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Interference of parathion with mitochondrial bioenergetics

António J.M. Moreno and Vitor M.C. Madeira

Centro de Biologia Celular, Departamento de Zoologia, Universidade de Coimbra, Coimbra (Portugal)

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The organophosphorus insecticide parathion depresses the phosphorylation efficiency of mitochondria as inferred from the decrease of RCR and ADP/O ratios. The transmembrane potential ($\Delta\Psi$) developed by energized mitochondria, and depolarization upon ADP addition are also decreased. Furthermore, repolarization is delayed and resumes at a slower rate. The inhibitory action of parathion on phosphorylation efficiency could be related with the following findings: (1) a direct effect on the succinate dehydrogenase-ubiquinone segment of the redox chain; (2) a direct action on the ATP synthetase complex; (3) partial inhibition of the phosphate transporter.

Introduction

Parathion (*O,O*-diethyl *O-p*-nitrophenylphosphorothioate) is a widely used organophosphorus insecticide owing to its effectiveness against insect pests. Unfortunately, it is also a chemical highly toxic to useful insects, humans and other animals.

The acute toxicity of organophosphorus insecticides has been predominantly assigned to dramatic effects on the nervous system subsequent to the inhibition of acetylcholinesterase [1,2]. Recently, the inhibition of acetylcholinesterase was also correlated to a potent antidipsogenic effect produced by stimulation of muscarinic pathways [3]. Parathion by itself is a poor inhibitor of the acetylcholinesterase unless it is previously metabolized by oxidative systems to paraoxon, its active metabolite. However, the symptoms of chronic intoxication induced by parathion not exclusively related to acetylcholinesterase inhibition are poorly understood.

The lipophilic nature of parathion facilitates its incorporation into biomembranes with consequent chemical and physical changes in their native properties [4,5]. These effects in membranes may partially explain physiological alterations not correlated with the inhibition of the cholinergic system [6–8].

It was previously reported that parathion lowers the temperature range of thermotropic transitions in liposomes [9]. This effect was correlated with a permeability increase of lipid membranes to non-electrolytes and to ion-ionophore complexes [10]. Additionally, the observed antihemolitic action in erythrocytes [5] may be consequence of membrane expansion upon incorporation of the insecticide.

Studies of parathion partition in native membranes showed a predominent incorporation of the insecticide in sarcoplasmic reticulum and mitochondria, but a limited incorporation in brain microsomes, erythrocyte ghosts and myelin [11]. The greater extent of parathion incorporation in sarcoplasmic reticulum and mitochondria was correlated with the low cholesterol content [11]. Additionally, a stimulation of sarcoplasmic reticulum calcium pump was originally reported by Antunes-Madeira and Madeira [12] and recently confirmed by Mobley et al. in skinned muscle fibers [13].

Studies of the effect of parathion in mitochondrial functions are scarce. Since the energetic metabolism in active tissues, e.g., liver, is of crucial metabolic importance, the present study of the effects of parathion on mitochondrial functions is intended to clarify the toxic action of the insecticide.

Material and Methods

Isolation of mitochondria

Mitochondria were isolated from rat liver by conventional methods [14] with some modifications. Homogenization media contained 0.25 M sucrose, 5 mM Hepes (pH 7.4), 0.5 mM EDTA and 0.1% defatted bovine serum albumin. However, EDTA and bovine serum albumin were omitted from the final washing medium, adjusted at pH 7.2. Mitochondrial preparations were tightly coupled with respiratory control ratios above 4 with succinate as respiratory substrate, and

Correspondence: A.J.M. Moreno, Centro de Biologia Celular, Departamento de Zoologia, Universidade de Coimbra, 3049 Coimbra, Portugal.

high degree of membrane intacteness as judged by the lack of oxidation of exogenous NADH. Protein was determined by the biuret method calibrated with bovine serum albumin [15].

