Alteration in Cell Permeability as a Mechanism of Action of Certain Quinone Pesticides

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ABSTRACT

The permeability of the Chlorella pyrenoidosa membrane was studied by following the efflux of "C-intracellular material from cells which had been allowed to incorporate "CO₂ photosynthetically. It was observed that the efflux increased upon treatment with low concentrations (3-30 μM) of 2,3-dichloro-1,4-naphthoquinone (dichlone), 2-amino-3-chloro-1,4-naphthoquinone (06K-quinone), and 2,3,5,6-tetrachloro-1,4-benzoquinone (chloranil). Dichlone caused a greater loss of intracellular material than chloranil or 06K-quinone. The rate of loss as well as the total loss of "C increased with an increase in the concentration of the quinones. In the dichlone-treated cells, the leakage was observed within 1 minute of the addition of the chemical and the effect on cell permeability was irreversible. Cells exposed to dichlone in the light or under anaerobic conditions released significantly greater amounts of "C-material than cells treated in the dark or under aerobic conditions. The aqueous ethanol-soluble fraction of the cell was found to be the source of the released material. The proportion of the ethanol-soluble "C that leaked out of the cell varied with the time of "C-assimilation prior to treatment with dichlone. In the dichlone-treated cells, practically all the "C-sucrose, alanine, glutamine, serine, and glycine leaked out, whereas glutamic, aspartic, succinic, and fumaric acids were lost only partially. Essentially no "C-lipids were lost from the cells during dichlone treatment.

The extreme rapidity of the effect of dichlone on permeability and the low concentrations at which dichlone acted suggest that the cell membrane may be a primary site of action of dichlone, and that the metabolic changes observed in dichlone-treated Chlorella may be due to the changes in the cell membrane structure.

Quinones have been reported to act on biological systems in a number of ways including interference with electron transport, inhibition of phosphorylation, inactivation of enzymes, and reaction with sulfhydryl and amino groups (11, 16). Owens (6) reported that the activity of substituted benzoquinones and naphthoquinones was related to the inhibition of enzymes and coenzymes with free sulfhydryl and amino groups. Black and Myers (1) postulated that the phytotoxic effect of 06K-quinone might be due to the inhibition of ATP and NADH production and catalytic oxidation of reduced pyridine nucleotide. In our studies concerning the mode of action of certain quinone pesticides in algae and isolated chloroplasts, we have observed that dichlone, chloranil, and 06K-quinone inhibited electron transport, photophosphorylation, and CO₂ fixation (13, 17). The same chemicals were also found to cause a drastic reduction in the chlorophyll content of Chlorella cells within a few hours after treatment (18). The bleaching effect of the quinones has suggested that inhibition of photosynthesis may not be the only explanation for their algalicidal activity. It is conceivable that quinones interact with chloroplast and cell membranes causing a disruption of the chloroplast and cellular structure, thereby resulting in a loss of chlorophyll.

Previous investigations on the mode of action of quinone pesticides have dealt primarily with their effect on cellular metabolism (7, 9, 12, 17). In order to have a better understanding of the nature of the toxic action of quinones on algae, it was decided to study the effect of these chemicals on the permeability of Chlorella cells. The response chosen for the study was the leakage of intracellular material from the cells. In this paper we present evidence that treatment of Chlorella with dichlone, chloranil, or 06K-quinone causes a rapid release of intracellular material, apparently as a result of changes in the cell membrane.

MATERIALS AND METHODS

Growth of Algae. Chlorella pyrenoidosa Chick (Emerson strain) was cultured in an inorganic medium (18) using a turbidostat (20) at 25 C with a stream of air containing 4% CO₂. The algae were grown at a light intensity of about 1000 ft-c provided by cool white fluorescent lamps. Cell density was kept constant at an absorbance of about 1.0 (680 nm) by the automatic addition of culture medium.

Preparation of "C-Labeled Cells. One hundred ml of cell suspension were removed from the culture vessel and placed in a 500-ml Erlenmeyer flask. One ml of NaH"CO₃ solution containing 100 μc was added to the suspension, and the flask was placed on a shaker. The cells were allowed to incorporate "C for 30 min at 25 C under a light intensity of about 1000 ft-c. The cells were harvested by centrifugation, washed, and resuspended in the growth medium to give an absorbance of about 1.0 at 680 nm. To determine the amount of "C incorporated in the cells, an aliquot of the cell suspension was filtered through a Millipore filter and washed with the growth medium; the filter was transferred to a scintillation vial for counting.

