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INHIBITION OF MITOCHONDRIAL ENERGY-LINKED REACTIONS BY 2,4-DIHYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE (DIMBOA), A HYDROXAMIC ACID FROM GRAMINEAE

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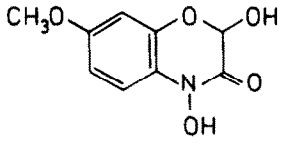
Abstract—DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) is the main hydroxamic acid isolated from maize extracts. It inhibited reversibly ATP synthesis, P_i-ATP exchange reaction and ATPase activity in submitochondrial particles from bovine heart. Half-maximal effects were obtained with 4, 2, and 6 mM DIMBOA respectively. At higher concentrations it also inhibited mitochondrial electron transport (I_{50} = 11 mM). Irreversible inactivation of mitochondrial electron transport, P_i-ATP exchange reaction and 8-anilino-1-naphthalene sulfonate energy-dependent fluorescence enhancement was also observed. These effects of DIMBOA on energy-linked mitochondrial reactions may explain the inhibitory action of DIMBOA on several aerobic organisms.

DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) is the main hydroxamic acid isolated from wheat extracts [1]. This and other hydroxamic acids, isolated from various Gramineae [2], have been suggested to play an important role in the defense of the plant against insects [3–6]. It has also been shown that they inhibit bacterial [7] and fungal [8] growth.



DIMBOA

Recently, it was reported that DIMBOA inhibits photosynthetic ATP synthesis in spinach chloroplasts, thus behaving as an energy transfer inhibitor [9]. Coupling factor CF_1 from the same origin is affected by DIMBOA in two different manners: (i) a reversible one that accounts for an uncompetitive inhibition of the ATPase activity, and (ii) an irreversible one that results in a progressive inactivation of the enzyme [10]. This last effect was suggested to be due to an irreversible modification of sulfhydryl groups in the enzyme molecule since DIMBOA is able to participate in reactions with sulfhydryl compounds forming addition and/or reduction products [11–13].

The above-described effects of DIMBOA on different organisms are indicative that hydroxamic acids can act, similarly to other products of secondary plant metabolism, as allelochemical agents, playing an important role in the interaction between different species or in the mechanism of defense against plant pathogens or predators [14, 15]. Only in a few cases is the molecular mechanism of this allelochemical action well known. One of the enzymic systems that must be considered as potential targets for the action of such allelochemical agents are the systems involved in mitochondrial energy metabolism [16, 17].

In this paper we discuss the effect of DIMBOA on electron transport and other energy-linked reactions in phosphorylating submitochondrial particles from bovine heart, as an attempt to contribute to the knowledge of the biochemical basis of its action on plant predators.

MATERIALS AND METHODS

Heavy bovine heart mitochondria were prepared as described [18]. Phosphorylating submitochondrial Mg^{2+}-ATP particles (SMP) were prepared from bovine heart mitochondria essentially as described [19].

Electron transport. Electron transport in SMP was determined following oxygen consumption with a Clark electrode connected to a Gilson oxygraph in a reaction medium containing 250 mM sucrose and 50 mM Tris–HCl (pH 7.5) using either 10 mM succinate, an NADH-regenerating system composed of 50 mM ethanol, 0.2 mM NADH and 125 I.U./ml of yeast alcohol dehydrogenase (EC 1.1.1.27) or 5 mM ascorbate *plus* 0.1 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) as oxidizable substrate. Rate values reported correspond to the slopes of linear traces drawn by the oxygraph.

Spectral studies. The redox state of the cytochromes of the respiratory chain under different conditions was studied spectrophotometrically in an Aminco DW-2a provided with a magnetic stirrer

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attachment and connected to a MIDAN-T microprocessor data analyzer.

ATPase activity. SMP (60 μg protein) were added to a reaction medium (1 ml) containing 180 mM sucrose, 2 μM carbonylcyanide *p*-trifluoro-methoxyphenyl hydrazone (FCCP) and 50 mM Tris–HCl (pH 7.5). After 5 min of preincubation, the reaction was started by adding ATP (4 mM final concn). The reaction was stopped 5 min later with 0.05 ml of 100% trichloroacetic acid (TCA). After centrifugation (10 min, 3000 rpm), aliquots were withdrawn from the supernatant fraction and analyzed for inorganic phosphate according to Sumner [20].

