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Short Term Toxicity Effect of Dimethoate on Transthylakoid pH Gradient of Intact *Synechocystis* sp. PCC 6803 Cells

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In recent times organophosphorus (OP) insecticides have been abundantly used to control the insect pests. The mechanism of action of these insecticides is fairly well understood for the target organisms. They are poisonous to the insects because of their ability to inhibit the enzyme acetylcholinesterase (AChE: EC 3.1.1.7) thereby disrupting the normal functioning of nerve transmission, producing hyperexcitability, convulsion, muscular paralysis, and respiratory failure (O'Brien 1967). The change in the fluidity of the lipid membrane caused by lowering of membrane transition temperature (Marcelja and Wolfe 1979), disorganisation of the phospholipid core (Antunes-Madeira et al. 1994) and inhibition of mitochondrial energy production (Yamano and Morita 1993) have also been observed. Many references are available on the influence of OP insecticides on growth and metabolism of cyanobacteria (for review see Mohapatra and Mohanty 1990). However, the exact mechanism of action of OPs on cyanobacterial photosynthesis especially on short term incubation is poorly known. On the other hand, it is established that 1 % of the amount of pesticides, applied in a field to control the disease, is taken by the target pests while the remaining part drifts into the soil affecting the microflora of which cyanobacteria constitute a major part (Gupta and Gupta 1980).

Dimethoate is a thion group containing OP insecticide with a carbamyl bond sensitive to hydrolysis. It is easily detoxified through oxidation, glutathione-dependent demethylation and hydrolysis (O'Brien 1967). The relatively rapid hydrolysis of the amide bond necessitates the evaluation of its immediate toxicity effects to non-target organisms. In this report we have presented the short term effect of dimethoate on thylakoid membrane of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 which is widely used as the standard organism for photosynthetic research.

MATERIALS AND METHODS

Axenic stock cultures of *Synechocystis* sp. PCC 6803 were grown in 250-mL batch culture vessels containing 100 mL of growth medium (Allen and Arnon 1955) and were kept in thermostatic water bath at 20 °C at a continuous irradiance of 35 µmol photon m^{-2} s^{-1} with constant aeration. Cultures in the mid-log phase of growth were diluted with sterile medium (pH = 7.2) to a cell density of 107 mL^{-1} 4 hr before the treatments and were used as initial inocula for the measurements.

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The active ingredient (99 % pure) of dimethoate [0, 0 - dimethyl -S-(-N-methyl-carbamoylmethyl)-phosphorodithioate] was supplied by Laboratory Chemicals, Riedel-deHaen, Augsburg, Germany. Stock solution (100 mM) of the insecticide was prepared with acetone (99.5 % pure) and was then diluted with sterile medium (acetone concentration of the stock: 1 % v/v). Freshly prepared stock solutions were always used to obtain the treatment doses (0.01 - 3 m*M*). ACMA (9-amino-6-chloro-2-methoxy acridine) was obtained from Schuchardt & Co, Hohenbrunn, Germany. Tentoxin, KCN and DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] were supplied by the Sigma Chemicals, Deisenhofen, Germany.

In order to detect the effect of dimethoate on photosynthetic electron transport chain, chlorophyll-a fluorescence measurements were done at 20 °C using a PAM - 101 chlorophyll fluorometer (H. Walz, Effeltrich, Germany) according to the procedure of Schreiber et al. (1986). Treatments were directly made in the chlorophyll fluorometer cuvette so as to achieve 1 mL volume of the culture (cell density 10^7 m L^{-1}). Initial fluorescence (F_0) was determined by applying only modulated measuring beam (0.026 µmol photon $m^{-2} s^{-1}$) while the maximum fluorescence (Fm) response of photosystem II (PS II) without driving photosynthesis and the generation of non-photochemical quenching (qN) were measured by applying short white light pulses (600 msec at an intensity of 2000 µmol photon $m^{-2} s^{-1}$) from a SCOTT KL 1500 electronic lamp for 1 hr with a dark period of 1 min between two consecutive pulses.

The light dependent quenching of ACMA fluorescence was measured by determining the effect of the insecticide on transthylakoid pH gradient of *Synechocystis* cells. Treatment was done with 2 mM of dimethoate (7-d LC_{50} = 1.97 ± 0.09 m*M* at 95 % confidence limit) for 30 min at 20 °C and then the treated cultures were condensed to a cell density of 7.2 x 10s mL^{-1} through centrifugation for 3 min at 3500 rpm. Equally dense samples were taken as controls. Five different sets of controls separately containing 100 µ*M* KCN, 10 µ*M* DCMU, 1 µ*M* tentoxin, and with medium pH 6.0 and 8.5 were taken. The treated culture was also supplemented with 100 µ*M* KCN immediately before detection of fluorescence emission. After 5 min of dark quenching, background light (1400 µmol photon $m^{-2} s^{-1}$), for generation of pH difference across the thylakoid membrane, was supplied from a SCHOTT KL 1500 electronic lamp for 2 min. Fluorescence emission was detected at 458 nm with excitation at a peak wavelength of 400 nm using a fluorescence spectrophotometer (F4010, Hitachi Ltd., Japan). The concentration of ACMA was 51 µ*M*. Unless otherwise indicated the measurements were made at a pH of 7.2.