Determination of "C-Efflux. Changes in cell permeability of Chlorella were studied by following the loss of radio-labeled material from the cells, prepared as described above. Appropriate volumes of quinone solutions in methanol were added to the "C-labeled cell suspension in 50-ml Erlenmeyer flasks so that the final concentration of methanol was 1% (controls

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2 Abbreviations: 06K-quinone: 2-amino-3-chloro-1,4-naphthoquinone; dichlone: 2,3-dichloro-1,4-naphthoquinone; chloranil: 2,3,5,6-tetrachloro-1,4-benzoquinone.
that leaked out of control cells was very small, indicating relative impermeability of the cell membrane. However, with cells treated with the above quinones, the efflux of $^3$C-material was markedly increased. The results also illustrate the rapidity of the action of the quinones since the effect was observed as early as 5 min after the addition of the quinones. Dichlone and chloranil had a more pronounced effect than 06K-quinone. Two hr after the addition of dichlone, chloranil, and 06K-quinone, the cells had lost 40, 28, and 8% of their total $^3$C, respectively.

The integrated first-order kinetics equation
\[ k = \frac{2.303}{t} \log \frac{a}{(a - x)} \]
was used to determine if the loss of $^3$C from the quinone-treated cells followed a first-order reaction. In this equation, $a = \text{maximum amount of the leakable } ^3\text{C, and } x = \text{amount of leaked } ^3\text{C material at time } t$. The efflux had reached a maximum in 2 hr which might also represent an equilibrium concentration. However, when these cells were centrifuged and resuspended in a fresh medium containing quinone, no further loss of $^3$C could be measured, indicating that the cells had released the maximum amount of radioactivity. A graph of the log $a/(a - x)$ against time yielded a straight line, suggesting that the efflux of $^3$C-material from the quinone-treated cells is a first-order kinetic process. Using the general form of the first-order equation, $dx/dt = k(a - x)$, the maximum rates of efflux from the cells treated with dichlone, chloranil, and 06K-quinone were calculated to be 3.11, 2.44, and 0.43 dpm/hr, respectively.

The $^3$C-leakage from the cells was dependent on the concentration of quinones in the medium. Figure 2 shows the effect of dichlone and chloranil concentration on the efflux of $^3$C-labeled organic compounds from Chlorella. The rate of loss as well as the total loss of $^3$C increased progressively with an increase in the concentration of quinones. The increase in $^3$C-loss was roughly linear when dichlone concentration was increased from 1.5 to 3 $\mu$M. When the dichlone or chloranil concentration was increased from 3 to 30 $\mu$M, the increase in

![Fig. 1. Release of intracellular $^3$C-material from Chlorella in the presence of different quinones. Chlorella cells were allowed to incorporate $^3$CO$_2$ for 30 min. $^3$C-Labeled cells were centrifuged, washed, and resuspended in a fresh medium. The cells were incubated with 30 $\mu$M quinone at 25 C under a light intensity of 500 ft-c. Samples of cell suspension were removed at intervals, filtered, and the release of $^3$C-material was measured by counting the radioactivity in the filtrate. One ml of cell suspension contained 2.48 $\times$ 10$^4$ dpm prior to quinone treatment.](image1)

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**Analysis of Cellular and Effluent Material.** The alcohol extract of untreated $^3$C-labeled cells was concentrated to dryness under a stream of air, and the residue was partitioned between water and petroleum-ether. The petroleum-ether layer was counted to determine the amount of $^3$C-lipids in the cell extract. The effluent from the treated cells was also extracted with petroleum-ether. Following ether extraction, aliquots of the cell extract and the effluent containing at least 50,000 dpm were chromatographed two-dimensionally on Whatman No. 1 filter paper as described by Pedersen et al. (10). Location of the labeled compounds on the chromatograms was detected by autoradiography. The radioactive compounds were identified by co-chromatography with authentic compounds. The radioactivity of each spot was determined by cutting it out and counting it in the liquid scintillation counter.

**RESULTS**

**Effect of Quinones on $^3$C-Efflux.** The leakage of $^3$C-material was measured from cells treated with dichlone, chloranil, and 06K-quinone. Figure 1 shows the loss of $^3$C from Chlorella treated with 30 $\mu$M dichlone, chloranil, or 06K-quinone relative to time of treatment. The amount of cellular material

![Fig. 2. Effect of quinone concentration on the leakage of $^3$C-material. The leakage of $^3$C-labeled Chlorella cells was determined in the presence of varying concentrations of dichlone and chloranil as described in Figure 1.](image2)


\(^{14}\)C-efflux was no longer proportional to the increase in quinone concentration.

The amount of \(^{14}\)C in the 80% ethanol-insoluble fraction of the cells remained constant before and after treatment (Table I). These findings indicate that the \(^{14}\)C incorporated in the ethanol-soluble fraction of the cell was the source of the released material.

Since dichlone caused the greatest loss of intracellular material, all further studies were focused on dichlone using a concentration of 30 \(\mu M\).

