Plant Physiol. (1994) 106: 1483–1488

Effects of 3,5-Dibromo-4-Hydroxybenzonitrile (Bromoxynil) on Bioenergetics of Higher Plant Mitochondria (*Pisum sativum*)[1]

Michela Zottini, Valeria Scoccianti, and Davide Zannoni*

Department of Biology, University of Bologna, Via Irnerio 42, 40126 Bologna-I Italy (M.Z., D.Z.); and Institute of Botany, University of Urbino, Via Bramante 28, 61029 Urbino-I Italy (V.S.)

The herbicide bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) was tested on mitochondria from etiolated pea (*Pisum sativum* L. cv Alaska) stems. This compound when used at micromolar concentrations (≈20 μM) inhibited malate- and succinate-dependent respiration by intact mitochondria but not oxidation of exogenously added NADH. Bromoxynil did not affect the activities of the succinic and the internal NADH dehydrogenases. Analyses of the effects induced by this herbicide on the membrane potential, ΔpH, matrix Ca^{2+} movements, and dicarboxylate transport demonstrated that bromoxynil is likely to act as an inhibitor of the dicarboxylate carrier. In addition, bromoxynil caused a mild membrane uncoupling at concentrations ≥20 μM. No effect on the ATPase activity was observed.

BX (3,5-dibromo-4-hydroxybenzonitrile) is a contact herbicide widely used by postemergence application to control seedling broad-leaved weeds in cereal crops (genera *Triticum, Oryza, Sorghum, Zea*), flax (*Linum vulgaris*), garlic (*Allium sativum*), onion (*Allium cepa*), and in newly sown turf (Worthing and Hance, 1991). BX belongs to the group of hydroxybenzonitriles, classified as herbicides with a broad spectrum of activity because they act in both photosynthesis and respiration (Worthing and Hance, 1991). Indeed, BX has been reported to bind isolated PSII reaction centers (Giardi et al., 1988), to inhibit respiratory electron transport (Worthing and Hance, 1991), and to be effective in uncoupling oxidative phosphorylation within plant tissues (Carpenter and Heywood, 1963; Wain, 1963). To our knowledge, however, there have been no reports about the specific effects of BX on isolated higher plant mitochondria. In this respect, we have examined the effects of variable concentrations of this herbicide on several bioenergetics parameters of plant mitochondria from etiolated pea (*Pisum sativum* L. cv Alaska) stems. The present work demonstrates that BX is a mild uncoupling agent of the mitochondrial Δψ and an inhibitor of the dicarboxylate carrier. Our conclusions with isolated plant mitochondria support and extend previous observations in intact plant tissues.

[1] This research was supported by Consiglio Nazionale delle Ricerche of Italy (Bilateral Project U.K./I).

* Corresponding author; fax 39–51–242576.

MATERIALS AND METHODS

Plant Material, Isolation of Mitochondria, and SMP

Etiolated pea (*Pisum sativum* L. cv Alaska) stems were obtained by growing plants for 7 to 8 d in the dark at 25°C and 70% RH. Mitochondria were isolated from pea stems as described in detail by Zottini and Zannoni (1993). SMP were prepared by following the sonication method as described by Moore and Proudlove (1983).

Assays

The Δψ was monitored by following the fluorescence signal variations of the lipophilic cation safranine 0 according to Zottini et al. (1993). All experiments were carried out at 28°C in a reaction assay consisting of 0.8 mg of mitochondrial proteins resuspended in 2 mL of 300 mM mannitol, 20 mM Hepes (pH 7.2).

The ΔpH was measured in SMP using the fluorescent dye ACMA (excitation = 410 nm, emission = 500 nm). All determinations were performed at 28°C with 0.1 mg of SMP resuspended in 2 mL of 300 mM mannitol, 50 mM glycyl-Gly (pH 8.2), 1 mM $MgCl_2$, 50 mM KCl, 0.1 mg/mL valinomycin, and 5 μM ACMA. The signal was quantified according to Huang et al. (1983) and Rottemberg (1979), assuming an SMP internal volume of 1.5 μL mg^{-1} protein as determined by Papa et al. (1973).

