

## INHIBITION OF ENERGY METABOLISM BY BENZOXAZOLIN-2-ONE

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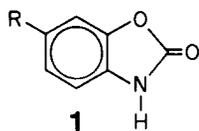
**Abstract**—1. The effects of the title compound (BOA) on energy-linked reactions in mitochondria were studied.

2. BOA inhibited electron transfer between the flavin and ubiquinone in Complex I, and ATP synthesis at the  $F_1$  moiety of the ATPase complex.

3. These results are discussed in relation to the toxicity of BOA towards a wide range of aerobic organisms.

### INTRODUCTION

Benzoxazolin-2-ones such as compounds **1** (Scheme 1) are decomposition products of hydroxamic acid glucosides naturally occurring in Gramineae such as maize, wheat and rye, their production starting when the plant tissue is injured (Hofman and Hofmanova, 1971).



BOA : R = H

MBOA : R = CH<sub>3</sub>O

The possible role of benzoxazolin-2-ones as host plant resistance factors towards various pests and pathogens has been investigated. Benzoxazolin-2-one (BOA) and its 6-methoxy derivative (MBOA) produce deleterious effects on various organisms.

MBOA is toxic to the cereal aphid *Metopolophium dirhodum* ( $I_{50} = 7$  mM) (Argandoña *et al.*, 1980). Benzoxazolin-2-ones inhibit growth of larvae of the European corn borer, *Ostrinia nubilalis* ( $I_{50} = 2.5$  mM) (Beck and Smissman, 1961) and of other insects such as *Bombyx mori* (Kubo and Kamikawa, 1983) and *Blatella germanica* and *Prodenia eridana* (Beck and Stauffer, 1957).

The growth of *Fusarium nivale*, a fungus pathogenic to rye, is completely inhibited by 3 mM BOA or MBOA (Wahlroos and Virtanen, 1958). Other plant pathogenic fungi such as *Fusarium moniliforme*, *Gibberella zeae*, *Pyrenochaeta terrestris*, *Diplodia zeae* (Whitney and Mortimore, 1959), *Sclerotinia trifoliorum* (Virtanen *et al.*, 1957) and *Penicillium chrysogenum* (Beck and Smissman, 1961) are also inhibited at concentrations ranging from 1 to 6 mM.

Plant pathogenic bacteria such as *Xanthomonas stewartii* are inhibited by MBOA at 4 mM (Whitney and Mortimore, 1961). In addition, both BOA and MBOA inhibit growth of *Staphylococcus aureus*,

*Pseudomonas fluorescens* and *Escherichia coli* at concentrations higher than 5 mM (Virtanen *et al.*, 1957).

The toxicity of benzoxazolin-2-ones towards such a wide range of aerobic organisms suggests that the energy metabolism might be a possible target for their action. We report herein the effects of BOA on mitochondrial electron transport and ATP synthesis.

### MATERIALS AND METHODS

Phosphorylating submitochondrial  $Mg^{2+}$ -ATP particles (SMP) were prepared from heavy bovine heart mitochondria (Smith, 1967) as described (Low and Vallin, 1963). Beef-heart  $F_1$  was prepared according to Knowles and Penefsky (1972). It was stored at liquid nitrogen temperature and processed before used as described (Roveri and Calcaterra, 1985).

#### Electron transport

Electron transport in SMP (0.3 mg protein) was determined following oxygen consumption with a Clark electrode connected to a Gilson oxygraph in a reaction medium containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.5), 50 mM ethanol, 125 I.U./ml of yeast alcohol dehydrogenase (EC 1.1.1.1) and NADH. Rates reported correspond to the slopes of linear traces drawn by the oxygraph.

#### Reduction of flavoproteins and cytochrome b in SMP

The reduction kinetics were followed by determining the absorbance changes in an Aminco DW-2a spectrophotometer (Dual wavelength mode) at 475 minus 510 nm (flavoproteins) or 563 minus 575 nm (cytochrome b). The reaction was carried out at 2°C in a medium containing 50 mM potassium phosphate (pH 7.5) and SMP (2.5 mg protein/ml).

