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BIOLOGICAL ACTIVITY OF THE TWO GEOMETRICAL ISOMERS OF METHOMYL ON MAIZE MITOCHONDRIA

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Key Word Index—*Zea mays*; Gramineae; maize; male sterility; mitochondria; *Helminthosporium maydis* race T toxin; Methomyl; geometric isomery; specific biological activity.

Abstract—Methomyl (*S*-methyl-*N*[(methylcarbamoyl)oxy]thioacetimidate), the active ingredient in lannate insecticide, gave a geometrical isomer after acid hydrolysis. An X-ray study showed that Methomyl and its isomer were respectively (*Z*)- and (*E*)-isomers. (*Z*)-Methomyl was active only on mitochondria from maize with a T (male sterile) cytoplasm: it stimulated NADH-sustained respiration and inhibited malate-sustained respiration. (*E*)-Methomyl showed no specificity and inhibited the oxidation of both substrates in mitochondria from both T and N (male fertile) maize. The specificity of the biological activity was related without ambiguity to the molecule conformation.

INTRODUCTION

Maize (*Zea mays* L.) with a mitochondrial gene for male sterility (Texas male sterile cytoplasm, or T) is highly susceptible to *Helminthosporium maydis* race T (HmT). A host-specific pathotoxin produced by HmT was purified and showed the same specificity as HmT [1]. A growing body of evidence suggests that mitochondria are a site of toxin action [2]. The insecticide Methomyl (*S*-methyl-*N*[(methylcarbamoyl)oxy]thioacetimidate) was found to mimic the action of HmT toxin both in plants [3] and on isolated mitochondria [4].

Purified Methomyl shows toxic and specific action against isolated mitochondria and on sprouting of corn seeds with a Texas cytoplasm [5]. We also carried out studies on the activity of Methomyl analogues on these two systems and found that the efficiency of Methomyl is governed by very stringent structural requirements and the extent of its electron conjugated system [6, 7].

The synthesis of carbamates gives in most cases two geometric isomers which cannot be separated by chromatography [7]. We have developed an alternative method involving acid hydrolysis of Methomyl which yields thiomethyl-methylsulfonate [8] and an unknown compound isolated with a 0.1–0.2% yield. The latter and commercial Methomyl were identified by X-ray analysis as (*E*)- and (*Z*)-isomers, respectively. The (*E*)-isomer is stable in any solution and can be recovered from solution without isomeric modification.

We have studied their action of N and T mitochondria to see whether the two isomers have the same biological activity.

RESULTS

Characterization of (E)-*Methomyl*

Spectroscopic data, in particular ^{1}H NMR and ^{13}C NMR data, indicated that the compound we obtained after acid hydrolysis of Methomyl is one of the two geometric isomers of Methomyl (Table 1). It is well known that the equilibrium $(Z) \rightleftharpoons (E)$ of diastereomeric carbamates is displaced to the left towards the (*Z*)-isomer which is more energetically favoured [9].

However, because of several rotational isomerisms, these spectroscopic data could not give precise indications about the structure of the two isomers. Single crystals of the (*E*)- and (*Z*)-isomers were examined by X-ray analysis. The final atomic parameters, including anisotropic thermal parameters, bond lengths and angles, and deviations of the atoms from molecular mean planes, for both isomers have been deposited at the Cambridge Crystallographic Centre. Figure 1 shows a perspective drawing of the two molecules. The geometry of the two molecules is clearly visible. In the (*E*)-isomer, the methyl group C-8 is slightly rotated out of the best molecular plane (0.16 Å), probably due to intermolecular forces. In the (*Z*)-isomer, the vicinity of the methyl groups C-8 and C-9 causes the aperture of the angle S–C(-7)–C(-9) (122°) and the tightening of N(-6)–C(-7)–H(-9) (115°). The common feature is the existence of an intramolecular hydrogen bond H-2 . . .N-6 [2.54 Å in (*Z*) and 2.57 Å in (*E*)], which contributes in both molecules to the resonance of the planar system, and consequently its rigidity.

Action of (E)- *and* (Z)-*Methomyl on* F_7T *and* F_7N *mitochondria*

Respiratory studies. A striking feature of (*E*)-Methomyl action was its ability to inhibit both electron transfer and phosphorylation in F_7N mitochondria, with all substrates tested (Fig. 2). Electron transfer was partially inhibited whereas phosphorylation was completely inhibited as shown by the lack of effect of a subsequent addition of ADP. CCCP (1 μM) could partly relieve the inhibition of NADH oxidation (Fig. 2A). Conversely, 5 mM (*Z*)-Methomyl had no action on the oxidative properties of N mitochondria (data not shown).