The free mitochondrial matrix Ca^{2+} was monitored using the fluorescent probe fluo-3 (Merritt et al., 1990). Fluo-3 fluorescence was measured in a Jasco (Easton, MD) FP-770 spectrofluorimeter at 505 nm excitation, 530 nm emission. Quantitative matrix Ca^{2+} determinations were performed as described by Williams (1990). Mitochondria, suspended at the final concentration of 3 mg/mL in 300 mM mannitol, 20 mM Hepes buffer (pH 7.2), 0.1% BSA, were loaded for 20 to 30 min at 29°C with 5 μM fluo-3/AM. The ATP hydrolysis was detected with the use of the ammonium molybdate reagent as described by Dufour et al. (1988).

O_2 uptake was measured in a Yellow Springs (Yellow Springs, OH) model 5331 oxygen electrode at 28°C. The

Abbreviations: ACMA, 9-amino-3-chloro-7-methoxyacridine; BX, bromoxynil; Δψ, membrane potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; NEM, *N*-ethylmaleimide; SMP, submitochondrial particles.

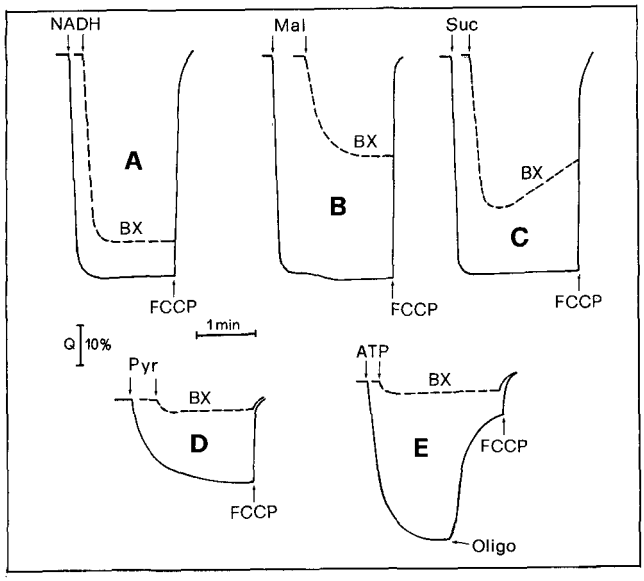

Figure 1. Generation of a mitochondrial $\Delta\psi$ as detected by the quenching of safranine 0 fluorescence coupled to NADH (A), malate (B), succinate (C), and pyruvate oxidations (D) and ATP hydrolysis (E) in the presence (interrupted line) or in the absence (continuous line) of BX. Pea stem mitochondria (0.265 mg/mL) were suspended in 0.3 M mannitol, 20 mM Hepes (pH 7.4), and 1 mM $MgCl_2$. Safranine 0 was added at a final concentration of 6 μM corresponding to 24 nmol mg^{-1} protein. Additions were succinate (Suc, 2 mM), malate (Mal, 10 mM), NADH (0.5 mM), pyruvate (Pyr, 5 mM), ATP (1 mM), and BX (8 μM). Where indicated, FCCP (360 ng mg^{-1} protein) and oligomycin (Oligo, 10 ng mg^{-1} protein) were added. The traces represent typical results of five different mitochondrial preparations.

suspension medium contained 0.3 M mannitol, 20 mM Hepes buffer/0.1% BSA (pH 7.2), and 0.5 to 1 mg/mL mitochondrial proteins.

Mitochondrial swelling as an indicator for metabolite uptake was measured by the addition of 50 to 60 μL of mitochondria (corresponding to about 1 mg of protein) to a 2-mL suspension containing 5 mM Tes buffer (pH 7.4), 0.1 mM EGTA, 1 μM antimycin A, and 100 mM NH_4^+ salt of the dicarboxylate indicated. When indicated, 5 mM NH_4^+ phosphate or 5 μM valinomycin was added. The decrease of A_{546} (at 25°C) was measured with an Hitachi photometer. NEM, mersalyl, and BX were preincubated for 2 min with mitochondria before the addition of phosphate. Protein content was determined by the Bradford procedure (Bradford, 1976) with BSA as the standard. BX (Aldrich Chemie) was dissolved in ethyl alcohol (99.7%).