Mitochondrial respiration

Reactions were carried out in a 1 ml water-jacketed closed chamber with magnetic stirring, at 25 °C. O_2 uptake was measured polarographically with a Clark oxygen electrode [16] connected to a suitable recorder. The standard respiratory medium contained 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM Hepes (pH 7.2) and 2 μM rotenone, when succinate was used as substrate. Parathion was added (a few μl) to the reaction medium supplemented with mitochondria (2 mg) from concentrated ethanolic solutions. All the other reagents were also added in limited amounts via a capillary enclosure. The respiratory control ratio (RCR) and ADP/O ratios were calculated according to Chance and Williams [17].

Determination of the transmembrane potential (ΔΨ)

A sensitive electrode of tetraphenylphosphonium prepared according to Kamo et al. [18] was used to determine the ΔΨ by monitoring the distribution of TPP^+ across the mitochondrial membrane. The voltage response of the TPP^+ electrode to log [TPP^+] was linear with a slope of 59 ± 1 at 25 °C, in good agreement with the Nernst equation. Reactions were carried out in a thermostated vessel, at 25 °C, with efficient magnetic stirring, in 1 ml of the standard respiratory medium supplemented with 8 μM TPP^+. The ΔΨ was estimated as indicated by Kamo et al. [18] and Muratsugu et al. [19] from the following equation (at 25 °C):

$$\Delta\Psi(\text{mV}) = 59 \log(v/V) - 59 \log(10^{\Delta E/59} - 1)$$

where v, V and ΔE are mitochondrial volume, volume of the incubation medium and deflection of the electrode potential from the baseline. An inner mitochondrial volume of 1.1 μl per mg protein was assumed [20].

No corrections have been made for the 'passive' binding contribution of TPP^+ to the mitochondrial membranes, since the purpose of the experiments was to show relative changes rather than absolute values; in consequence, we can anticipate some overestimation for ΔΨ values. Control experiments have shown that 8 μM TPP^+ do not interfere with mitochondrial respiration and phosphorylation activities. Under our conditions, interferences begin to appear above 20 μM TPP^+.

Enzyme activities

Succinate dehydrogenase activity was measured polarographically at 25 °C in 1 ml of the standard reaction medium supplemented with 5 mM succinate, 2 μM rotenone, 0.5 μg antimycin A, 1 mM KCN, 0.12 mg Triton X-100, and 0.4 mg mitochondria. The reaction was initiated by the addition of 1 mM PMS (phenazinemethosulfate). NADH dehydrogenase activity was measured similarly with 10 mM pyruvate plus 2.5 mM malate, 1 μg antimycin A, 1 mM KCN, 1 mM malonate, 0.12 mg Triton X-100 and 0.4 mg mitochondria. Controls with added NADH were made to rule out any direct oxidation of PMS by NADH formed from pyruvate/malate. Cytochrome oxidase activity was measured polarographically at 25 °C in 1 ml of the standard reaction medium supplemented with 2 μM rotenone, 10 μM cytochrome *c*, 0.13 mg Triton X-100, and 0.42 mg mitochondria. The reaction was initiated by the addition fo 5 mM ascorbate plus 0.5 mM TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine).

Oxidation of NADH by the redox chain of disrupted mitochondrial (sonication) was measured polarographically at 25 °C in 1 ml of the standard respiratory medium without phosphate supplemented with 1 μg oligomycin and 1 mg of disrupted mitochondria. The reaction was initiated by the addition of 0.1 mM NADH.

ATPase activity was measured by the determination of liberated H^+ according to Madeira et al. [21]. The reaction was carried out in 2 ml of the standard respiratory medium lightly buffered with 0.5 mM Hepes (pH 7.2), and supplemented with 2 μM rotenone and 3 mM Mg-ATP. The reaction was initiated by the addition of 1 mg of mitochondria, and parathion was added 5–6 min later. The addition of oligomycin (1 μg) at the end of the assays completely abolished H^+ production.

Mitochondrial swelling

Mitochondrial osmotic volume changes were measured by the apparent absorbance changes at 540 nm with a spectrophotometer linked to a suitable recorder. The reactions were carried out at 25 °C in 3 ml of the appropriate isoosmotic media as indicated in the legends to figures.