Table 1. ^{1}H NMR and ^{13}C NMR spectral data for (*E*)- and (*Z*)-Methomyl ($CHCl_3$, TMS as internal standard)

	^{1}H NMR	^{13}C NMR
(*Z*)-Methomyl*	2.18 (*s*, 3H, Me-C)	13.4 (*q*, MeC)
	2.33 (*s*, 3H, Me-S)	18.9 (*q*, MeS-)
	2.81 (*d*, J = 4.8 Hz, MeNH)	27.6 (*q*, MeNH)
	5.88 (*m*, 1H, =NH)	155.2 (*s*, C=N)
		160.7 (*s*, C=O)
(*E*)-Methomyl	2.13 (*s*, 3H, Me-C)	13.4 (*q*, MeC)
	2.34 (*s*, 3H, Me-S)	18.2 (*q*, MeS)
	2.85 (*d*, J = 4.8 Hz, MeNH)	27.7 (*q*, MeNH)
	6.03 (*m*, 1H, NH)	156.4 (*s*, C=N)
		160.7 (*s*, C=O)

*Previously published ^{13}C NMR data must be corrected [5].

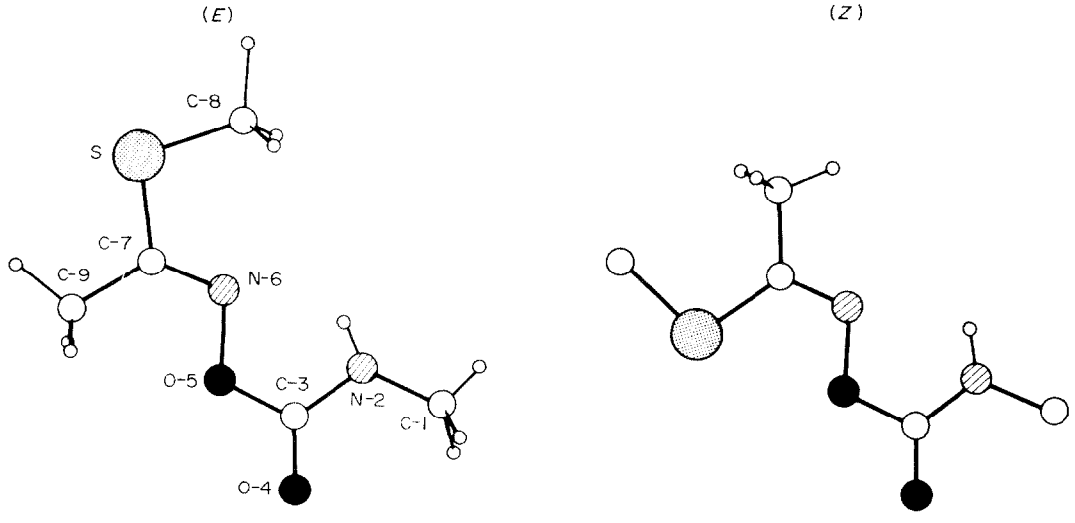

Fig. 1. Perspective drawing of the two geometrical isomers of Methomyl.

Figure 3 shows that the effects of (*E*)-Methomyl on F_7T mitochondria were the same as on those of F_7N— inhibition of both electron transfer and phosphorylation. (*E*)-Methomyl (5 mM) did not prevent further stimulation of NADH oxidation by 5 mM (*Z*)-Methomyl (Fig. 3A); however, the stimulation thus obtained was less than that brought about by (*Z*)-Methomyl alone. A lower (*E*)-Methomyl concentration had little effect: 22% stimulation of state 4 oxygen consumption and no conspicuous effect on either phosphorylation or state 3 (NADH was the substrate).

(*Z*)-Methomyl had the same effect on F_7T mitochondria as those already reported for Methomyl [4], namely stimulation of NADH (Fig. 3A) and succinate oxidations, and inhibition of malate oxidation (data not shown).

'Swelling studies'. Figure 4 shows the effects of (*E*)-and (*Z*)-Methomyl on the absorbance of a suspension of T mitochondria. Upon addition of (*Z*)-Methomyl a sharp decrease in absorbance followed by a slower decrease was observed in all three media (iso-osmotic mannitol, KCl and NH_4Cl solutions buffered at pH 7.2). By contrast, (*E*)-Methomyl brought about an initial absorbance increase followed by a quick decrease.