RESULTS AND DISCUSSION

Effects of BX on $\Delta\psi$ and ΔpH

To define the effect of BX on bioenergetics of pea stem mitochondria, the $\Delta\psi$ and ΔpH values generated by the oxidation of NADH, pyruvate, malate, succinate, and by ATP hydrolysis were examined. Figure 1 (dotted traces) shows that low concentrations of BX (10 μM) did not affect significantly the $\Delta\psi$ induced by oxidation of external NADH (trace A), but that this herbicide was quite effective in inhibiting the $\Delta\psi$ with malate and pyruvate as respiratory substrates (traces B and D) and also when the $\Delta\psi$ was generated by ATP hydrolysis (trace E). Notably, the inhibitory effect of BX on the $\Delta\psi$ generated by succinate respiration (trace C) was time dependent and was evident only after 2 min of the substrate addition. The reason for this phenomenon is unknown. The severe inhibition of $\Delta\psi$ generated by ATP hydrolysis (trace E) might be due to uncoupling properties of BX, as proposed by Wain (1963), and/or to inhibition of the ATPase complex.

To clarify this point, the ATPase activity by pea stem SMP was tested as a function of variable concentrations (from 5–20 μM) of BX. The results (not shown) indicated that BX does not inhibit the ATPase activity of pea stem mitochondria to support the role of BX as uncoupling agent. These data, along with those of Figure 1, suggest that the herbicidal activity of BX in isolated plant mitochondria is due to both uncoupling of oxidative phosphorylation (trace E), as previously observed in intact plant tissues (Wain 1963), and to inhibition of respiration (traces in B, C, and D). This inhibition, however, does not seem to be located at the electron transport level, because oxidation of cytosolic NADH is not affected by BX (trace A, see also below).

On the other hand, the striking difference between the effects of BX on $\Delta\psi$ generated by NADH and the effects observed with malate, succinate, and pyruvate as respiratory substrates and with ATP (traces B, C, D, and E) might be explained by a higher efficiency of the NADH-dependent respiratory system in maintaining a consistent $\Delta\psi$ in the presence of 10 μM BX (see Fig. 5). The pattern of the mitochondrial $\Delta\psi$ generated by NADH (■), malate (△), and succinate (●) as a function of increasing concentrations of BX is shown in Figure 2. It is noteworthy that the values reported were operationally taken after 2 min of the substrate addition and in the presence of the protonophore nigericin (50 ng/mg protein) to emphasize the effect of BX on $\Delta\psi$. Apparently,

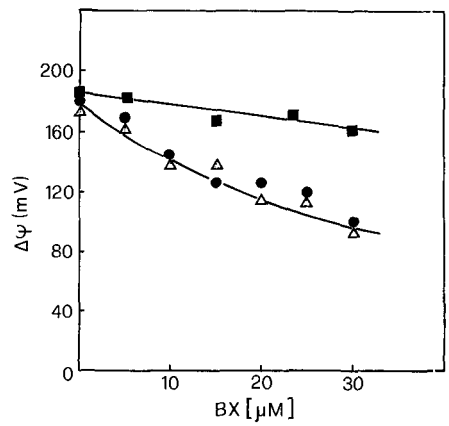

Figure 2. Mitochondrial $\Delta\psi$ as a function of increasing concentrations of BX. Mitochondria (0.265 mg/mL) were energized by oxidation of 0.2 mM NADH (■), 2 mM succinate (●), or 10 mM malate (△). Conditions were as for Figure 1 except for the presence of nigericin (50 ng mg^{-1} protein) in the assay before substrate addition (see text for further details).

the $\Delta\psi$ was strongly repressed (90-mV drop) by BX only with malate or succinate as respiratory substrates, whereas the $\Delta\psi$ generated by NADH oxidation was only slightly inhibited (20-mV decrease) by 15 μM BX. To fully analyze the effect of BX on the electrochemical proton gradient, the mitochondrial ΔpH (expressed as ZΔpH) generated by ATP hydrolysis as a function of variable BX concentrations was also tested. As shown in Figure 3, a considerable inhibition of the ZΔpH (from 210 to 160 mV) that follows ATP hydrolysis by SMPs becomes evident only at 30 μM BX. This latter result suggests that BX is a poor proton translocator when used at concentrations $\leq$10 μM.

