown,under strictly anaerobic conditions,in a Yeast extract - 10% glucose medium (medium R) as described in detail by Sels et al. (1965),with the single modification that ergosterol (20mg/ml) and Tween 80 (4.4g/L) replaced wheat germ oil. The cell yield in the stationary phase of growth at 26° reached 4.3 $\pm$ 0.1mg protein/ml.

Adaptation of the stationary phase cells to oxygen was achieved, as a rule,at 28° in 2% glucose-buffer at pH 6.3 (equally adequate as pH 4.2 buffer) in the absence of any significant cell growth.

When oxygen is introduced,anaerobic yeast cells derive their energy from fermentation with breakdown of glucose;under such conditions,the repression of the acceleration of the respiration rate by glucose is at a maximum. However,this repression phenomenon is of limited amplitude if we examine the adaptability of anaerobically galactose-grown cells aerated with various fermentable (supplemented or not with ethanol) carbon sources. This aspect of the problem will be discussed elsewhere;anyhow, the treatment of unadapted yeast cells with BZI,which reduces drastically the adaptability to oxygen,imposes the choice of a fermentable carbon source i.e. the availability of an energy supply.

Figure 1 shows that the acceleration of the rate of O_2 uptake (i.e. $AO_2 = d^2O_2/dt^2$ which is expressed for convenience as $\mu lO_2/h^2$/mg protein) behaved as a constant equal to 28 $\mu lO_2/h^2$/mg protein for at least 4 hours. Irrespective of the delay between the introduction of O_2 and the beginning of treatment,inhibition was found to be immediate and almost complete - 90% for curve A,otherwise 80% - as soon as 9mM BZI was added at pH 6.3 (BZI is ineffective at pH 4.2).

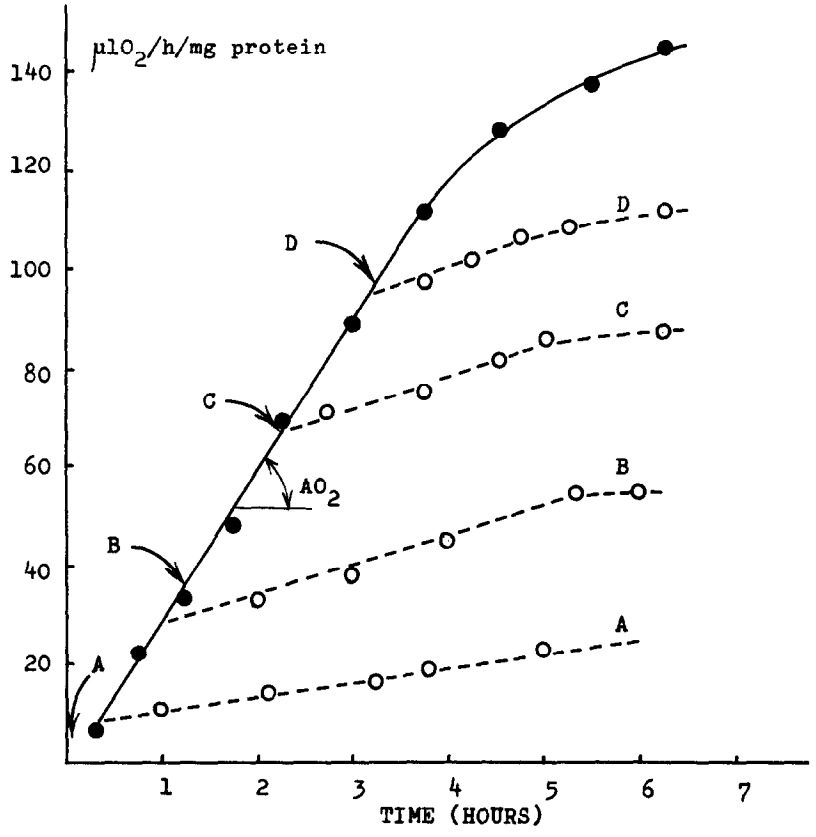

Fig.1 - Inhibition of the increase of respiration rate by BZI during adaptation of yeast by oxygen.

Anaerobic stationary phase cells, harvested and washed at 0°, were resuspended immediately in 2% glucose-maleic acid/K maleate buffer (0.1M -pH 6.3) and aerated in the manometric flask at 28° (Warburg constant volume respirometer); the concentration of yeast was 0.4mg protein/ml. BZI, at a final concentration of 9mM, was added at the introduction of oxygen (curve A) or after different intervals of time since the start of aeration : after 75 min. (curve B), after 135 min. (curve C) and after 195min. (curve D). The respiration rates of suspensions treated with BZI are corrected for the inhibition of respiratory activity (A, B, C and D) (21% inhibition with 9mM BZI, see Figure 2).