Figure 5 shows the effects of (*E*)- and (*Z*)-Methomyl on F_7N mitochondria. (*Z*)-Methomyl had no action. (*E*)-Methomyl triggered absorbance increases that were slower than with F_7T mitochondria. No subsequent absorbance decrease was observed in iso-osmotic mannitol. In iso-osmotic KCl and NH_4Cl a slow decrease occurred which was much slower than that observed with F_7T mitochondria.

DISCUSSION

Because of the method of preparation, we obtained only small amounts of (*E*)-Methomyl and thus could not carry out all the biochemical experiments that were necessary to characterize its action fully. Nevertheless, we can draw a few conclusions from the experiments performed.

(*Z*)-Methomyl has the same action on F_7T mitochondria as, what is commonly referred to as, 'Methomyl' (stimulation of NADH and succinate oxidations, inhibition of malate oxidation, decrease in the absorbance

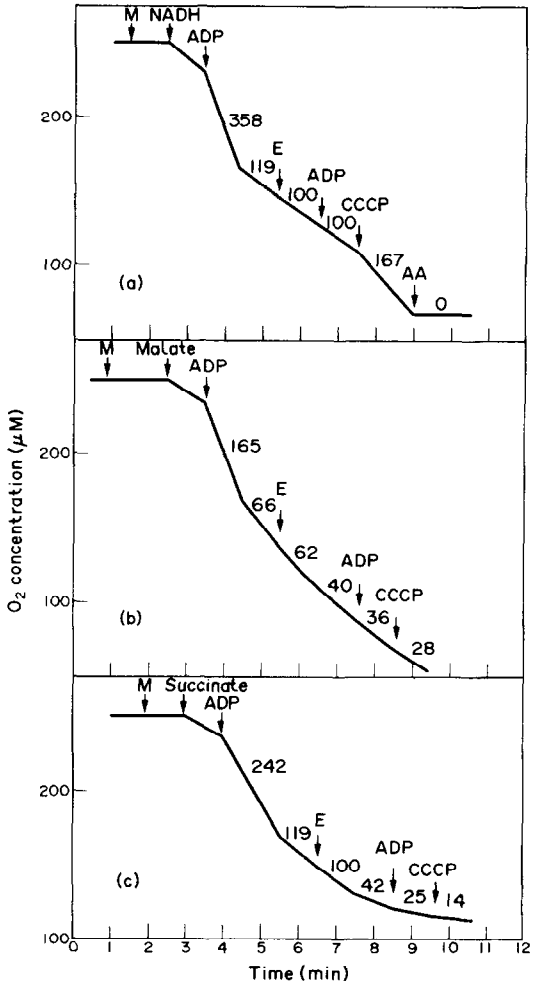

Fig. 2. Action of (*E*)-Methomyl on F_7N mitochondria oxidizing various substrates. Additions were: ADP, 0.4 μmol (except for malate, 0.6 μmol); (*E*)-Methomyl (*E*), 5 mM; antimycin A (AA), 0.5 μM. 0.36 mg mitochondrial protein (except for malate, 0.72 mg). The results presented in the figure come from one of three experiments which gave similar results.