The experiments were done twice taking duplicates each time. The comparisons among the means have been done through regression analysis and the regression coefficients have been included in the text. Standard statistical methods for calculation of confidence limit (p = 0.05), coefficient of variations (cv) and standard errors have been followed(Gomez and Gomez 1984).

RESULTS AND DISCUSSION

The changes in Fm and qN of actively growing *Synechocystis* cells in response to 2 m*M* of dimethoate have been represented in Figure 1. The insecticide caused reduction of Fm and increase of qN with increase in incubation time (data not shown). We also observed an increasing trend of F_0 and qN with increase of pesticide level in the cultures (Fig. 2).The concentration dependent increase of F_0

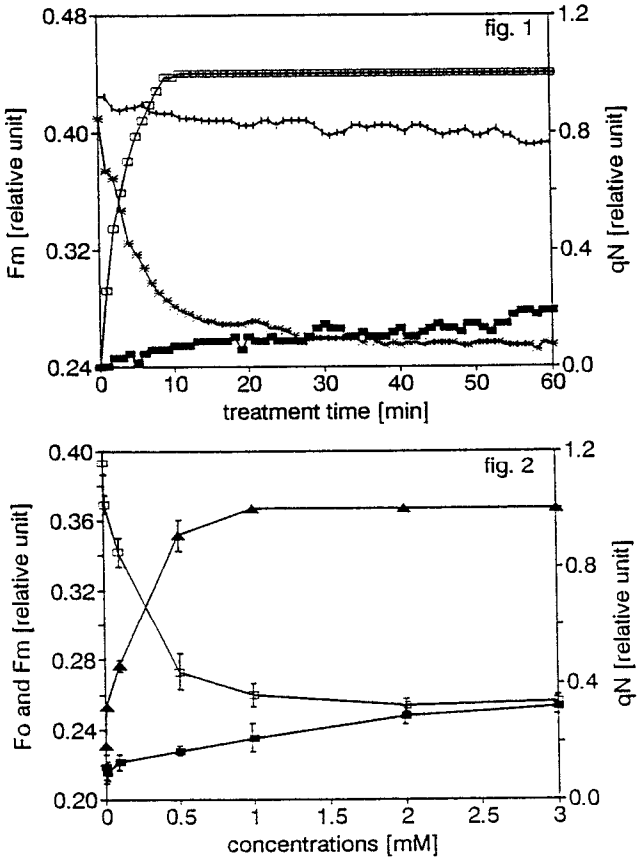

Figure 1. Effect of dimethoate (2 m*M*) on Fm and qN of intact *Synechocystis* cells. The data points are the means of 4 replicates. Standard errors have been omitted to increase the clarity of the Figure. Control: (i) Fm, (✱) qN; treated: (□) Fm, (■) qN.

Figure 2. Change in (■) F_0, (□) Fm, and (▲) qN of *Synechocystis* at different concentrations of dimethoate. F_0 represents the mean value of the last 10 pulses taken from each replicate while Fm and qN are the means of those recorded with last (60th) saturating pulse. Vertical bars are the standard errors.

was found to be insignificant at p = 0.05 (r^2 = 0.011; n = 240) while the acceleration of qN and reduction of Fm were significantly correlated with log concentrations of dimethoate (r^2 = 0.89 and 0.88 for Fm and qN, respectively; n = 24). At 0.01 and 0.1 m*M* a biphasic effect on Fm was recorded (Fig. 3); an immediate decrease coupled with increase of qN during first 10 min of treatment followed by a slow response and further decrease thereafter. On the other hand, a drastic decrease of Fm was observed during first 20 min of treatment with ≥ 1 m*M* of the chemical. Similarly qN showed a rapid increase at these concentrations (Fig. 2). It reached at 1.0 within first 10 min of treatment at 2 m*M* (Fig. 1) and after 18 min at 1 m*M* (not shown) indicating a strong inhibition of PS II activity through decrease of intrathylakoid pH.