Effects of BX on Mitochondrial Ca^{2+} Movements

It has been demonstrated that plant mitochondrial Ca^{2+} uptake is strongly repressed by uncoupling agents and by the sulfhydryl-inhibitor group reagent mersalyl (Rugolo et al., 1990; Silva et al., 1992). Figure 4 illustrates the effects of BX on mitochondrial Ca^{2+} transport as monitored by the fluorescent dye fluo-3. Both succinate and NADH were able to raise the matrix free Ca^{2+} concentration from a basal value of approximately 150 to 170 nM to a maximal value of approximately 300 nM with NADH (trace a) and 280 nM with succinate (trace d). However, although the Ca^{2+} uptake was drastically repressed by the uncoupling agent FCCP with both substrates (traces c and f), the herbicide BX (5 μM) inhibited the free Ca^{2+} movements only in the presence of succinate (compare trace b, NADH, with trace e, succinate). Thus, in contrast to FCCP, the uncoupling effect of 5 μM BX does not seem to be sufficient to inhibit Ca^{2+} uptake by pea stem mitochondria.

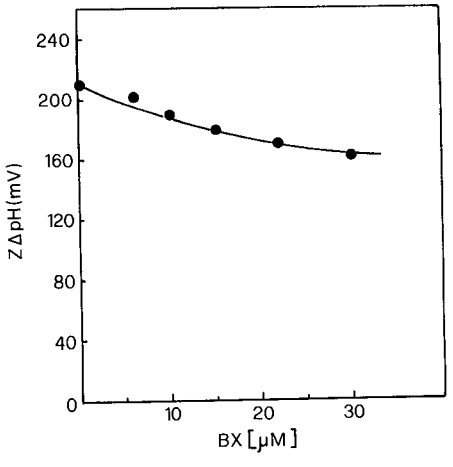

Figure 3. Effect of BX on the ΔpH as determined by distribution of the fluorescent dye ACMA in pea stem SMP energized by ATP hydrolysis. Data are expressed as ZΔpH (mV). SMP (0.3 mg/mL) were incubated in 0.3 M mannitol, 50 mM glycil-Gly (pH 7.8), 1 mM $MgCl_2$, 50 mM KCl, and valinomycin (330 ng mg^{-1} protein). ACMA was used at a concentration of 5 μM (corresponding to 17 nmol mg^{-1} mitochondrial protein).

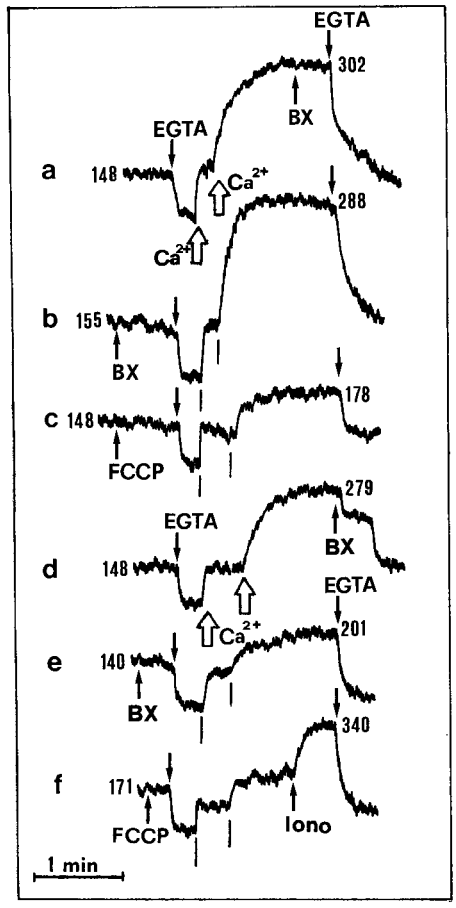

Figure 4. Effects of BX on Ca^{2+} movements into fluo-3-loaded mitochondria from *P. sativum* stems. Fluo-3-loaded mitochondria (0.3 mg/mL) were resuspended in 2 mL of 0.3 mannitol, 20 mM Hepes (pH 7.2). Respiratory substrates were NADH (0.5 mM; experiments in a, b, and c) and succinate (2.5 mM; experiments in d, e, and f). Where indicated (arrows from the top to the bottom), EGTA (first addition, 1 mM; second addition, 5 mM), BX (3 μM), FCCP (0.75 μM), ionomycin (Iono, 2 μM), and Ca^{2+} (open large arrows and vertical bars below, two additions up to 0.5 mM external free Ca^{2+}) were added. Numbers along the traces represent the concentrations of mitochondrial matrix-free Ca^{2+} (nM).