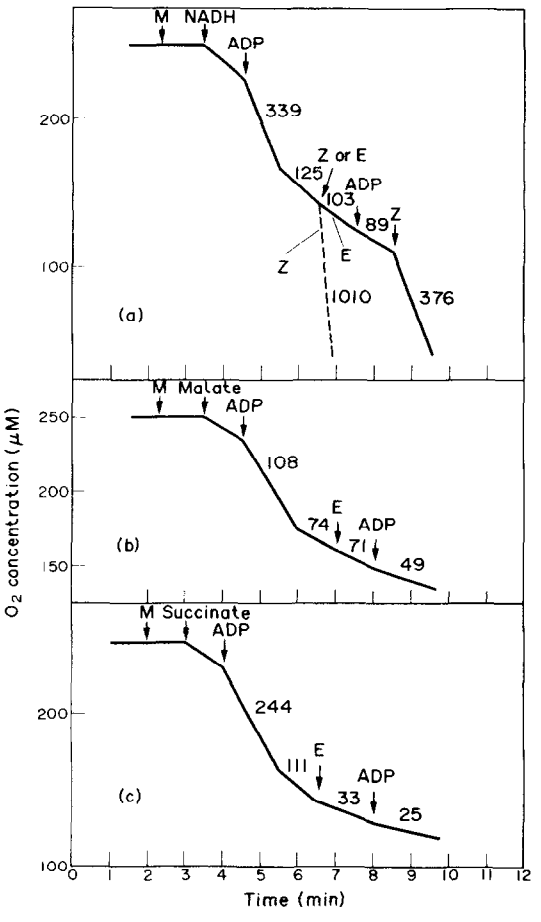

Fig. 3. Action of (*E*)-Methomyl on F_7T mitochondria oxidizing various substrates. Additions and protein concentrations were the same as in Fig. 2. (*Z*)-Methomyl (*Z*), 5 mM. The results presented in the figure come from one of three experiments which gave similar results.

of mitochondrial suspensions). This is as expected since (*Z*)-Methomyl is the major (> 99.5 %) isomeric form found in commercial 'Methomyl'. Similarly, the lack of effect of (*Z*)-Methomyl on F_7N mitochondria is as expected.

(*E*)-Methomyl action is strikingly different: (1) It is equally effective on F_7T and F_7N mitochondria and (2) it appears to act in a different way from (*Z*)-Methomyl since it does not stimulate (indeed inhibits) NADH oxidation and it triggers an increase (instead of a decrease) in the absorbance of mitochondrial suspensions.

We can infer that: (1) (*Z*)-Methomyl acts at the same active site as HmT toxin whereas (*E*)-Methomyl does not (the active site is present only in F_7T mitochondria); (2) (*E*)-Methomyl acts at another site which is present in both F_7T and F_7N mitochondria and with which (*Z*)-Methomyl does not interact.

The structure of (*Z*)-Methomyl shows that the arrangement of the O-4, O-5 and S-1 atoms defines an electron-rich region with great potency for extramolecular interactions. It is probably responsible for the interaction of (*Z*)-Methomyl with its active site in F_7T mitochondria. It explains why in our previous work [6] all Methomyl analogues in which the sulphur atom was removed had no Methomyl-like action. Conversely, substitutions at the other end of the molecule (C-1) retained some Methomyl activity (provided they were not too bulky) because they did not alter the O,O,S arrangement defined above. The (*E*)-Methomyl molecule does not contain this structure and this might explain why it does not have the selective action of (*Z*)-Methomyl.

The results of the respiratory studies show that (*E*)-Methomyl inhibits both electron transfer and phosphorylation in both F_7T and F_7N mitochondria. NADH oxidation is inhibited to a lesser extent than succinate and malate oxidations since CCCP relieves the inhibition of NADH oxidation but not the inhibition of succinate and malate oxidation. In agreement with this is the ability of (*Z*)-Methomyl to stimulate NADH oxidation after it has been inhibited by (*E*)-Methomyl. However, the stimulation brought about by (*Z*)-Methomyl in these conditions is less than when it is applied alone (Fig. 3A), indicating a significant inhibition of electron transfer by

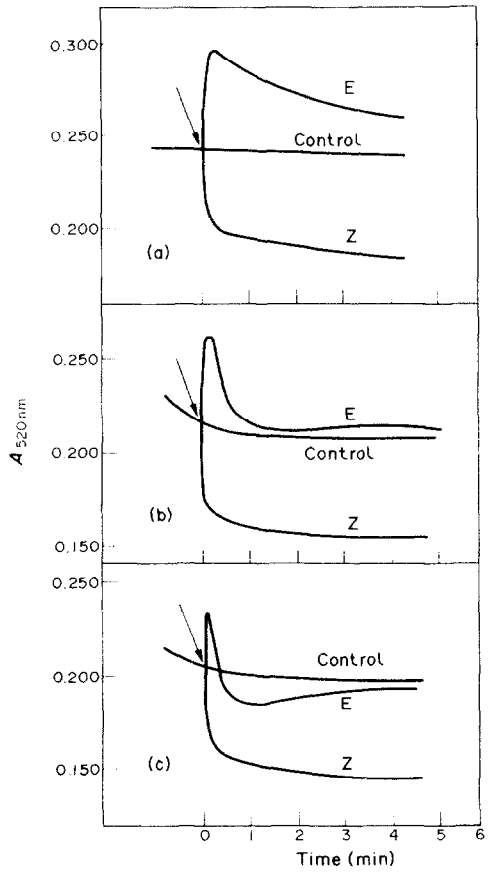

Fig. 4. Action of (*E*)- and (*Z*)-Methomyl on the absorbance of F_7T mitochondria suspensions. Suspension media are described in the text; A, iso-osmotic mannitol; B, iso-osmotic KCl; C, iso-osmotic NH_4Cl. Additions were 5 mM (*E*)- or (*Z*)-Methomyl. The results presented in the figure come from one of two experiments which gave similar results.