ATP synthesis. The reaction was measured in a medium containing (final concn) 180 mM sucrose, 1 mM $MgCl_2$, 10 mM succinate, 0.5 mM EDTA, 3 μM rotenone, 50 mM glucose, 2 mM ADP, 5 I.U. of yeast hexokinase (EC 2.7.1.1) and 50 mM Tris–HCl, pH 7.4. SMP (0.4 mg protein) were added to this medium and incubated for 5 min. ATP synthesis was started by adding 5 μmoles P_i, 2×10^6 cpm carrier free $^{32}P_i$ and 3 μmoles $MgCl_2$. The final volume was 1 ml. The preincubation and the reaction were carried out under aerobiosis obtained with a gyratory waterbath shaker. After 5 min the reaction was stopped and inorganic phosphate was quantitatively precipitated essentially as described by Sugino and Miyoshi [21]. After separating the ppt by centrifuging for 10 min at 3000 rpm, aliquots were withdrawn and analyzed for [^{32}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 $MgCl_2$, 50 mM Tris–HCl (pH 7.5), 10 mM P_i and 2×10^6 cpm of carrier free $^{32}P_i$. After 5 min the reaction was stopped by adding Sugino and Miyoshi's reagent [21] and, after centrifuging at 3000 rpm for 10 min, the supernatant fraction was analyzed for [γ-^{32}P]ATP by Cerenkov counting.

Energy-linked 8-anilino-naphthalene sulphonate (ANS) fluorescence enhancement. Measurements were carried out in a Perkin–Elmer 650-40 fluorescence spectrophotometer. ANS was excited at 380 nm and fluorescence was measured at 480 nm essentially as described by Ferguson *et al.* [22].

Irreversible inactivation of SMP by DIMBOA. SMP (10 mg/ml) were incubated in a reaction medium containing 180 mM sucrose and 50 mM Tris–HCl, pH 7.5. After 5 min of preincubation, DIMBOA was added and aliquots were withdrawn at different time intervals and diluted (at least 100 times) in the appropriate reaction media in order to measure succinate oxidation, NADH oxidation, P_i-ATP exchange reaction, ATPase activity, or energy-linked ANS fluorescence enhancement.

Protein determinations were carried out using a modified biuret procedure [23].

DIMBOA was isolated as previously described [10], and dissolved in dimethyl sulfoxide (DMSO) prior to its addition to SMP. Controls with the solvent (less than 2%) were performed for all the biochemical reactions studied. Measurements were carried out at 25°. Rates are the average of determinations in duplicate which agreed within 10%.

RESULTS

DIMBOA inhibited electron transport from succinate to oxygen in submitochondrial particles (SMP) from bovine heart. The dependence of the inhibition on DIMBOA concentration followed a sigmoidal curve (Fig. 1A). Hardly any effect was observed at concentrations below 4 mM. Similar results are obtained for NADH and ascorbate + TMPD oxidations (data not shown). In all cases 50% inhibition was obtained with about 11 mM DIMBOA.

Inhibition by DIMBOA of ATP synthesis and P_i-ATP exchange reaction catalyzed by SMP was more effective and attained completeness. In both cases hyperbolic titration curves were obtained (Fig. 1B)