When a photoautotrophic cell or a chloroplast is illuminated, the excitation energy is

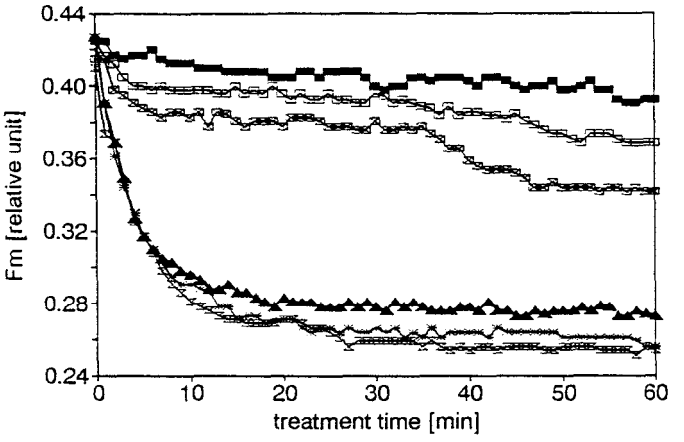

Figure 3. Time dependent effect of dimethoate on Fm of *Synechocystis*. Dimethoate concentrations (m*M*): (■) 0, (□) 0.01, (⊠) 0.1, (▲) 1, (✱) 2, (x) 3.

trapped by charge separation at the photosynthetically active chlorophylls P680 followed by electron transfer to quinone (Q_A). The electron is subsequently transferred to a plastoquinone (Q_B) molecule bound to the D_1 protein of PS II and thereafter to cytochrome - b_{559}. An increase in lumen pH energizes thylakoid membrane and results in shrinkage of plastoquinone pool (Schreiber et al. 1986). This causes a reduction of bound Q_B movement coupled with decreased photochemical activity of the PS II reaction centers and an increase in non-photochemical quenching (Mullineaux and Holzwarth 1993). In the present experiment the decrease of Fm and simultaneous increase of qN at different dimethoate concentrations is certainly due to low pH of the thylakoid lumen. Thus it can be hypothesised that dimethoate caused inhibition of thylakoid ATPase (more specifically the hydrophobic CF_0) increasing proton concentration inside the thylakoid lumen and decreasing Fm. Bulte et al. (1990) have reported that in the cells in which ATP synthesis is blocked, the fluorescence emission of PS II is also decreased because of an extensive reduction of PQ pool upon inhibition of ATP synthesis. Complete elimination of variable fluorescence at ≥ 1 m*M* of the chemical within the first few minutes of treatment proves that a high gradient of pH across the thylakoid membrane is built up at these concentrations.

In intact *Synechocystis* cells, in which both thylakoid and cell membranes are functionally active, the behavior of the ACMA fluorescence was different from that reported with spheroplasts (Matthijs et al. 1985) and thylakoid vesicles (Padan and Schuldiner 1978) of cyanobacteria. First a considerable and prolonged quenching immediately after addition of ACMA occurred due to its movement into the cells caused by differences in pH across the cell membrane and its protonation inside the cell cytosol (Fig. 4). The initial dark quenching appeared greater in dimethoate treated culture compared to that found in untreated one (Fig. 4A). Such results were consistently observed in all the replicates (cv = 4.31 % and 1.25 % among the replicates of control and treated samples, respectively). After turning on the light an initial quenching (about 18 sec) followed by a fluorescence enhancement was observed in the treated culture while in the control no change in the fluorescence response was noticed during first 20 sec though the enhancement followed thereafter. In samples separately containing DCMU (Fig. 4B) and tentoxin (Fig. 4C) no appreciable increase in fluorescence in response to light was observed