**Effect of Light and Oxygen on Efflux.** It has been suggested that quinones may cause the formation of free radicals in the cell (19). It is possible that these radicals may be responsible for the disruption of the membrane, thereby resulting in an increased permeability. Since light and oxygen presumably increase the rate of free radical formation, the above hypothesis

<table>
<thead>
<tr>
<th>Treatment Time</th>
<th>(^{14})C Released</th>
<th>(^{14})C Remaining in the Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/ml suspension (\times 10^{4})</td>
<td>dpm/ml cell suspension (\times 10^{4})</td>
</tr>
<tr>
<td>min</td>
<td>ETHANOL-SOLUBLE</td>
<td>ETHANOL-INSOLUBLE</td>
</tr>
<tr>
<td>0</td>
<td>14.8</td>
<td>11.5</td>
</tr>
<tr>
<td>60</td>
<td>7.2</td>
<td>11.2</td>
</tr>
<tr>
<td>120</td>
<td>5.1</td>
<td>11.3</td>
</tr>
</tbody>
</table>

would be supported if it could be shown that the leakage of cellular material was greater in light and in the presence of \(O_2\) than in dark and under anaerobic conditions. The release of \(^{14}\)C-material was measured from the cells which were treated with dichlone \((a)\) in the light and dark, and \((b)\) in the presence and absence of air. Figure 3 shows that the cells exposed to dichlone under anaerobic conditions lost considerably greater amounts of \(^{14}\)C-material than the cells incubated with dichlone in the presence of air. Also, the loss of \(^{14}\)C was higher from the cells exposed to dichlone in the light than from the cells which were treated with quinone in the dark. The effect of light on the efflux appeared to be more pronounced under anaerobic conditions than in the presence of air.

**Reversibility of the Effect of Dichlone on Permeability.** Figure 4 shows the results of an experiment designed to determine if dichlone-induced permeability can be reversed by removal of the quinone. The \(^{14}\)C-labeled cells were exposed to dichlone for 1, 5, and 10 min, washed, and resuspended in dichlone-free medium. These cells continued to lose \(^{14}\)C; the degree of loss varied with the time the cells were exposed to dichlone prior to washing. The cells which were in contact with dichlone for 5 or 10 min before washing lost more \(^{14}\)C than the cells which were treated with dichlone for 1 min. These findings indicate that dichlone-induced permeability changes could not be reversed by washing, suggesting either that the cells had been damaged or that dichlone was irreversibly bound to the cells even after 1 min treatment.

**Effect of Size of Ethanol-soluble Fraction.** Since it was shown that the ethanol-soluble fraction of the cell was the source of the released material, it was of interest to determine whether there was a relationship between the ethanol-soluble \(^{14}\)C-pool in the cell and the quinone-induced efflux. Cells containing different levels of ethanol-soluble \(^{14}\)C-material were prepared by allowing the cells to fix \(^{14}\)CO\(_2\) for varying periods.
Table II. Dichlone-induced ¹⁴C-Leakage as Affected by Changes in the Time of ¹⁴CO₂ Fixation Prior to Dichlone Treatment

Chlorella cells were allowed to incorporate ¹⁴CO₂ for varying periods. The labeled cells were filtered, washed, and resuspended in the medium containing 30 μM dichlone, and ¹⁴C-leakage was measured. The amount of total as well as ethanol-soluble ¹⁴C in the cells was determined in each treatment prior to the addition of dichlone.

<table>
<thead>
<tr>
<th>¹⁴CO₂ Fixation Time</th>
<th>Release of ¹⁴C-Material in the Presence of Dichlone (Added after ¹⁴CO₂ Fixation for Time Indicated)</th>
<th>¹⁴C Released</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum ¹⁴C lost</td>
<td>%</td>
</tr>
<tr>
<td>min</td>
<td>dpm/ml cell suspension × 10⁻²</td>
<td>%</td>
</tr>
<tr>
<td>2</td>
<td>7.21</td>
<td>88.8</td>
</tr>
<tr>
<td>10</td>
<td>8.76</td>
<td>68.9</td>
</tr>
<tr>
<td>30</td>
<td>8.09</td>
<td>56.2</td>
</tr>
<tr>
<td>60</td>
<td>7.41</td>
<td>45.9</td>
</tr>
<tr>
<td>90</td>
<td>6.68</td>
<td>40.3</td>
</tr>
</tbody>
</table>

Table III. Composition of the Ethanol-soluble Fraction of Untreated Chlorella and of the Effluent from the Dichlone-treated Cells

Chlorella cells labeled as a result of varying periods of ¹⁴CO₂ fixation were prepared as described in the text. Aliquots of cell suspension were extracted with ethanol while the remaining cells were treated with 30 μM dichlone for 2 hr. The ethanol-soluble fraction and the effluent were extracted with petroleum-ether to remove ¹⁴C-lipids and then analyzed for ¹⁴C-products by two-dimensional paper chromatography and autoradiography.