#### ATP synthesis

The reaction was measured in a medium containing (final conc.) 180 mM sucrose, 1 mM  $MgCl_2$ , 10 mM succinate, 0.5 mM EDTA, 3  $\mu$ M rotenone, 50 mM glucose, 2 mM ADP, 15 I.U. of yeast hexokinase (EC 2.7.1.1) and 50 mM Tris-HCl pH 7.4. SMP (0.25 mg protein) were added to this medium and incubated for 5 min. ATP synthesis was started by adding 5  $\mu$ mol  $P_i$ ,  $2 \times 10^6$  cpm carrier free  $^{32}P_i$  and 3  $\mu$ mol  $MgCl_2$ . The final vol. was 1 ml. The preincubation and the reaction were carried out under aerobiosis obtained with a girtatory waterbath shaker. After 5 min the reaction

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was stopped and inorganic phosphate was quantitatively precipitated essentially as described by Sugino and Miyoshi (1964). After separating the ppt. by centrifuging for 10 min at 3000 rpm, aliquots were withdrawn and analyzed for ( $^{32}\text{P}$ )glucose-6-phosphate by Cerenkov counting in a Beckman 8100 liquid scintillation counter.

#### $P_i$ -ATP exchange reaction

SMP (1 mg protein) were added to 1 ml of a reaction medium containing 180 mM sucrose, 10 mM ATP, 10 mM  $\text{MgCl}_2$ , 50 mM Tris-HCl (pH 7.5), 10 mM  $P_i$  and  $2 \times 10^6$  cpm of carrier free  $^{32}\text{P}_i$ . After 5 min the reaction was stopped by adding Sugino and Miyoshi's reagent (1964) and after centrifuging at 3000 rpm for 10 min the supernatant was analyzed for ( $\gamma$ - $^{32}\text{P}$ )ATP by Cerenkov counting.

#### ATPase activity of SMP and of $F_1$

ATPase activity was determined spectrophotometrically as described (Pullman *et al.*, 1960) in a reaction medium (2 ml) containing 100 mM sucrose, 1 mM  $\text{MgCl}_2$ , 4 mM phosphoenolpyruvate, 0.125 mM NADH, 60 I.U. pyruvate kinase (EC 2.7.1.40), 50 I.U. lactate dehydrogenase (EC 1.1.1.27), 40 mM Tris-HCl (pH 8.0) and Mg-ATP. The reaction was started by adding  $F_1$  (3  $\mu\text{g}$  protein) or SMP (0.1 mg protein) to the reaction medium. In the latter case, the medium also contained 3  $\mu\text{M}$  rotenone.

#### General

Protein determinations were carried out according to Lowry *et al.* (1951).

BOA (Aldrich Chem. Co.) was recrystallized twice before use and dissolved in dimethylsulfoxide prior to its addition to the reaction medium. The solvent (less than 2%) had no effect on any of the biochemical reactions studied.

Measurements were carried out at 30°C, except when indicated.

Rates informed are the average of duplicate experiments which agreed within 10%.

### RESULTS AND DISCUSSION

BOA completely inhibited electron transport from NADH to oxygen in SMP (Fig. 1). This effect can be located at the level of the mitochondrial respiratory chain since similar results were obtained in the presence of uncouplers (data not shown). In addition, since succinate oxidation was not inhibited it can be suggested that BOA inhibits electron transport from NADH to ubiquinone (complex I of the respiratory chain).

A more precise location was accomplished by following the absorbance changes produced by addition of NADH or NADH plus succinate, in the flavoprotein spectral region (475 minus 510 nm): (i) NADH-dependent absorbance decrease in SMP was partially inhibited by BOA. Maximal inhibition (about 50%) was obtained with 10 mM BOA (Fig. 2B), a concentration which almost completely inhibited cytochrome *b* reduction (Fig. 2A); (ii) subsequent addition of succinate to NADH-reduced BOA-inhibited SMP (Fig. 2B) increased the absorbance change to a level similar to that observed in the absence of BOA; (iii) similar results were obtained with rotenone (Fig. 2B), a known inhibitor that blocks electron transport from FMN to ubiquinone in complex I, and (iv) the addition of rotenone to NADH-reduced BOA-inhibited SMP did not produce any extra inhibition (Fig. 2B).