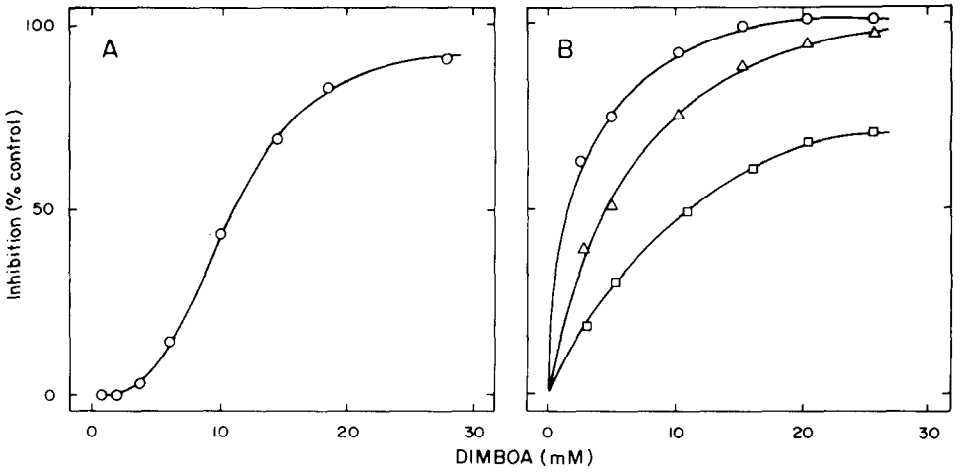

Fig. 1. Effect of DIMBOA on mitochondrial electron transport and other energy-linked reactions. (A) Mitochondrial electron transport (○) was measured as indicated in Materials and Methods using succinate as oxidizable substrate. (B) ATP synthesis (○), P_i-ATP exchange reaction (△) and ATPase activity (□) were measured as indicated under Materials and Methods. Activities for controls were 292 natoms/min·mg protein, and 315, 102, and 521 nmoles/min·mg protein respectively.

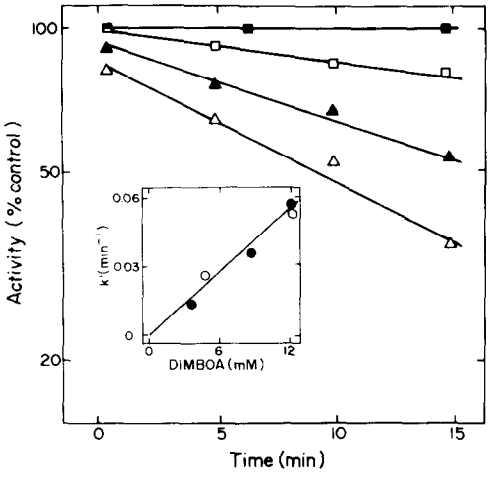

Fig. 2. Irreversible inactivation of mitochondrial electron transport by DIMBOA. SMP were incubated with 0 (■), 4 (□), 9 (▲) and 12.5 mM (△) DIMBOA as described in Materials and Methods. At the indicated time intervals, aliquots were withdrawn and diluted in the media for determining electron transport from succinate to oxygen. The pseudo-first-order rate constants (k') calculated from the slopes of the semilogarithmic plots are shown in the inset (●). Open circles (○) correspond to similarly determined values obtained from inactivation plots of electron transport from NADH to oxygen. Activities for controls were 278 (succinate) and 161 (NADH) natoms/min · mg protein.

that allowed the determination of I_{50} values (4 and 2 mM DIMBOA respectively) from double-reciprocal plots of percent inhibition versus DIMBOA concentration. DIMBOA also inhibited ATPase activity but the maximal inhibition that could be obtained was about 70% (Fig. 1B). Half-maximal effect was exerted by 6 mM DIMBOA.

The above-mentioned effects were produced almost instantaneously. When succinate oxidation was measured after incubating SMP with DIMBOA, a progressive inactivation was observed (Fig. 2). This must be attributed to an irreversible reaction because it persisted after 100-fold dilution and the activity of diluted samples did not change after standing for 30 min. This inactivation followed pseudo-first-order kinetics with a rate constant (k') that depended linearly on DIMBOA concentration (Fig. 2, inset). Similar results were obtained when NADH oxidation was measured with DIMBOA-treated SMP (see open circles in Fig. 2, inset). The inactivation of succinate and NADH oxidations by DIMBOA was protected completely by 10 mM succinate (Fig. 3). Spectral studies showed that the reduction of cytochromes *b*, *c* and *a* by succinate was blocked almost completely by preincubation of SMP with DIMBOA (Fig. 4, spectra B and D). However, under the same conditions, cytochromes *c* and *a* were reduced by ascorbate + TMPD (Fig. 4, spectra F and C).