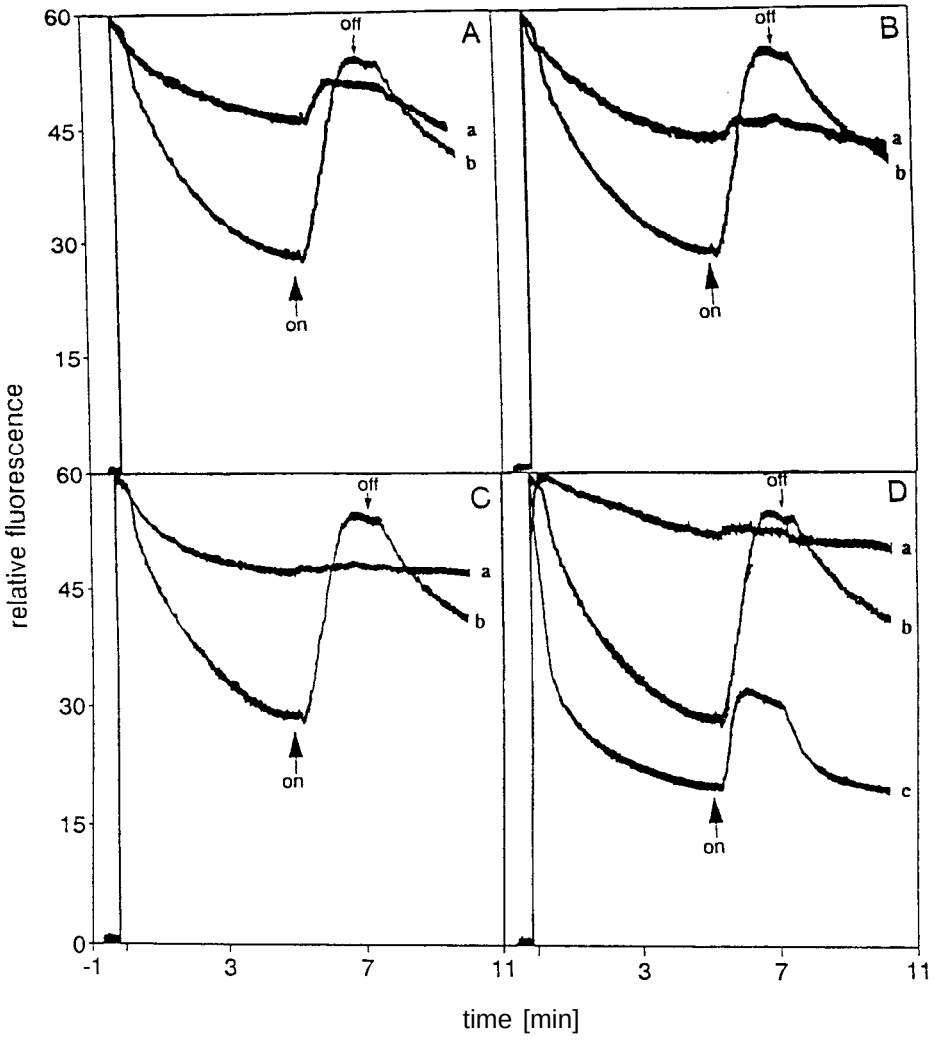

Figure 4. - Effect of dimethoate on light induced ACMA fluorescence changes of intact *Synechocystis* cells. ACMA (5 µ*M*) was added after 1 min of initialization of the culture. The positions with "on" and "off' signs indicate turning on and turning off of background light. The letter "a" represents the control with 100 µ*M* KCN, 10 µ*M* DCMU, 1 µ*M* tentoxin, and medium pH 6.0 in sector A, B, C and D, respectively. The culture treated with dimethoate 30 min before ACMA addition has been represented as "b". The letter "c" of sector D denotes the control with medium pH 8.5.

indicating that the quenching/enhancement in the presence of light was the effect of light induced transmembrane proton gradient. Since DCMU and tentoxin collapsed the proton gradient across the thylakoid membrane, no pH change was induced by light and no movement of fluorescent probe took place through the cell membrane.

Manipulation of the pH of the medium immediately before the fluorescence measurement showed that initial dark quenching increased with alkalization of the

medium (Fig. 4D) and the fluorescence values reached after 5 min of dark quenching showed significant positive correlation ($p = 0.01$) with medium pH ($r^2 = 0.86$; $n = 12$). These results suggest that the high fluorescence quenching in the treated culture, compared with that of the control, during initial dark phase, was due to high pH difference between the medium and the cell protoplasm (Fig. 4A). Such a transmembrane pH difference was probably built up due to the effect of dimethoate on proton and proton/ion channels of the cell membrane.

In intact cells, ACMA shows dualistic fluorescence response to illumination; at the level of thylakoid a quenching (protonation of ACMA in the thylakoid lumen) and at the level of cell membrane an enhancement (deprotonation due to alkalization of cytosol). When light is applied, the pH gradient across the thylakoid membrane is built up and ACMA moves into the thylakoid causing a quenching (Matthijs et al. 1985). The acidification of the lumen causes increase of cytosolic pH (Hansen et al. 1993) due to which ACMA moves out of the cell. Since volume of the thylakoid lumen is much lower than that of the cytosol, the fluorescence recovery becomes more than quenching (Matthijs et al. 1985). Absence of light-induced fluorescence increase in DCMU and tentoxin treated cultures provides the conclusion that the higher light-induced recovery of fluorescence in dimethoate treated cultures (see Figure 4) is certainly caused by the increase in acidity of thylakoid lumen. It is concievable that such situation occurs because of inhibition of thylakoid ATPase by dimethoate through disordering effect of the insecticide on the lipid core of the thylakoid membrane as reported in mitochondria (Yamano and Morita 1993).

The present study showed that dimethoate affected PS II activity and photophosphorylation of *Synechocystis* at all the tested concentrations. Such an immediate effect of the insecticide on this organism and on other soil cyanobacteria can be expected in the field conditions. It would result in reduction of soil cyanobacterial population and decrease of soil fertility.

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