Half of the absorbance change at 475 minus 510 nm can be attributed to reduction of the FMN

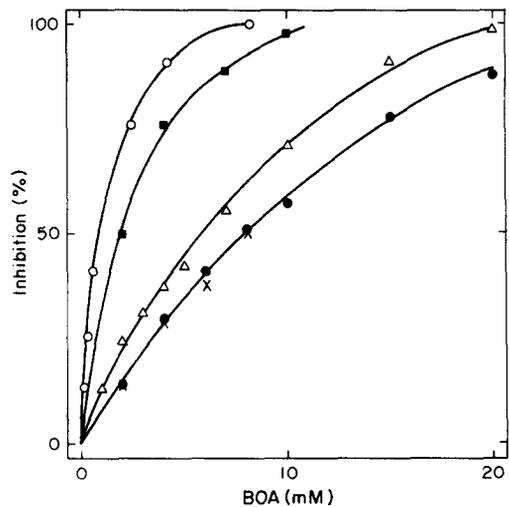


Fig. 1. Inhibition by BOA of several mitochondrial energy-linked reactions. The reactions measured (particular conditions in parenthesis) were:  $P_i$ -ATP exchange reaction ( $\circ$ ), ATP synthesis driven by succinate oxidation ( $\blacksquare$ ), NADH oxidation ( $\triangle$ , 0.3 mM NADH), ATP hydrolysis catalyzed by SMP ( $\bullet$ ; 1 mM Mg-ATP) or by  $F_1$  ( $\times$ ; 0.07 mM Mg-ATP). Activities for controls were 102, 315 nmol/min mg protein, 450 nat/min mg protein, 1.25 and 25  $\mu\text{mol}/\text{min}$  mg protein, respectively.

moiety of NADH dehydrogenase (Hatefi, 1985), which is the first acceptor of electrons from NADH. Therefore, it can be concluded that FMN is in the substrate side of the BOA-sensitive site. The nature of the electron transport chain components responsible for the other half of the absorbance change and their relative contribution has not been clearly established yet. Nevertheless, it has been reported (Boveris and Stoppani, 1970) that the major contribution to the signal corresponds to flavoprotein reduction (FMN of NADH dehydrogenase and FAD of succinate dehydrogenase). In addition, under our experimental conditions, NADH was able to completely reduce succinate, since the same extent of absorbance decrease was obtained in the presence of either NADH or NADH plus succinate (data not shown). Therefore, most of the BOA-sensitive NADH-dependent absorbance decrease could be attributed to FAD and ubiquinone reduction. Hence, FAD and ubiquinone would be located on the oxygen side of the BOA-sensitive site. Since BOA and rotenone exerted similar effects and the addition of rotenone to BOA-inhibited SMP did not produce any extra inhibition, it can finally be concluded that BOA exerts its action at or very near to the rotenone-sensitive site.

ATP synthesis driven by succinate oxidation was also inhibited by BOA (50% inhibition was obtained with 2 mM BOA, Fig. 1). This effect cannot be attributed to inhibition of electron transport since, as stated above, succinate oxidation was insensitive to BOA. This result together with the inhibition of  $P_i$ -ATP exchange reaction and ATPase activity (Fig. 1) in SMP indicated that a second probable site of action of BOA is the ATPase complex. Furthermore, since ATPase activity of the soluble mitochondrial ATPase ( $F_1$ ) was also inhibited by BOA (Fig. 1), the