Figure 2 demonstrates that the almost complete inhibition of adaptability (AO_2) of yeast in the presence of 9mM BZI is not directly correlated with the simultaneous small inhibition (about 20%) of the respiratory activity (QO_2). Simultaneously, the intensity of the aerobic fermentation of glucose, measured in the same way by the direct method (not represented in the Figure), is also only slightly decreased (70% efficiency with 10mM BZI).

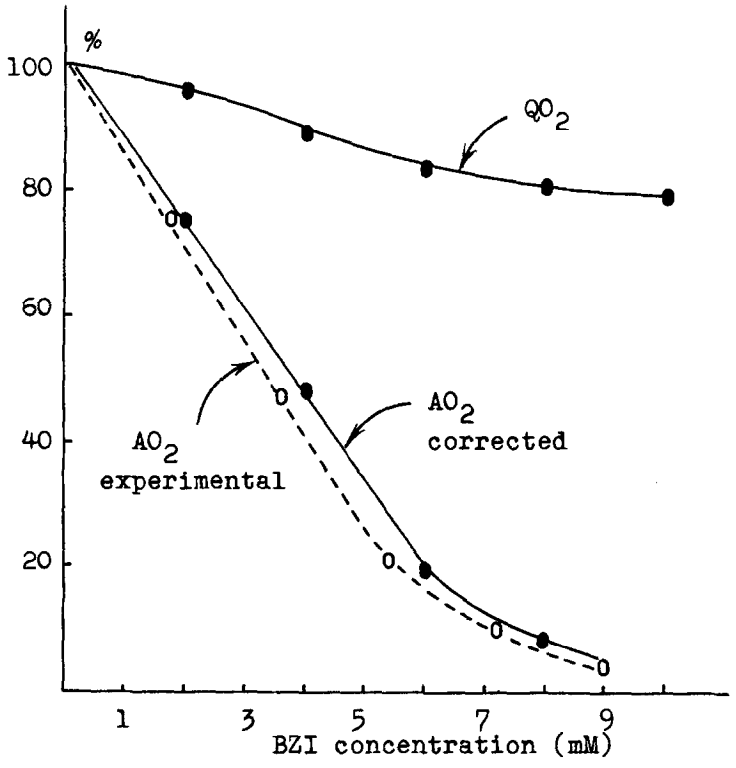

Fig.2 - Inhibition of the respiration rate (QO_2) and of the acceleration of the respiration rate (AO_2) as a function of BZI concentration.

(i) Yeast cells were grown aerobically at 26° in a 1% Yeast extract -1% Peptone-0.1% KH_2PO_4 medium, containing 2% glucose as the carbon source. Cells were harvested in the stationary phase of growth (3.5mg yeast protein/ml), washed at 0°, and resuspended in 2% glucose-maleic acid/K maleate buffer (0.1M-pH 6.3) containing various concentrations of BZI. The respiration rates (QO_2), which were compared by the manometric technique at 28°, are expressed as the percentage of the rate measured in the absence of BZI.

(ii) The accelerations of the respiration rate (AO_2, see text) of the anaerobically grown stationary phase cells were measured, as shown in the legend of Fig.1, in 2% glucose buffer (pH 6.3) containing BZI at various concentrations. AO_2's are estimated relatively to the AO_2 of uninhibited yeast and corrected owing to the inhibition of QO_2 (QO_2 curve).

The following experiments provided futher information about special sensitivity of the respiratory adaptation process.

As shown in Figure 3, we first confirmed that respiratory adaptation was restored with an initially increased velocity when yeast cells, pretreated with 9mM BZI, were transferred into fresh aeration buffer at pH 4.2.