Chemicals

All chemicals were of analytical grade for research. Parathion (98% pure), purchased from Supelco, was dissolved in ethanol. Ethanol alone in appropriate volumes was added to the controls.

Results

Fig. 1 summarizes the effect of parathion on RCR (respiratory control ratio) and ADP/O ratios of mitochondria utilizing succinate as respiratory substrate. Parathion in the concentration range of 0.1–1.0 mM depresses both ADP/O and RCR ratios. The highest inhibition induced by parathion in both activities is about 50% of the control. Data suggest that parathion effectively depresses either phosphorylation efficiency

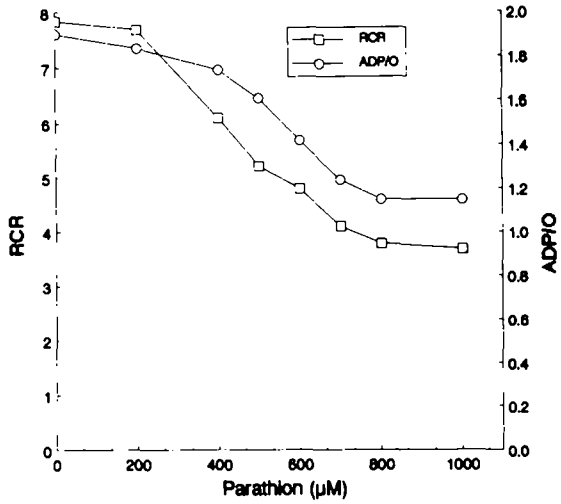

Fig. 1. Effect of parathion on mitochondrial respiratory indexes (RCR and ADP/O). Mitochondria (2 mg protein) incubated in 1 ml of the respiratory medium for 2.5 min in state 4 were supplemented with ADP (0.15 mM) to induce state 3 conditions.

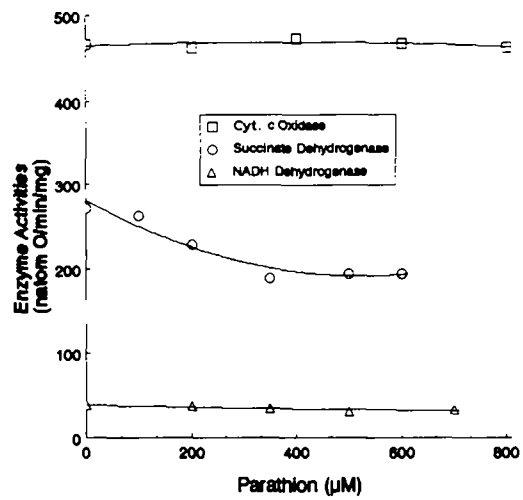

Fig. 2. Effect of parathion on cytochrome oxidase, succinate dehydrogenase, and NADH dehydrogenase of mitochondria. Experimental conditions were as described in Material and Methods. The only action of parathion on the redox chain is clearly related to the partial inhibition of the succinate dehydrogenase-ubiquinone segment.

of mitochondria and/or the activity of redox chain enzymes.

The effects of parathion on the enzymatic activity of mitochondrial redox chain are depicted in Fig. 2. No effect was detected on the terminal cytochrome *c* oxidase segment of the redox chain; similarly, it barely affects NADH dehydrogenase. However, a clear inhibitory effect on succinate dehydrogenase is induced. Therefore, parathion has a localized effect within the succinate dehydrogenase flavoprotein-ubiquinone segment of the redox chain. Nevertheless, the effect of parathion on phosphorylation efficiency of mitochondria does not exclusively result from the inhibition of succinate dehydrogenase segment of the mitochondrial redox chain. Thus, the phosphorylation activity is inhibited by about 50%, but succinate dehydrogenase by only about 25%, and the inhibition profiles are quite different.