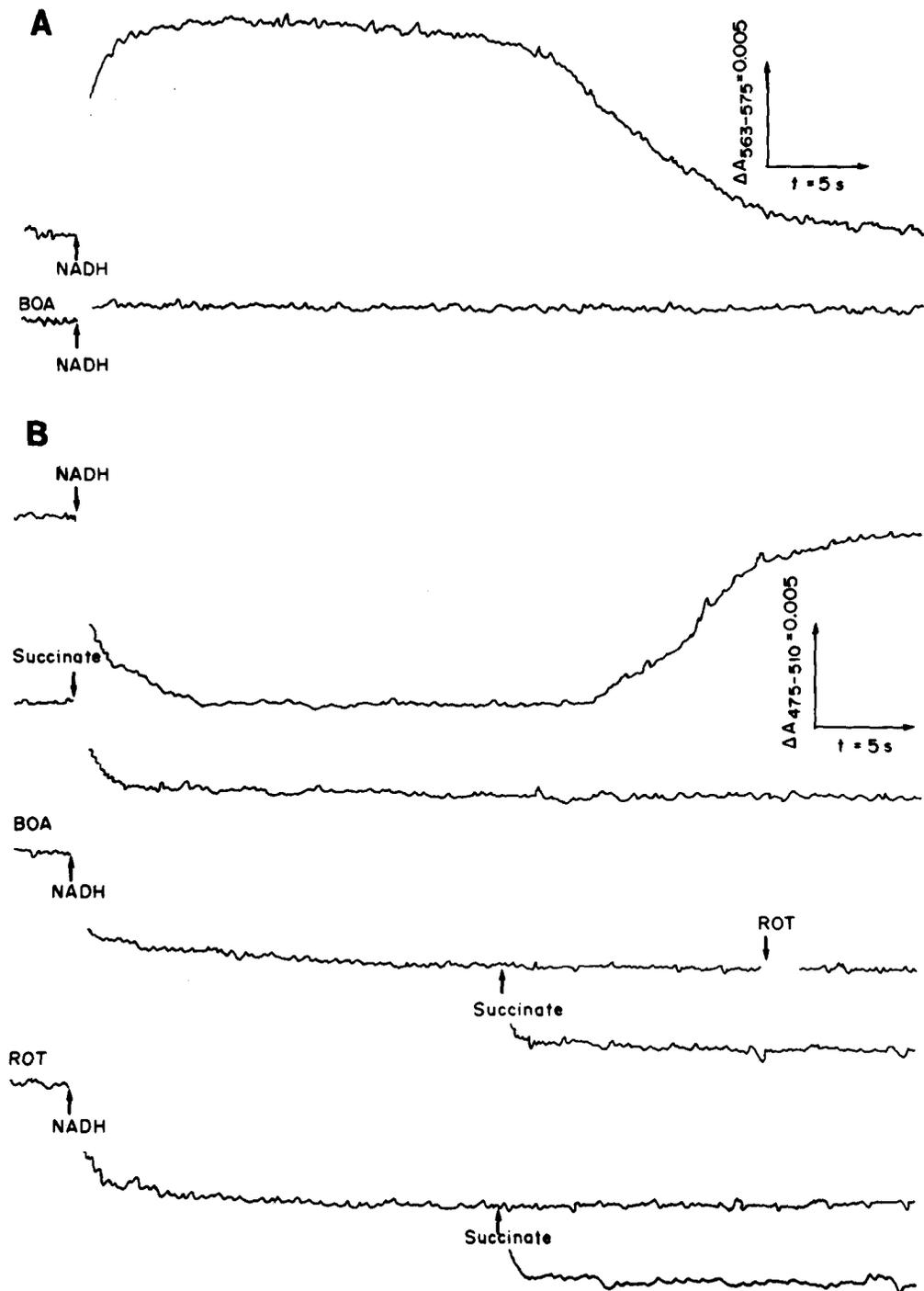


Fig. 2. Kinetics of reduction by NADH of flavin and cytochrome *b* in SMP. The time course of cytochrome *b* (A) and flavin (B) reduction and reoxidation was determined as described under Materials and Methods. Additions were NADH (0.45 mM), ROT (3  $\mu$ M rotenone), BOA (10 mM) and succinate (10 mM).

likely site of action of BOA is at the  $F_1$  moiety of the ATPase complex. BOA behaved as an uncompetitive inhibitor with respect to ATP for the ATPase activity of isolated  $F_1$  ( $K_i = 3$  mM, Fig. 3) and as a non-competitive inhibitor with respect to ATP in SMP ( $K_i = 8$  mM, Fig. 4). Hence it can be concluded that the binding of BOA does not occur at the active site of the ATPase complex. The differences between the inhibitions by BOA of the ATPase activities of

isolated  $F_1$  and of SMP, could reflect different conformational states or different mechanisms of soluble and membrane bound  $F_1$ , viz. in isolated  $F_1$  only the kinetically competent  $F_1$ -ATP complex would bind BOA, whereas in membrane-bound  $F_1$ , both  $F_1$  and the  $F_1$ -ATP complex would be equally able to bind BOA.