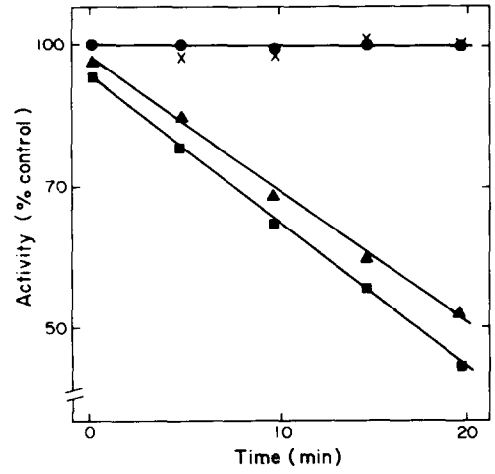

Fig. 3. Protection by succinate of mitochondrial electron transport against inactivation by DIMBOA. SMP were treated with 15 mM DIMBOA in the absence (■ and ▲) or in the presence of 10 mM succinate (● and ×) and then assayed for succinate oxidation (▲ and ●) or NADH oxidation (■ and ×) essentially as described in Materials and Methods. When NADH oxidation was measured, 1 mM malonate was added in order to specifically inhibit succinate oxidation. Activities for controls were 284 (succinate) and 172 (NADH) natoms/min · mg protein.

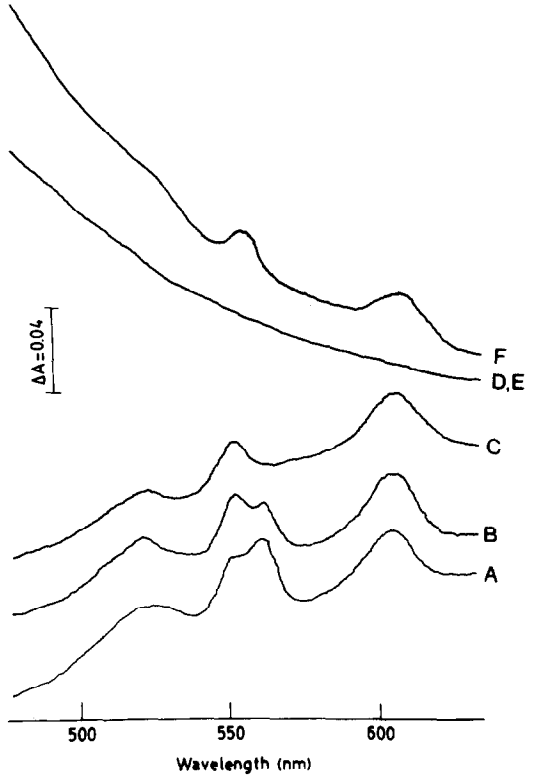

Fig. 4. Difference spectra (reduced *minus* oxidized) of SMP under various conditions. Traces shown correspond to the difference between spectra of SMP (1.9 mg protein/ml) treated as explained below and untreated oxidized SMP. The reaction medium (1.5 ml) was 50 mM potassium phosphate (pH 7.5). Treatments were as follows: (A) $Na_2S_2O_4$, (B) 10 mM succinate, (C) 5 mM ascorbate + 0.1 mM TMPD, (D) incubation of SMP with 20 mM DIMBOA for 15 min and further addition of 10 mM succinate, (E) incubation of SMP with 20 mM DIMBOA for 15 min, without addition of reducing agents, and (F) incubation of SMP with 20 mM DIMBOA for 15 min and further addition of 5 mM ascorbate + 0.1 mM TMPD.