The effect of parathion on the mitochondrial capability for oxidative phosphorylation was further investigated by following the transmembrane potential ($\Delta\Psi$) developed by mitochondria energized with succinate. Traces obtained with the TPP^+ electrode representative of the fluctuations of the transmembrane electrical potential ($\Delta\Psi$), negative inside, are depicted in Fig. 3. Upon succinate addition, mitochondria develop a $\Delta\Psi$ of about 215 mV (negative inside). The potential suddenly decreases upon addition of ADP (initiation of state 3)

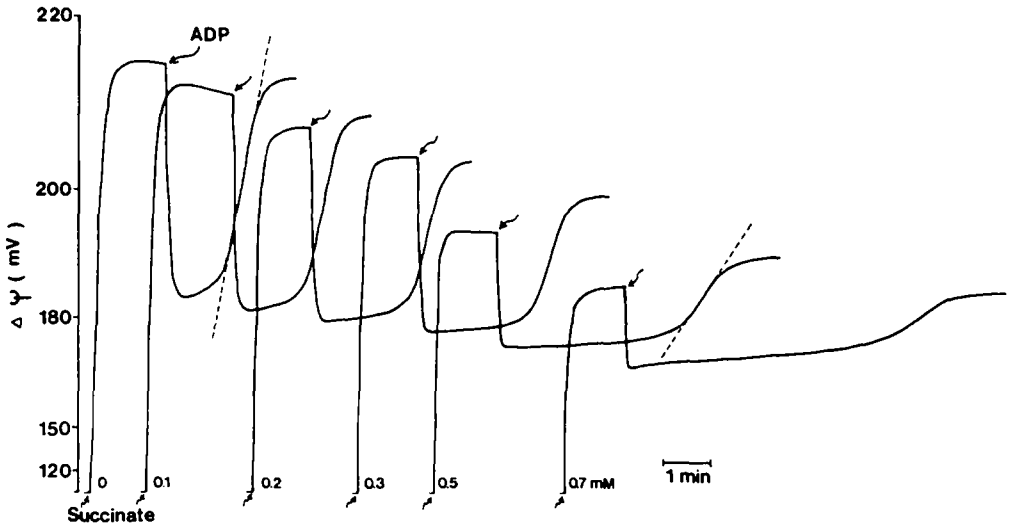

Fig. 3. Effect of parathion on the mitochondrial transmembrane electrical potential ($\Delta\Psi$). Mitochondria (0.6 mg protein) in 1 ml of the standard respiratory medium supplemented with 8 μM TPP^+ were energized with 5 mM succinate; addition of ADP (0.15 mM) induced state 3 condition. Parathion concentrations are indicated on traces. Note that parathion depresses the total developed $\Delta\Psi$, the depolarization induced by ATP, the amount of recovered $\Delta\Psi$ upon repolarization and it increases the time required for initiation of recovery.

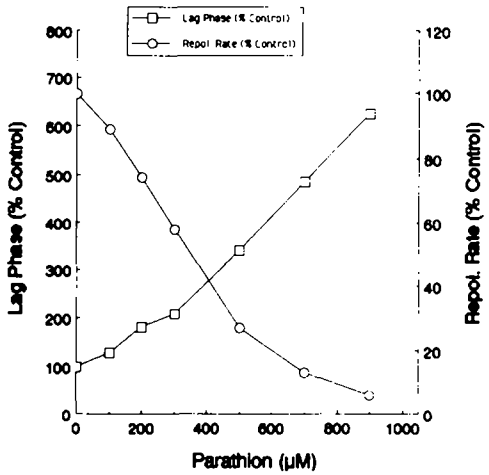

Fig. 4. Effect of parathion on the lag phase preceding repolarization, and on the rate of repolarization. Experimental conditions were as described for Fig. 3.