Effects of BX on Respiratory Activities in Intact Mitochondria and SMPs

In Figure 5 the polarographic traces of malate, succinate, and NADH oxidations by pea stem mitochondria in the presence or in the absence of various inhibitors are shown. It is apparent that a significant (10–30%) amount of respiration insensitive to antimycin A but sensitive to salicylhydroxamic acid is present (traces B and D); in addition, a certain effect of BX (20 μM) on succinate and malate oxidation is seen (37 and 30% inhibition, respectively; traces B and G), whereas the respiratory activity catalyzed by exogenously added NADH was 22% stimulated by this herbicide (trace E). A very low respiratory activity (BX insensitive, not shown) was observed with pyruvate, whereas no respiration was seen with Gly (not shown). In this latter case, the lack of Gly-

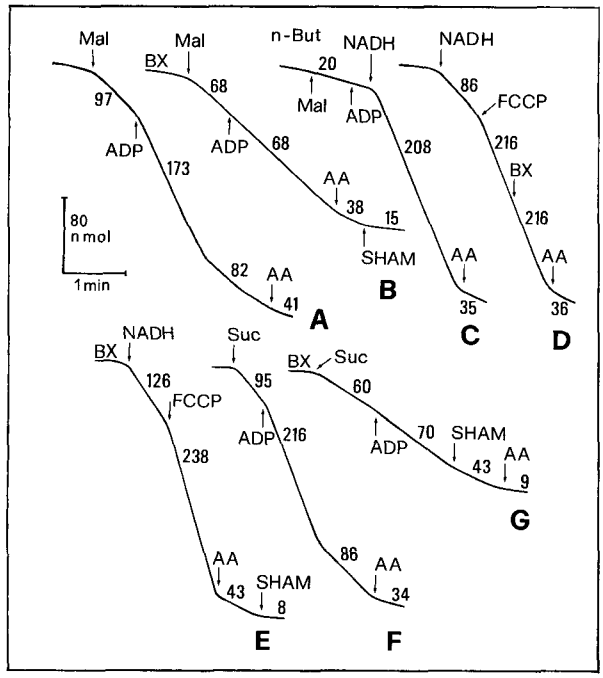

Figure 5. Oxidation of malate (A, B, and C), NADH (D and E), and succinate (F and G) by pea stem mitochondria as a function of various inhibitors. Assay conditions were as described in "Materials and Methods." Additions and abbreviations: antimycin A (AA, 1 μM), FCCP (360 ng mg^{-1} protein), *n*-butylmalonate (n-But, 10 mM), salicylhydroxamic acid (SHAM, 1 mM), succinate (Suc, 5 mM), malate (Mal, 10 mM), NADH (0.5 mM), ADP (224 μM), BX (8 μM). In A, B, and C, thiamine pyrophosphate (0.2 mM) and NAD (0.1 mM) were also present. Numbers along the traces are nmol O_2 min^{-1} mg^{-1} protein.

dependent respiration might be due to the fact that Gly-decarboxylase is light induced and that it is virtually absent in mitochondria from etiolated plant tissues (Kromer and Heldt, 1991). Figure 5 (trace C) also shows that malate respiration is 80% inhibited by *n*-butylmalonate; conversely, the sulfhydryl reagents NEM and mersalyl were slightly effective in inhibiting malate respiration (30 and 35% inhibition, respectively) (not shown).

It has previously been established that succinic and NAD(P)H dehydrogenases deliver reducing equivalents to the ubiquinone/bc_1 oxidoreductase complex (Møller and Lin, 1986). Thus, the results of Figure 5 suggest that the inhibition of malate and succinate respiration by BX might reflect two possible sites of inhibition: (a) the succinic and NADH (internal) dehydrogenase complexes, and/or (b) the mitochondrial dicarboxylate carrier. To select between these possibilities we tested the oxidative activities by SMP to avoid the involvement of the dicarboxylate carrier. The results shown in Table I indicate that the succinate dehydrogenase (measured as 2,6-dichloroindophenol reduction in the presence of both 1 μM antimycin A and 1 mM CN^-) and the NADH oxidation are insensitive to BX, whereas the succinate-Cyt *c* oxidoreductase and succinate oxidase activities are slightly (10–12%) inhibited by BX. This latter result might be due to contribution of

Table I. *Respiratory activities of pea stem SMP*

The data are the mean values of two preparations. Additions: NADH (0.2 mM); succinate (2 mM); EGTA (1 mM); rotenone (Rot, 24 μM); BX (10 μM); horse-heart Cyt c (50 μM); 2,6-dichloroindophenol (DCIP, 0.3 mM).