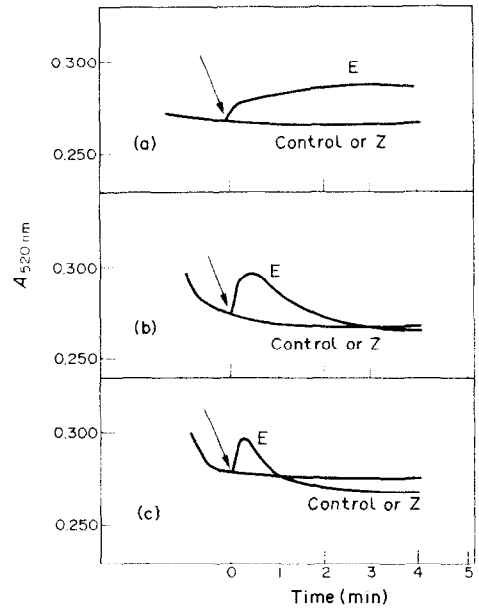

Fig. 5. Action of (*E*)- and (*Z*)-Methomyl on the absorbance of F_7N mitochondria suspensions. Suspension media (A, B, C) and additions were the same as in Fig. 4. The results presented in the figure come from one of two experiments which gave similar results.

(*E*)-Methomyl. At this stage, it is too early to suggest a mechanism for the multiple action of (*E*)-Methomyl.

When F_7T mitochondria suspended in either iso-osmotic mannitol, iso-osmotic NH_4Cl or iso-osmotic KCl are treated with (*E*)-Methomyl, a sharp decrease in absorbance is observed (Fig. 4). This action is similar to that of 'Methomyl' [6]. It is tempting to interpret it as the result of an increase in the mitochondrial membrane permeability to the osmoticant. Its entry into the mitochondria, together with that of osmolarity water, would trigger a swelling of the organelles and an absorbance decrease. However, Fig. 4 shows that treatment with (*E*)-Methomyl induces an increase in absorbance of the mitochondrial suspensions. It is difficult to explain why mitochondria would shrink in these conditions. Hence, it is more probable that (*Z*)- and (*E*)-Methomyl modify the optical density of F_7T mitochondria without necessarily inducing volume changes. Even though these results are difficult to interpret, they show that (*E*)- and (*Z*)-Methomyl have opposite initial effects on the absorbance of mitochondrial suspensions.

(*E*)-Methomyl has the same optical effects on F_7N mitochondria as on F_7T mitochondria (Fig. 5). However, the absorbance differences are less sharp. In this respect, (*E*)-Methomyl seems to be slightly more effective against F_7T mitochondria than against F_7N ones; but this is unimportant compared with the selectivity exhibited by (*Z*)-Methomyl (no action at all on F_7N mitochondria).

Finally, this study provides a clear-cut example in which the specificity of a biological action is related to the conformation of the molecule.

EXPERIMENTAL

^1H NMR (Jeol PMX-60 with internal lock): $CDCl_3$, TMS as int. standard. ^{13}C NMR (v/v internal reference). (Varian CFT-20): Fourier transform mode.

(*Z*)-*Methomyl.* Methomyl was isolated from a commercial soln of 'lannate' after removal of solvents *in vacuo*. It was recrystallized (× 3) from EtOH to give colourless crystals, mp 78.5–79.5°.

Synthesis of (E)-*Methomyl.* Purified Methomyl (10 g) was dissolved in 30 ml 15% chlorhydric soln. The soln was stirred at 50° for 5 hr, and then extracted with CH_2Cl_2. The CH_2Cl_2 was washed with H_2O, dried (K_2CO_3) and the solvent removed *in vacuo*. The resultant bad smelling oil (6.5 g) was dissolved in hexane and Et_2O. After recrystallization of Methomyl, the mother liquor was collected and chromatographed on silica gel H. Elution with hexane–Et_2O (1:4) followed by hexane–Et_2O (1:1) gave Me_2SO_2 and Methomyl with the majority of the new compound, respectively. TLC on silica gel HF 254 double-developed with Et_2O gave two spots, R_f 0.56 and R_f 0.25 (starting Methomyl). Chromatography of the material of R_f 0.56 on plates of HF 254 (1.25 mm), with Et_2O–EtOAc (9:1) gave 17 mg.