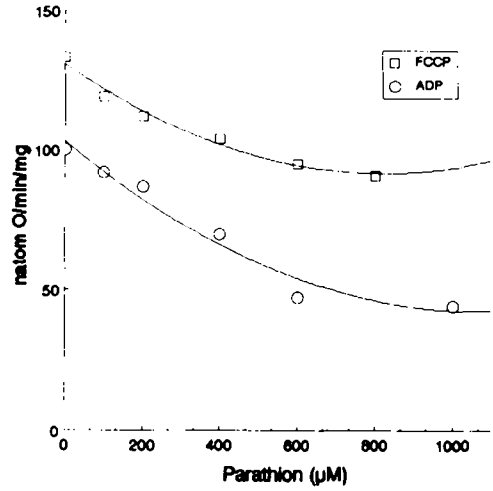

Fig. 5. Effect of parathion on respiratory rates of mitochondria. Mitochondria (1 mg protein) were incubated in1 ml of the respiratory standard medium. The ADP-stimulated respiration was initiated by the addition of 2 mM ADP. The FCCP-stimulated respiration was initiated by the addition of 1.5 μM FCCP. ADP or FCCP were added 2 min after the initiation of state 4 respiration induced by 5 mM succinate.

to about 184 mV; after a short lag phase, the mitochondrial membrane repolarizes close to its state 4 value. However, when parathion is present, mitochondria developed a $\Delta\Psi$ consistently lower with increasing concentrations of the insecticide. Furthermore, the depolarization following ADP addition is decreased as a function of parathion concentration, the rate of repolarization on recovery from state 3 is progressively decreased, and the lag phase preceding repolarization steadily increases (Fig. 4). The rate of repolarization is defined by the slope of the broken lines in Fig. 3.

At the highest parathion concentration under study, the $\Delta\Psi$ reached only 184 mV after succinate energization, and the depolarization induced by ADP, 14 mV, was substantially lower as compared to 31 mV in the absence of parathion; additionally, the repolarization is significantly delayed and the recovery is only partial. These absolute fluctuations of the $\Delta\Psi$ vary among the mitochondrial preparations, but the qualitative changes are maintained.

The effects of parathion on FCCP-induced respiration (Fig. 5) reflect the interaction of the insecticide with the mitochondrial redox chain. This interaction is also reflected in the O_2 consumption of disrupted mitochondrial membranes oxidizing exogenous NADH as respiratory substrate (Table I). These experiments further show that parathion affects the electron transfer along the respiratory chain and confirm the results of Fig. 5. However, state 3 respiration is more sensitive to parathion inhibition than FCCP-stimulated respiration (Fig. 5), indicating that parathion not only depresses electron transport, but also directly affects reactions linked to the phosphorylation pathway.

Parathion directly acts on the ATP synthetase complex, since the addition of oligomycin to mitochondria, in the presence of the insecticide, during the lag phase preceding repolarization, immediately restores the normal state 4 membrane potential (Fig. 6). This observation indicates that, in addition to redox chain inhibition, parathion induces some sort of energy dissipation linked to the ATP synthetase complex, probably by disrupting the coupling between F_0 and F_1 components of the ATP synthetase. This interpretation is in accordance with the stimulation effect of parathion on the mitochondrial ATPase activity (Table II) which can not be explained in terms of membrane uncoupling of CCCP type.

The effect of parathion on the ATPase activity is concentration-dependent and is consistent with the extension of the potential lag phase preceding repolarization (Table II). Additionally, stimulated ATPase is oligomycin-sensitive, since the addition of the specific ATPase inhibitor to mitochondria, in the presence of the pesticide, immediately stops any production of pro-

TABLE I

Effect of parathion on NADH oxidation by sonicated rat liver mitochondria

Mitochondrial suspension was sonicated until it optimally oxidized a known amount of exogenous NADH.

Parathion (μM)	NADH oxidation (natom O/min per mg protein)	% inhibition
0	30.0	0.0
100	26.5	25.3
400	20.4	32.0
500	17.5	41.7
600	17.3	42.3
800	17.6	41.3
1000	17.7	41.0