Electron Donor and Additions	Electron Acceptor	Rate[a]	Percent of Control
NADH	O_2	3.1	100
EGTA	O_2	2.5	80
EGTA, Rot	O_2	1.7	55
BX	O_2	3.1	100
Succinate	O_2	2.2	100
BX	O_2	1.9	90
Succinate	Cyt C[b]	47.4	100
BX	Cyt C[b]	41.7	88
Succinate	DCIP[c]	156.5	100
BX	DCIP[c]	156.5	100

[a] Expressed as μmol of substrate reduced h^{-1} mg^{-1} protein. [b] Measured in the presence of 1 mM CN^-. [c] Measured in the presence of 1 mM CN^- plus 1 μM antimycin A.

"right-side-out" SMP, as indicated by 20% EGTA-sensitive NADH respiration that is possibly linked to the activity of the external Ca^{2+}-dependent NADH dehydrogenase (Møller et al., 1981; Rugolo et al., 1991). Therefore, these data tend to restrict the possible inhibitory site of BX on the mitochondrial dicarboxylate carrier.

Metabolite Uptake

To test the effect of BX on the dicarboxylate carrier, we examined the permeability to NH_4^+ malate. Pea stem mitochondria did not swell significantly when added to a 100-

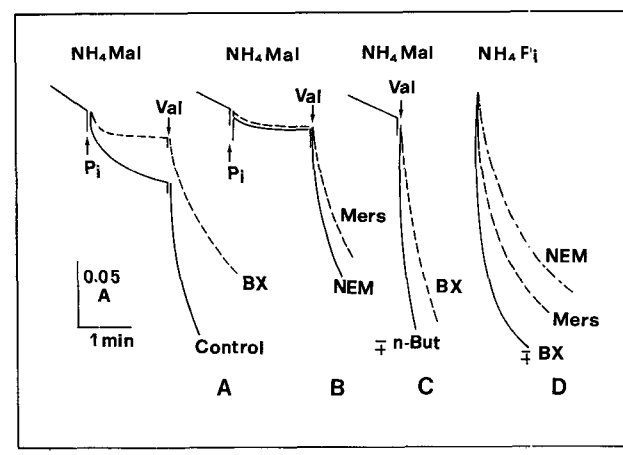

Figure 6. Swelling of pea stem mitochondria in 100 mM NH_4^+ malate (A, B, and C) and NH_4^+ phosphate (D). When indicated, 5 mM NH_4^+ phosphate (NH_4P_i) or 5 μM valinomycin (Val) were added. The swelling was initiated by the addition of mitochondria. Inhibitor abbreviations and concentrations: BX (20 μM), mersalyl (Mers, 20 μM), NEM (2 mM), *n*-butylmalonate (n-But, 10 mM). In C and D, ∓n-But and ∓BX indicate that the control was not affected by *n*-butylmalonate or BX, respectively. Further details are described in "Materials and Methods."

mM NH_4^+ malate solution (Fig. 6A, continuous line), and this little swelling was only slightly enhanced by addition of phosphate. Conversely, a rapid and consistent swelling was induced by subsequent addition of 5 μM valinomycin. This demonstrates that in pea stem mitochondria the major route of malate uptake is not by malate/phosphate antiport, as in mammalian mitochondria (Chappel and Haarhoff, 1967), but by electrogenic uniport. Notably, our results confirm previous observations in mitochondria from pea leaves and etiolated pea shoots (Zoglowek et al., 1988). By including BX in the medium, an inhibition of both Pi- and valinomycin-dependent malate uptake (interrupted traces) was seen; conversely, the effect of the sulfhydryl group inhibitors NEM or mersalyl was more evident on transport of malate induced by Pi (upper part of traces in Fig. 6B), although some effect on valinomycin-induced malate uptake was apparent with mersalyl (Fig. 6B, interrupted trace). Interestingly, *n*-butylmalonate, a strong inhibitor of the Pi-dependent malate uptake in mitochondria from mammalian tissues (Palmieri et al., 1971) and also of the malate- and succinate-dependent respiration of pea stem mitochondria (Fig. 5), did not affect significantly the valinomycin-dependent uptake of malate (Fig. 6C, continuous trace). This result, which confirms previous observations in mitochondria from etiolated pea shoots (Zoglowek et al., 1988), might be due to far less sensitivity of the plant mitochondrial dicarboxylate/carrier complex toward this substrate analog. Indeed, equimolar concentrations of substrate and inhibitor are generally required to inhibit the dicarboxylate transport in plant tissues (Day and Wiskich, 1984).