According to several expts, the yield of the first compound varied from 0.1 to 0.2%. Recrystallizations of the mixture from

hexane–Et_2O or hexane–Me_2CO gave crystals (mp 48–49°) which were used for the X-ray analysis.

X-ray studies. (a) *Common features.* Cell dimensions and intensity data were measured with a graphite monochromated 4-circle diffractometer, using CuKα ($\lambda = 1.5418$ Å) radiation. Diffraction data were recorded in the 0–68° theta range, using the $\omega/2\theta$ scan technique. The background was measured on both sides of every reflection during a time equal to the scan time. The acceptance level for the observed intensities was twice the estimated standard deviation based on counting statistics. Lorentz and polarization corrections were applied, but none for absorption. The structures were solved by direct methods [10] and refined by the full-matrix least-squares method [11], including the anisotropic thermal parameters for non-hydrogen atoms.

(b) *(Z)-Isomer.* Single crystals of the (*Z*)-isomer suitable for *X*-ray analysis were obtained from a soln of EtOH–H_2O (1:1). The size of the crystal used for the data collection was *ca* 0.08 × 0.3 × 0.4 mm^3. The crystals were monoclinic, $P2_1/n$, with $a = 13.964$ (7), $b = 9.662$ (5), $c = 6.083$ (4) Å, and $\beta = 91.51$ (8)°. The calculated specific gravity was $d_x = 1.313$ g/cm^3 for four molecules in the cell. From 1503 recorded reflections, 993 were observed. Amongst the 10 hydrogen atoms, only four were located on difference Fourier maps and included in structure factors calculations, but not refined. The final *R*-factor was 6.9%.

(E)-*Isomer.* Single crystals of this isomer were grown from a mixture hexane–Me_2CO (4:1). The crystal size was 0.6 × 0.7 × 0.3 mm^3. The space group was $P2_1/c$, with $a = 9.623$ (6), $b = 10.282$ (7), $c = 9.084$ (6) Å, and $\beta = 109.85$ (8)°. $d_x = 1.273$ g/cm^3 for $Z = 4$. From 1520 unique reflections, 951 were observed. All hydrogen atoms were located and the final *R*-factor was 8.6%.

Preparation of mitochondria. Mitochondria were isolated from epicotyls of F_7T (male sterile) and F_7N (male fertile) maize (*Zea mays* L.) lines. The plants were grown on eight layers of wet germination paper for 5 days at 25° in the dark. The epicotyls (*ca* 15 g fr. wt) were ground with a mortar and pestle in a medium consisting of 0.4 M mannitol, 0.1 M morpholinopropanesulphonate buffer (MOPS), 1 mM EGTA and 2 mg/ml bovine serum albumin, pH 7.6. After straining through four layers of cheesecloth, the homogenate was centrifuged at 2000 *g* for 10 min, and the resulting supernatant fraction at 10000 *g* for 10 min. The pellet from the second centrifugation was resuspended in grinding medium, then centrifuged at 10000 *g* for 10 min. The resulting pellet contained 50–80 mg protein/ml and was kept on ice under an argon stream. Argon was used to prevent the progressive loss of sensitivity of T mitochondria to HmT toxin due to ageing in the presence of O_2: Pham and Gregory [12] showed that N_2 or dithiothreitol prevent this.

Assay of the two Methomyl isomers. Respiratory studies were carried out at 25° with a Clark-type O_2 electrode (Rank Brothers, Cambridge, U.K.). The reaction medium was 300 mM mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KPi, pH 7.2 or 6.5 (malate oxidation). The assay mixtures contained: 12.5 mM succinate or 2 mM NADH and 0.15 mg/ml mitochondrial protein, and 50 mM malate, 1.25 mM NAD and 0.3–0.5 mg/ml mitochondrial protein.

Swelling studies were performed with a Beckman Acta III spectrophotometer at 520 nm. Mitochondria (*ca* 0.15 mg protein/ml) were suspended in a medium containing 300 mM mannitol and 10 mM MOPS buffer (pH 7.2) and A_{520} variations were recorded after addition of the compound under study.

Unless mentioned, all expts presented here were repeated at least three times.

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