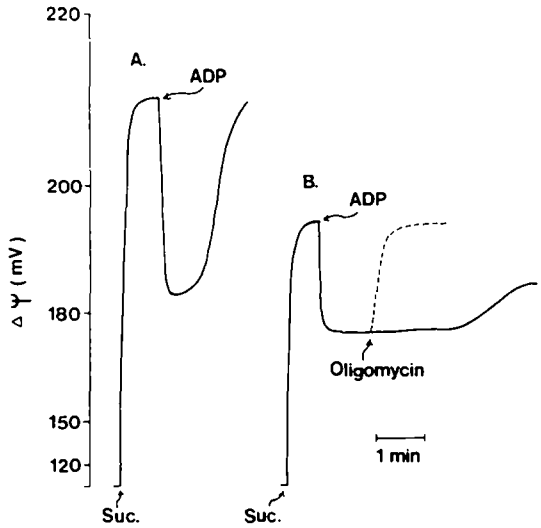

Fig. 6. Effect of oligomycin on transmembrane potential ($\Delta\Psi$) of mitochondria in the presence of parathion. Experimental conditions were as described for Fig. 2. (A) Control; (B) 500 μM parathion added. Where indicated, 1 μM oligomycin was added. Note that the addition of oligomycin induces a prompt and complete recovery of the total $\Delta\Psi$ observed before ADP addition.

tons. Clearly, the observed delay in potential repolarization may be related to a direct effect of parathion on the mitochondrial ATP synthetase complex.

Non-respiring mitochondria swell when suspended in ammonium phosphate (Fig. 7) due to the entrance of NH_3 and $H_2PO_4^-$ transported into mitochondria in co-transport with a proton by the phosphate carrier [22]. *N*-ethylmaleimide (NEM), a specific inhibitor of

TABLE II

Effect of parathion on ATPase activity of rat liver mitochondria

Parathion (μM)	ATPase activity (nmol ATP hydrolyzed/min per mg protein)
0	8.6
200	9.6
300	11.8
500	21.2
700	27.5
CCCP (5 μM)	51.8

the phosphate carrier, prevents mitochondrial swelling (Fig. 7). Apparently, parathion causes a partial inhibition of phosphate transport into mitochondria, since the rate of mitochondrial swelling is clearly depressed when the pesticide is present. The depression of phosphate transport may also contribute to the decreased rate of ATP synthesis.

A decreased membrane fluidity induced by parathion could partially explain the inhibition of phosphate translocator. However, parathion does not depress, rather it accelerates, the rate of swelling induced by valinomycin of mitochondria suspended in an isoosmotic KNO_3 medium (Fig. 7). Since transport of the complex valinomycin-K^+ occurs through the membrane lipid continuum, it appears that parathion increases the membrane fluidity, in agreement with previous descriptions [5,10,21]. Therefore, the effect of parathion on phosphate transport has to be explained in terms of a