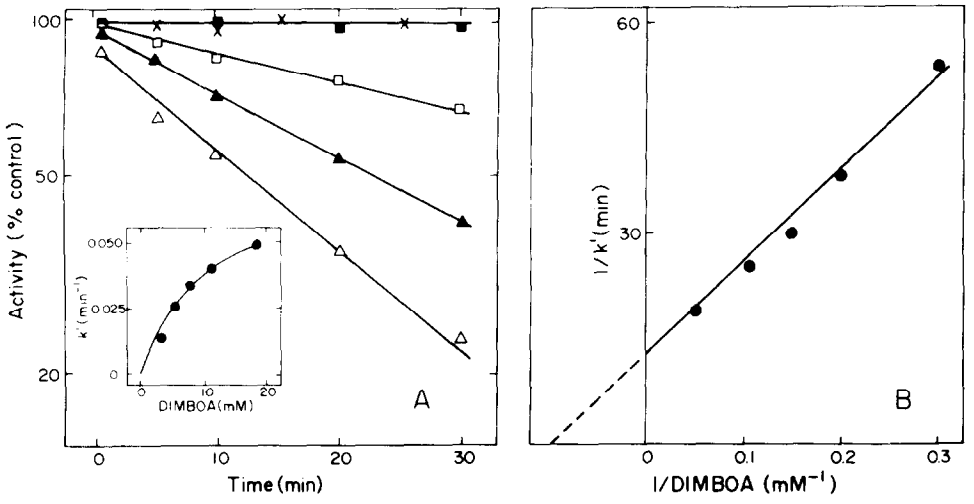

Fig. 5. Irreversible inactivation of P_i-ATP exchange reaction. Experimental conditions were similar to those explained in the legend to Fig. 2 except that in this case P_i-ATP exchange reaction was measured (see Materials and Methods). DIMBOA concentrations were: 0 (■), 3 (□), 7 (▲) and 18 mM (△). (×) corresponds to SMP treated with 20 mM DIMBOA and afterwards assayed for ATPase activity as indicated under Materials and Methods. In the inset are plotted the pseudo-first-order rate constants (k') determined from the slopes of the semilogarithmic plots shown. Activity for control was 98 nmoles/min · mg protein.

Preincubation of SMP with DIMBOA also provoked an exponential inactivation of the P_i-ATP exchange reaction (Fig. 5A). The pseudo-first-order rate constants, in this case, were dependent in a hyperbolic manner on DIMBOA concentration (Fig. 5A, inset) since the double-reciprocal plot of k' versus DIMBOA concentration yielded a straight line that intersected the y-axis at a value significantly different from zero (Fig. 5B). Conversely, when ATPase activity was measured with DIMBOA-treated SMP, no inactivation could be observed (see Fig. 5A).

DISCUSSION

DIMBOA exerted several actions on mitochondrial energy metabolism. At concentrations above 5 mM it inhibited electron transport from NADH, succinate and ascorbate + TMPD to oxygen, acting at the level of the respiratory chain since similar results were obtained in the presence of uncouplers (data not shown). These results suggest that the site of action of the inhibitor can be tentatively located in the cytochrome chain after the site of entry of electrons from ascorbate + TMPD. Since it was not possible to observe an instantaneous change in the redox state of succinate-reduced SMP by addition of DIMBOA in concentrations inhibiting electron transport more than 80% (data not shown), it can be suggested that the reversible inhibition of electron transport is produced between cytochrome a and O_2.

At lower concentrations, DIMBOA inhibited ATP synthesis, P_i-ATP exchange reaction and ATPase activity. These effects cannot be attributed to the action of DIMBOA on electron transport and are probably exerted at the mitochondrial ATPase complex, since the ATPase activity was also inhibited by DIMBOA. This inhibition was reversible since a 100-fold dilution restored the original activity.

When these effects are compared to the reversible effects described on ATP synthesis in chloroplasts [9], several differences can be pointed out: (i) in chloroplasts DIMBOA behaved exclusively as an energy transfer inhibitor whereas in SMP it also behaved as an electron transport inhibitor, and (ii) chloroplasts were twice as sensitive to DIMBOA as SMP.

A second category of effects probably related with the known reactivity of DIMBOA (see Refs. 11 and 13) resulted in the irreversible inactivation of some mitochondrial functions. Electron transport either from succinate or from NADH to oxygen were similarly inactivated upon incubation of SMP with DIMBOA. The inactivation followed pseudo-first-order kinetics, suggesting that the activity is suppressed after reaction of one group in the enzymatic complex with DIMBOA. From the kinetic data it can be postulated that DIMBOA reacted with some component in the respiratory chain following a simple second-order reaction and resulting in an irreversible inactivation of mitochondrial electron transport (see reaction scheme I in Fig. 6). From the inset in Fig. 2 the second-order rate constant (k) can be estimated to be equal to 4.4 M^{-1} min^{-1}. The electron transport chain component modified by DIMBOA is probably located at the level of the cytochrome chain since: (i) NADH and succinate oxidation were equally inactivated, and (ii) succinate was able to completely protect both reactions against inactivation by DIMBOA (see Fig. 3). The protection by succinate cannot be explained by binding of succinate to succinate dehydrogenase since this enzyme is not in the path of electrons from NADH to oxygen and hence is unlikely to be the electron