These findings suggest that BX inhibits to some extent the dicarboxylate carrier, whereas NEM and mersalyl, which are potent inhibitors of the phosphate transport, have only a secondary effect on the phosphate/malate antiport of pea stem mitochondria. This suggestion was confirmed by examining the effect of these inhibitors on swelling in NH_4^+ phosphate. Ammonium phosphate induces a rapid swelling of plant and mammalian mitochondria (Chappel and Haarhoff, 1967; Zoglowek et al., 1988). Traces in Figure 6D indicate that swelling of pea stem mitochondria was inhibited by mersalyl and NEM but not by BX.

CONCLUSIONS

The present work unequivocally demonstrates that the herbicide BX has a dual effect on bioenergetics of higher plant mitochondria. (a) It acts as inhibitor of the dicarboxylate carrier, since the electron transport activities catalyzed by malate and succinate are inhibited by this herbicide only in intact mitochondria. Indeed, succinate respiration in SMP is not affected by BX. In addition, the $\Delta\psi$ generated by malate and succinate oxidation, but not by NADH oxidation, is decreased by BX. We have also been able to demonstrate that the accumulation of Ca^{2+} ions into the matrix space is strongly repressed when mitochondria are energized by succinate respiration but not by NADH respiration. Analysis of the mitochondrial permeability in isoosmotic solution of NH_4^+ malate has demonstrated that swelling of the mitochondria due to uptake of malate is rate limited by BX, which confirms its effect on the dicarboxylate carrier. (b) BX, when used at concentrations ≥20 μM, also affects the mitochondrial $\Delta\psi$ by acting as a mild protonophore. This latter conclusion is in line with evidence that the NADH-dependent respiration in intact mitochondria is 22% stimulated by this herbicide. Conversely, under uncoupled respiratory state-3 (0.15 μM FCCP), addition of BX does not affect the rate of NADH respiration (not shown).

The results and conclusions summarized in points a and b partially support and extend an earlier report by Wain (1963) indicating that the herbicidal capacity of BX is due to uncoupling of oxidative phosphorylation within plant tissues. On the other hand, it is important to note that the herbicide, after its application as a spray, is extensively metabolized by growing plant tissues (Buckland et al., 1973a, 1973b). Studies on metabolism of hydroxybenzonitriles in growing cereal crops have demonstrated that BX undergoes a complex metabolic pathway resulting from initial hydrolysis of the cyano groups to the amide and carboxylic acid with subsequent decarboxylation to 2,6-dibromphenol and debromination (Buckland et al., 1973b). Although the metabolites identified in this latter study represented only a small proportion (11%) of the herbicide applied, it is evident that the actual herbicidal properties of the molecule might be considerably masked by the simultaneous presence of several metabolic products. On this basis, it is presumable that the reported effects of BX on respiratory electron transport within plant tissues (Worthing and Hance, 1991) are not due to the molecule itself (see Table I) but, most probably, to its metabolic products. In fact, the present work demonstrates that the mitochondrial oxidative metabolism is damaged by BX through inhibition of the mitochondrial dicarboxylate carrier and uncoupling of the membrane electrochemical potential.

ACKNOWLEDGMENTS

We wish to thank E. Manaresi and P. Politi for their skillful assistance during the course of the mitochondrial isolation and purification procedures.

Received July 6, 1994; accepted August 16, 1994.