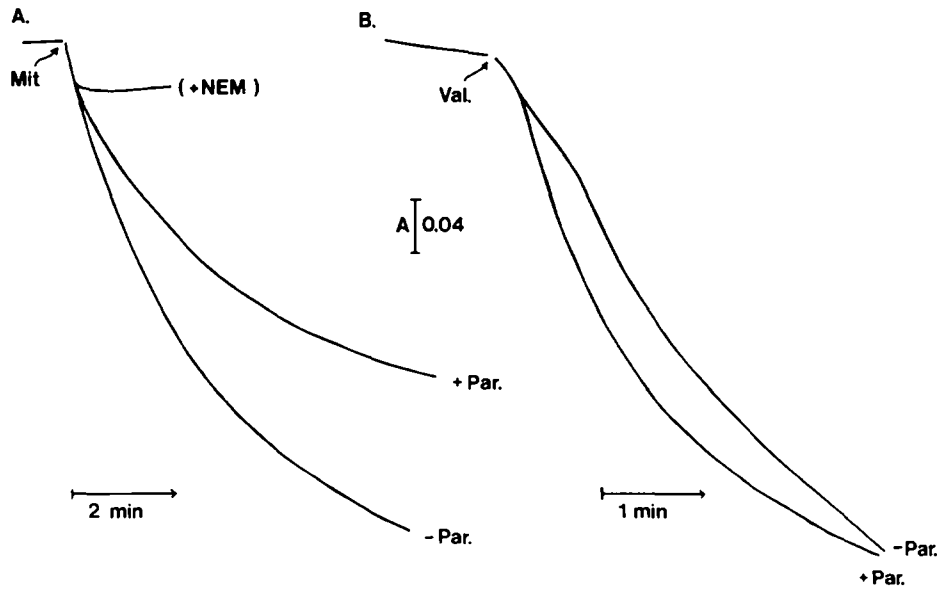

Fig. 7. (A) Effect of parathion on swelling of mitochondria suspended in isoosmotic NH_4PO_4. Mitochondria (1 mg protein) were suspended in 3 ml of 135 mM NH_4PO_4, 5 mM Hepes, 0.1 mM EDTA, 2 μM rotenone (pH 7.2) at 25 °C. When present, added parathion was 600 μM and NEM 0.6 mM. (B) Effect of parathion on valinomycin-induced swelling of mitochondria suspended in isoosmotic KNO_3. Mitochondria (1 mg protein) were suspended in 3 ml of 135 mM KNO_3, 5 mM Hepes, 0.1 mM EDTA, 2 μM rotenone (pH 7.2) at 25 °C. When present, added parathion was 600 μM. Note that parathion decreases the rate of swelling induced by the entrance of NH_4PO_4 into mitochondria, but it increases the rate of swelling induced by the entrance of KNO_3 (with K^+ translocated by valinomycin).

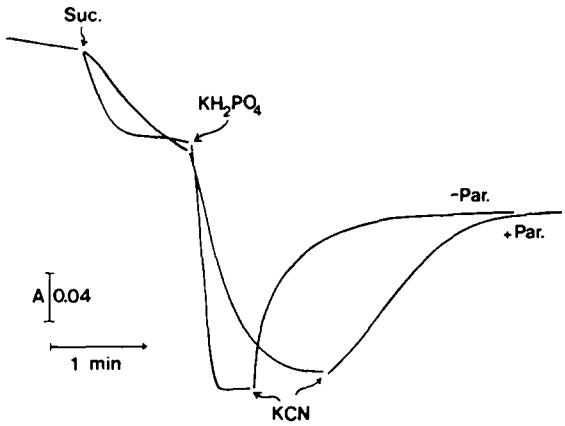

Fig. 8. Effect of parathion on osmotic volume changes of mitochondria. Mitochondria (1 mg protein) were incubated in 3 ml of the standard respiratory medium without phosphate, and supplemented with 2 μM rotenone and 1 μg valinomycin. Additions of 5 mM succinate, 5 mM KH_2PO_4 and 1 mM KCN were as indicated. When present, added parathion was 600 μM. Note that parathion decreases the rate of swelling and shrinkage as consequence of partial inhibition of the phosphate carrier, and that the osmotic changes are reversible.

direct interaction of the insecticide with the phosphate carrier.

The effects of parathion, within the concentration range under study, are rather specific and are not consequence of physical damage exerted on mitochondrial membranes since concentrations up to 1.0 mM do not promote any oxidation of exogenous NADH. Mitochondrial inner membrane is impermeable to exogenous NADH [24] and NADH dehydrogenase activity is accessible only from the matrix side [25]. Thus, exogenous NADH is not oxidized unless intact mitochondria are physically disrupted. Therefore, the absence of NADH oxidation clearly indicates that parathion does not perturb the physical integrity of the membranes. Mitochondrial volume changes measured essentially according to Gealy et al. [26] (Fig. 8) reflect the osmotic relations subsequent to solute fluxes across mitochondrial inner membrane and are reversible. Therefore, disruptive compounds would abolish the characteristic reversibility of osmotic changes. Reversible osmotic volume changes of respiring mitochondria suspended in the standard medium (phosphate-free) supplemented with valinomycin are represented in Fig. 8. Upon addition of phosphate, a marked swelling occurs due to the massive entrance of potassium phosphate into mitochondria. The inhibition of respiration by KCN induces the efflux of potassium phosphate from mitochondria resulting in osmotic shrinkage. The presence of parathion (up to 1.0 mM) decreases the rates of swelling and shrinkage, but the reversibility is not affected. Therefore, parathion has no disrupting actions on mitochondrial membranes. The reduced rates of swelling and shrinking, when parathion is present, reflect the inhibitory action of the insecticide upon the phosphate carrier as discussed previously.