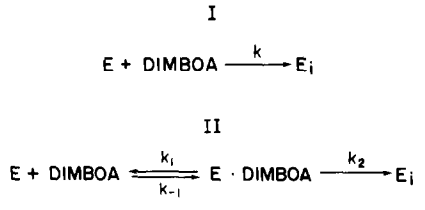

Fig. 6. Irreversible inactivation of mitochondrial reactions by DIMBOA. E represents the unmodified fully active enzyme form. E_i represents an inactive form, either an adduct of the enzyme and DIMBOA or an irreversibly modified enzyme form. E·DIMBOA represents a reversible intermediate whose dissociation constant (K_i) equals k_{-1}/k_1.

transport chain component affected by DIMBOA. Therefore, succinate protection must be exerted in an indirect way probably by changes in the redox state of some electron transport chain component located in the cytochrome chain near the point of entry of electrons from succinate dehydrogenase. The change in redox state of such a component could be accompanied by changes in nucleophilicity and/or reducing power [11, 13] of the groups involved in the irreversible modification. Further experimental evidence allowed the location of the site of action of DIMBOA as an irreversible inhibitor of the respiratory chain: (i) ascorbate + TMPD oxidation was not inactivated upon incubation of SMP for 30 min with 20 mM DIMBOA and (ii) cytochromes *b*, *c* and *a* cannot be reduced by succinate in DIMBOA-treated SMP (Fig. 4, spectrum D), whereas cytochromes *c* and *a* can be reduced by ascorbate + TMPD (Fig. 4, spectrum F). Therefore, the site of inactivation by DIMBOA must be located before cytochrome *c* and after succinate dehydrogenase (see above). Whether the appearance of a chromophore upon incubation of SMP with DIMBOA (Fig. 4, spectrum E) is related to the inactivation of electron transport by DIMBOA is still a matter of study.

The inactivation of the P_i-ATP exchange reaction cannot be explained by reaction scheme I, since the hyperbolic dependence of the pseudo-first-order rate constants with DIMBOA concentration indicates that a non-covalent intermediate or an easily-reversible covalent intermediate is formed prior to the irreversible modification (see reaction scheme II in Fig. 6). From the minus reciprocal of the x-intercept in Fig. 5B the dissociation constant (K_i) for the reversible intermediate can be estimated to be equal to 7.5 mM and, from the minus reciprocal of the y-intercept in the same figure, the value of k_2 (rate constant for the irreversible step) can be estimated as equal to 0.1 min^{-1}. The site modified by DIMBOA that is responsible for the P_i-ATP exchange inactivation must necessarily be different from the one responsible for electron transport inactivation, since both inactivations followed kinetically different reaction schemes.

An interesting point is that the ATPase activity of SMP was not inactivated at all by DIMBOA, indicating that the mitochondrial ATPase complex was not modified or that it was modified in such a way that the enzyme complex was still able to hydrolyze ATP. However, this ATP hydrolysis was not able to build a proton gradient through the mitochondrial inner membrane since the P_i-ATP exchange reaction and the ATP-dependent ANS fluorescence enhancement (data not shown) were inactivated completely when SMP were preincubated for 30 min with 20 mM DIMBOA. The inactivation of these two energy-linked (ATP-driven) reactions can be explained by an irreversible uncoupling of ATP hydrolysis and proton gradient build-up whose mechanism is still a matter of study.

An additional difference between the effect of DIMBOA on mitochondria and on chloroplasts is that ATPase activity in chloroplasts was irreversibly inactivated by DIMBOA whereas mitochondrial ATPase activity was not. This difference is probably related to the fact that, in the β-subunit of chloroplast coupling factor CF_1, there are essential cysteine residues (see Refs. 10, 11 and 13). Conversely, it has been recently confirmed that β-subunit from mitochondrial ATPase does not contain cysteine residues [24].

In summary, the actions of DIMBOA on SMP, described in this paper, suggest that mitochondrial metabolism is a good candidate for explaining the biological action of these hydroxamic acids on organisms that depend largely on mitochondrial ATP synthesis. From the point of view of the toxicity of DIMBOA, probably its most relevant effects on mitochondrial energy metabolism were the irreversible inactivation of electron transport and the irreversible uncoupling of the ATP-driven energy-linked reactions. Either effect would produce an inactivation of mitochondrial ATP synthesis leading to increased ineffectiveness of the energy metabolism of aerobic organisms.

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