By adding CYCLO (25 µg/ml) during the pretreatment with BZI or by

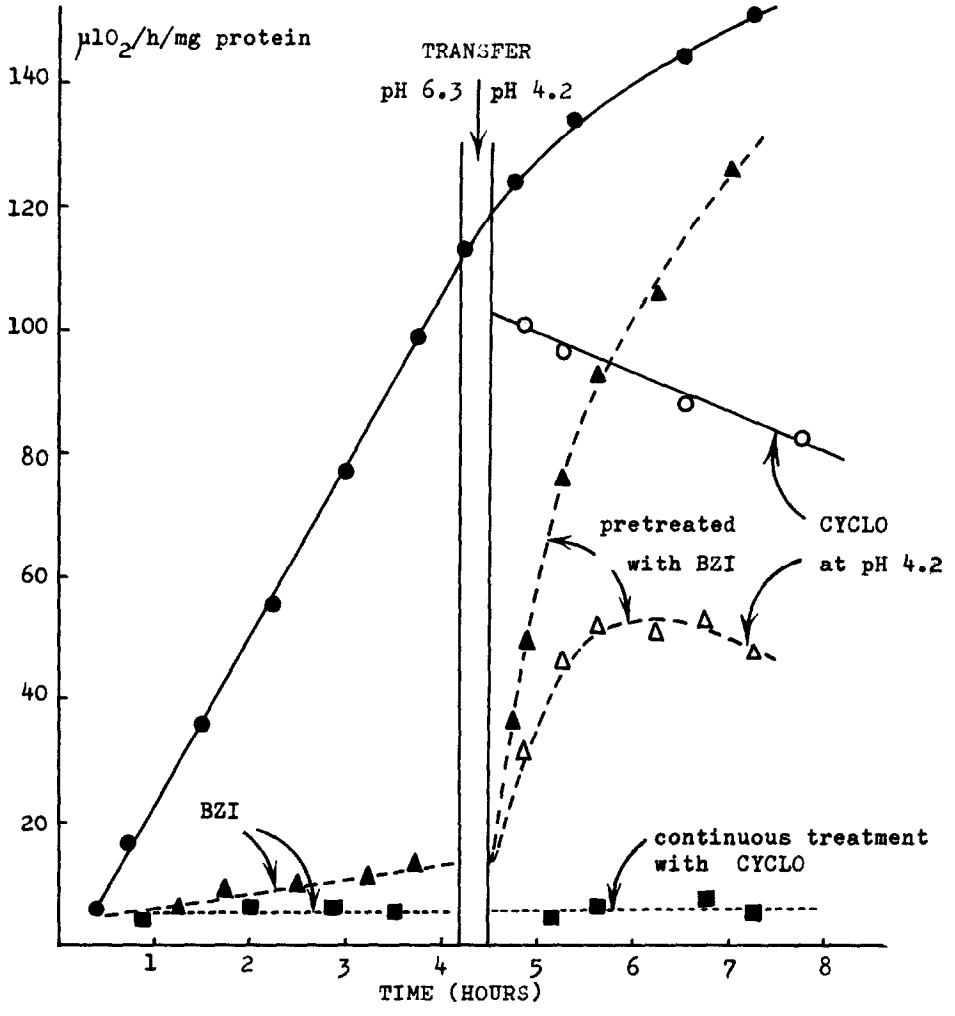

Fig.3 - Time course of the conversion of inactive macromolecular precursors into respiratory holoenzymes after pretreatment of yeast with BZI.

Anaerobic stationary phase cells were adapted to oxygen at pH 6.3 (see legend of Fig.1) in the absence (full circles) or in the presence of 9mM BZI (full triangles); in a third suspension, CYCLO (25 µg/ml) was present simultaneously with BZI (squares). 4 hours later, the yeast cells were harvested by centrifugation and resuspended (TRANSFER pH 6.3 ⟶ pH 4.2) in 2% glucose-succinic acid/K succinate buffer (0.05M-pH 4.2) supplemented or not with CYCLO (25 µg/ml). O_2-induced cells (reference sample) were aerated with CYCLO (open circles) while BZI-pretreated cells were reincubated at pH 4.2 with (open triangles) or without (full triangles) CYCLO. One yeast suspension received a continuous treatment with CYCLO (squares).

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delaying the addition of the antibiotic till the time when the yeast cells were transferred to pH 4.2, we demonstrated that the increased AO_2 mainly