The above discussed effects of BOA resemble somehow the effects described for hormonal steroids

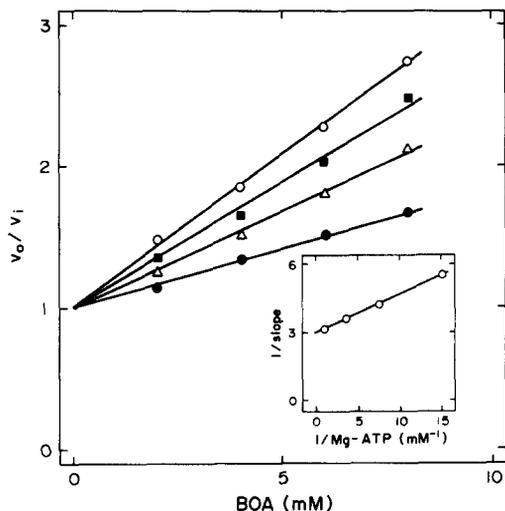


Fig. 3. Uncompetitive inhibition by BOA of ATPase activity of soluble mitochondrial ATPase. Experimental conditions were as described under Materials and Methods. Mg-ATP concentrations were 0.065 (●), 0.130 (△), 0.260 (■) and 1 (○) mM. In the figure,  $v_o/v_i$  values are plotted against BOA concentrations, with  $v_o$  equal to the velocity in the absence of inhibitor and  $v_i$  equal to the velocity in the presence of the corresponding concentration of inhibitor. In the inset, the reciprocal of the slopes of the  $v_o/v_i$  vs (BOA) lines are plotted against the reciprocal of Mg-ATP concentration, according to the procedure described by Roveri (1985) for linear uncompetitive inhibitors. The value for  $K_i$  was determined from the intercept on the  $y$ -axis as 3 mM.

and related substances (Vallejos and Stoppani, 1967; Boveris and Stoppani, 1970). However, a difference must be pointed out: BOA inhibited ATP synthesis

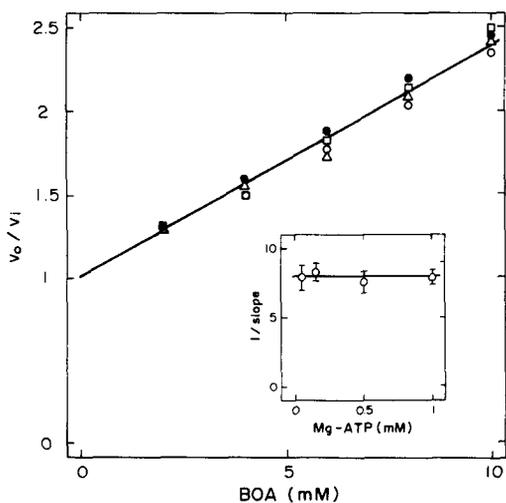


Fig. 4. Non-competitive inhibition of ATPase activity in SMP. ATPase activity was measured as described under Materials and Methods at 0.05 (□), 0.150 (●), 0.50 (○) and 1 (△) mM Mg-ATP. The figure shows the line correlating all the points simultaneously since the slopes of lines corresponding to different substrate concentrations did not differ significantly (see inset). The plot shown in the inset identifies a non-competitive inhibitor, as described by Roveri (1985). The points correspond to the slopes and the bars to the standard deviation of the slopes, obtained by linear regression analysis of  $v_o/v_i$  values as a function of (BOA) at the different particular Mg-ATP concentrations.

by acting directly on the ATPase complex and not in the energy coupling.

Finally, the effects of BOA on mitochondrial electron transport from FMN to ubiquinone and on ATPase complex would result in an impairment of ATP synthesis in organisms largely dependent on oxidative phosphorylation for their energy requirements, thus providing a possible explanation for reported deleterious effects caused by BOA.

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