Discussion

Although the protonic nature of mitochondrial coupling between electron transport and ATP synthesis is widely recognized, there is no universal consensus about the coupling mechanism. The delocalized Mitchell hypothesis of chemiosmotic coupling involves a bulk phase electrochemical proton gradient ($\Delta p = \Delta\mu_H \cdot / F = \Delta\Psi - Z\Delta pH$) as the sole and obligatory link between electron transport and ATP synthesis [27]. The localized theory of Williams assigns to the mitochondrial membrane a more active role on energy storage, and postulates the existence of proton conducting structures within the membrane responsible for direct functional interactions between redox and ATP synthetase components [28]. On the other hand, semilocalized theories [29] postulate proton pathways laterally aligned along the membrane between the energy sources and the ATP synthetase complexes. The theories opposing the delocalized Mitchellian hypothesis assign to the bulk proton gradient a marginal role as a minor storage device on energy transduction. The transmembrane electrical potential ($\Delta\Psi$) is the main component of the electrochemical gradient accounting for more than 90% of the total energy and is interchangeable with ΔpH under particular conditions [30]. Therefore, determinations of $\Delta\Psi$ are of major importance in studies of mitochondrial oxidative phosphorylation.

In our studies, the TPP^+ electrode has proved very useful to the determinations of $\Delta\Psi$ with advantage to other methods [31,32], particularly since it permits a rapid, sensitive, and continuous registration of $\Delta\Psi$ fluctuations without artifactual interference of parathion.

The fact that parathion inhibits state 3 and RCR by similar amounts (50%) further supports the suggestion that the insecticide energetically dissociates the ATP synthetase complex without membrane uncoupling in the sense the uncouplers (e.g., CCCP) act. If the only action were the inhibition of the electron transfer rate, state 3 would be accordingly depressed, but RCR would be essentially unaltered.

In addition to the inhibitory effect on electron transport, the partial inhibition of phosphate transport and the direct action on the ATP synthetase complex further accounts for the depression of energy transduction induced by the insecticide. The interaction with the ATP synthetase is tentatively assigned to a disruption effect on ATP synthetase coupling through the alteration of the functional link of F_0 and F_1 components of the ATP synthetase complex. Actually, the prompt recovery of the $\Delta\Psi$ after the addition of oligomycin during the lag phase preceding repolarization strongly supports this proposal.

Parathion increases the rate of ATP hydrolysis an effect also observed with the classical uncouplers of

oxidative phosphorylation [33]. However, unlike the uncouplers, the increase of mitochondrial ATPase activity induced by parathion is not related to any classical uncoupler action since the following events were observed: (a) state 4 respiration is not significantly affected; (b) oligomycin inhibition of stimulated ADP respiration is not reverted; (c) mitochondria suspended in isoosmotic NH_4NO_3 do not swell, indicating that permeability to protons is not affected.

The decrease in the efficiency of mitochondrial energy transduction could also be related to the inhibition of the ADP–ATP exchange. However, the relatively positive phosphorus atom of parathion excludes this possibility, since the binding to the ADP–ATP exchanger involves at least three negative charges of the known inhibitors [34].

Our results are in conflict with the reported lack of effect of parathion on mitochondrial oxidative phosphorylation in beetle tissues [1]. However, no detailed research has been carried out about the insecticide action on mammalian mitochondrial bioenergetics, although it has been reported that no significant effects have been achieved on mitochondrial RCR, ADP/O and ATPase activity of rats fed with methylparathion, a less toxic dimethyl analogue of parathion [35].

Probably the main toxic effect of parathion in organisms is related with its anticolinesterase action, since the effects can be reversed by administration of oximes and other drugs favoring dissociation of the parathion-enzyme complex [1]. However, inhibitory action on mitochondrial oxidative phosphorylation may also additionally concur for the chronic toxicity of the pesticide, although the effective concentrations in our study can not easily be correlated with the LD_{50} obtained with experimental mammals [1,2], since the distribution of parathion in tissues will depend on several parameters, namely the partition coefficients [11].

Acknowledgement

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