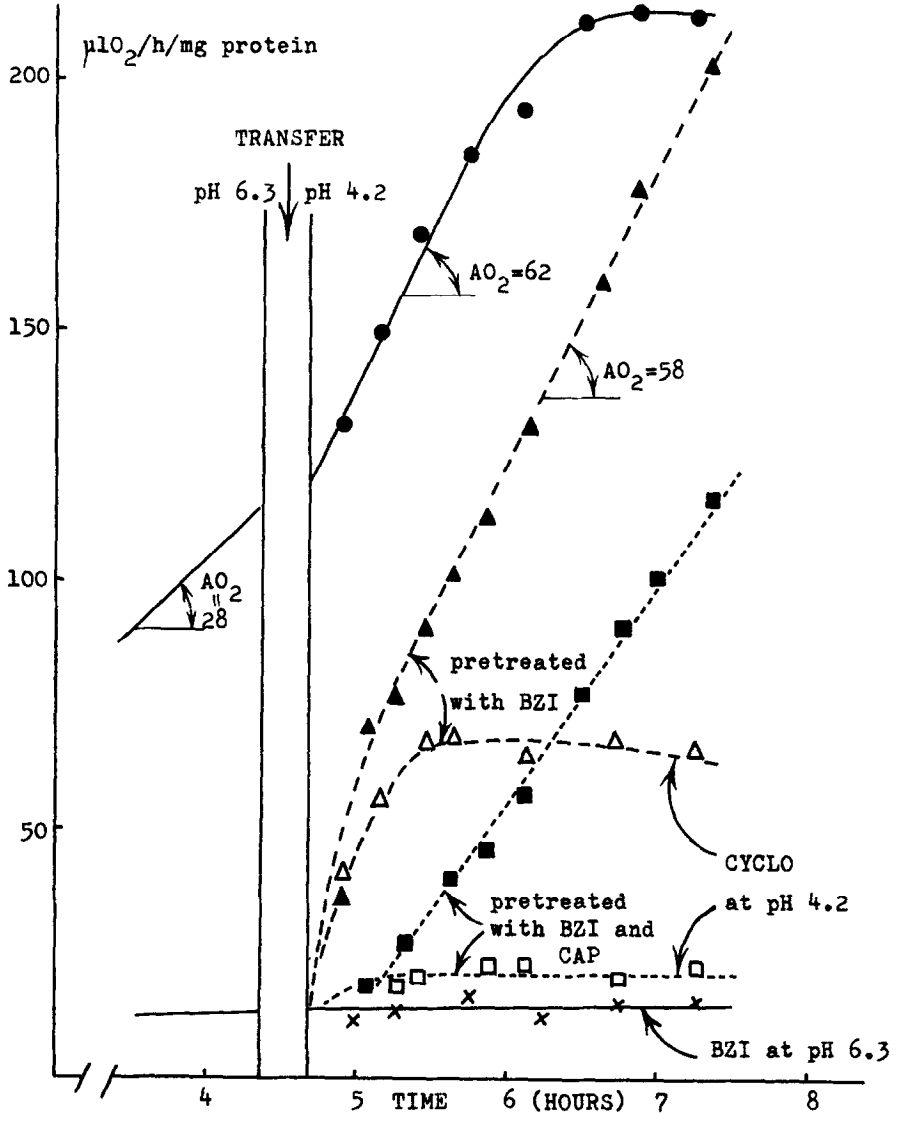

Fig.4 - Inhibition by chloramphenicol of the production of respiratory precursors synthesized during treatment with BZI.

The protocol of the experiment is similar to that of Fig.3, except that 9mM BZI is used in combination or not with CAP (4mg/ml) which replaces CYCLO. The yeast cells, aerated during 4 hours at pH 6.3, were washed with buffer at pH 6.3 before they were transferred to pH 4.2 buffer containing 2% v/v ethanol as the carbon source. The yeast suspensions reincubated at pH 4.2 were : (i) O_2-induced cells (full circles), the reference system (ii) yeast pretreated with BZI alone (full triangles) and (iii) cells pretreated simultaneously with BZI and CAP (full squares). The last two yeast suspensions i.e. (ii) (open triangles) and (iii) (open squares) were also reincubated at pH 4.2 with CYCLO (25 µg/ml). A yeast suspension received a continuous treatment with BZI at pH 6.3 (x).

corresponds to the transformation of macromolecular precursors into functional respiratory holoenzymes; these precursors are futhermore synthesized from free amino acids during the aerobic incubation with BZI. The conversion of the catalytically inactive precursors into holoenzymes restores respiratory efficiency to about 50% of that of the uninhibited cells; the persistant loss in respiratory efficiency is due to the general toxicity of BZI for protein anabolism,as measured by the incorporation of free radioactive amino acids (Sels et al.,1966;Sels,1967).

In the experiment described on Figure 4, CAP (4mg/ml),a more specific and less toxic inhibitor,replaced CYCLO during the preincubation with 9mM BZI. Ethanol was also used instead of glucose in the transfer medium in order to amplify the AO_2's (AO_2 of the reference suspension was close to 60 $\mu lO_2/h^2/mg$ protein) as well as the final respiration rates (QO_2 exceeds 200 $\mu lO_2/h/mg$ protein). CAP works with the same efficiency as CYCLO in abolishing completely the de novo synthesis of those respiratory apoproteins which can be activated and which may accumulate during incubation with BZI.

The group of catalytically inactive precursors of respiratory enzymes,which accumulate in yeast adapting to oxygen in the presence of BZI, comprises the respiratory apoproteins whose synthesis or transformation into functional holoenzymes is under the control of the mitochondrial protein synthesizing system (sensitive to CAP). Our studies of the differential effects of BZI on the biosynthesis of individual hemoproteins, which will be described in detail elsewhere,also favorize this conclusion. Simultaneously,the mode of action of CAP emerges distinctly as the inhibition of the de novo synthesis of the apoprotein moiety of some of the respiratory components having an enzymatic or structural function,since no readily activated apoenzymes were found to accumulate in the presence of CAP.

The mechanism of the accelerated formation of respiratory holoenzymes after the treatment of yeast with BZI is clearly different from the transient acceleration of the de novo synthesis of hemoproteins specific to zygotes from crosses between complementary respiratory deficient mutants of S.cerevisiae (Sels and Jakob,1967).

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