LITERATURE CITED

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Buckland JL, Collins RF, Henderson MA, Pullin EM (1973a) Radiochemical distribution and decline studies with bromoxynil octanoate in wheat. Pestic Sci **4:** 689–700

Buckland JL, Collins RF, Pullin EM (1973b) Metabolism of bromoxynil octanoate in growing wheat. Pestic Sci **4:** 149–162

Carpenter K, Heywood BJ (1963) Herbicidal action of 3,5-dialogeno-4-hydroxybenzonitriles. Nature **200:** 28–29

Chappel JB, Haarhoff KH (1967) The penetration of the mitochondrial membrane by anions and cations. *In* EC Slater, Z Kaniuga, L Wojtczak, eds, Biochemistry of Mitochondria. Academic Press, London, pp 75–91

Day DA, Wiskich JT (1984) Transport processes of isolated plant mitochondria. Physiol Veg **22:** 241–261

Dufour J-P, Amory A, Goffeau A (1988) Plasma membrane ATPase from yeast *Schizosaccharomyces pombe*. Methods Enzymol **157:** 511–533

Giardi MT, Marder, Barber J (1988) Herbicide binding to the isolated photosystem II reaction centre. Biochim Biophys Acta **934:** 64–71

Huang CS, Kopacz SJ, Lee CP (1983), Mechanistic differences in the energy-linked fluorescence decreases of 9-aminoacridine dyes associated with bovine heart submitochondrial membranes. Biochim Biophys Acta **722:** 107–115

Kromer S, Heldt HW (1991) Respiration of pea leaf mitochondria and redox transfer between the mitochondria and extramitochondrial compartment. Biochim Biophys Acta **1057:** 42–50

Merritt JE, McCarthy SA, Davies PA, Moores KE (1990) Use of Fluo-3 to measure cytosolic Ca^{2+} in platelets and neutrophils. Biochem J **269:** 513–519

Møller IM, Johnston SP, Palmer JM (1981) A specific role for Ca in the oxidation of exogenous NADH by Jerusalem artichoke (*Helianthus tuberosus*) mitochondria. Biochem J **194:** 487–495

Møller IM, Lin W (1986) Membrane-bound NAD(P)H dehydrogenases in higher plant cells. Annu Rev Plant Physiol **37:** 309–334

Moore AL, Proudlove MO (1983) Mitochondria and sub-mitochondrial particles. In JL Hall, AL Moore, eds, Isolation of Membranes and Organelles from Plant Cells. Academic Press, London, pp 153–184

Palmieri F, Prezioso G, Quagliariello E, Klingenberg M (1971) Kinetic study of the dicarboxylate carrier in rat liver mitochondria. Eur J Biochem **22:** 66–74

Papa S, Guerrieri F, Simone S, Lorusso M, La Rosa D (1973) Mechanism of respiration-driven proton translocation in the inner mitochondrial membranes. Biochim Biophys Acta **292:** 20–38

Rottemberg H (1979) The measurement of membrane potential and ΔpH in cells, organelles and vesicles. Method Enzymol **55:** 547–569

Rugolo M, Antognoni F, Flamigni A, Zannoni D (1991) Effects of polyamines on the oxidation of exogenous NADH by Jerusalem artichoke (*Helianthus tuberosus*) mitochondria. Plant Physiol **95:** 157–163

Rugolo M, Pistocchi R, Zannoni D (1990) Calcium transport in higher plant mitochondria (*Helianthus tuberosus*). Physiol Plant **79:** 297–302

Silva MAP, Carnieri EGS, Vercesi AE (1992) Calcium transport by corn mitochondria. Evaluation of the role of phosphate. Plant Physiol **98:** 452–457

Wain RL (1963) 3, 5-Dihalogeno-4-hydroxybenzonitriles: new herbicides with molluscidial activity. Nature **200:** 28

Williams DA (1990) Quantitative intracellular calcium imaging with laser-scanning confocal microscopy. Cell Calcium **11:** 589–597

Worthing CR, Hance RJ (1991) The Pesticide Manual. The British Crop Protection Council, Farnham, pp 100–101

Zoglowek C, Kromer S, Heldt HW (1988) Oxalacetate and malate transport by plant mitochondria. Plant Physiol **87:** 109–115

Zottini M, Mandolino G, Zannoni D (1993) Oxidation of external NAD(P)H by mitochondria from taproots and tissue cultures of sugar beet (*Beta vulgaris*). Plant Physiol **102:** 579–585

Zottini M, Zannoni D (1993) The use of Fura-2 fluorescence to monitor the movement of free calcium ions into the matrix of plant mitochondria (*Pisum sativum* and *Helianthus tuberosus*). Plant Physiol **102:** 573–578