<table>
<thead>
<tr>
<th>¹⁴C Product</th>
<th>¹⁴CO₂ Fixation Time Prior to Dichlone Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 Min</td>
</tr>
<tr>
<td></td>
<td>Ethanol-soluble fraction</td>
</tr>
<tr>
<td></td>
<td>dpm/ml cell suspension × 10⁻²</td>
</tr>
<tr>
<td>Sucrose</td>
<td>29.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.6</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.8</td>
</tr>
<tr>
<td>Serine and glycine</td>
<td>6.2</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>5.5</td>
</tr>
<tr>
<td>Lipids</td>
<td>21.1</td>
</tr>
<tr>
<td>Succinic acid + fumaric acid</td>
<td>1.2</td>
</tr>
</tbody>
</table>

¹ Untreated cells.
² Dichlone-treated cells.
³ Not detected.

Prior to treatment with dichlone. The ethanol-soluble pool of the cell is expected to vary with the time of CO₂ fixation by the cell. Table II shows the effect of dichlone on the loss of ¹⁴C from the cells with varying ¹⁴C-pool sizes produced by ¹⁴C-assimilation for periods ranging from 2 to 90 min before treatment with dichlone. In these studies, the amount of ethanol-soluble ¹⁴C in the cell increased with an increase in the time of CO₂ fixation up to 10 min; thereafter, it gradually decreased with a further increase in the ¹⁴CO₂ fixation period. The loss of ¹⁴C from the cells which had fixed ¹⁴C for varying periods prior to quinone treatment followed the same general trend as the level of ethanol-soluble ¹⁴C in the cell. However, the changes in the ¹⁴C-efflux were not directly proportional to the changes in the level of the ethanol-soluble pool. For example, the cells which were incubated with H¹⁴CO₂ for 30 min lost considerably less ¹⁴C than the cells which had incorporated ¹⁴CO₂ for 10 min in spite of the fact that the levels of ethanol-soluble ¹⁴C in these cells before dichlone treatment did not differ markedly. It was further observed that the percentage of the total amount of ethanol-soluble ¹⁴C which leaked out of the cells decreased with an increase in the time of ¹⁴CO₂ fixation prior to quinone treatment.

Nature of Effluent Material. The composition of the ethanol-soluble fraction of untreated Chlorella which had fixed ¹⁴CO₂ for varying periods was determined and compared with that of the effluent from the cells treated with dichlone for 120 min (Table III). The results show that dichlone caused a quantitative release of sucrose, alanine, glutamine, serine, and glycine from the cell. Glutamic and aspartic acids were also released but the proportion of the two compounds lost from the cell decreased with an increase in the time of ¹⁴CO₂ fixation prior to quinone treatment. About 60% of the succinic and fumaric acids was lost; the loss of the two organic acids could be measured only in the cells which had fixed ¹⁴CO₂ for 10 min, since their pool size had decreased in the cells which had fixed ¹⁴CO₂ for longer periods. ¹³C-lipids, which represented a major proportion of the ethanol-soluble fraction of the cell, were not released by dichlone treatment. A radioactive spot near the origin of the paper chromatogram of the treated Chlorella cells appeared to be an artifact due to a high salt concentration in the medium and was not considered in the ¹³C-distribution study.

DISCUSSION

The findings of the present study have demonstrated that dichlone, at low concentrations, causes a rapid and non-reversible loss of intracellular material from Chlorella. This effect occurs at approximately the same concentrations at which cell growth, oxygen evolution, and CO₂ fixation in Chlorella are inhibited (17, 18). This similarity, together with the extreme rapidity of the dichlone effect on permeability, suggest that its primary effect may be on the permeability of the cell membrane. Since the membrane is the structural unit mainly concerned with the maintenance of selective permeability of the cell, it is reasonable to assume that this may be the principal site of action of dichlone.

The ¹³C-material released by Chlorella upon treatment with dichlone was derived from the ethanol-soluble fraction of the cell. However, not all of these ethanol-soluble compounds leaked out as a result of quinone treatment. The findings demonstrate that the membrane of dichlone-treated cells is not open to the efflux of all cellular material but permits only the release of sucrose, organic acids, and most amino acids. This selective loss may explain why the proportion of ethanol-soluble ¹³C which leaked out of the cell decreased with an increase in the time of ¹⁴CO₂ fixation (Table II). Since lipids are not released by dichlone treatment, an increase of ¹³C incorporation in lipids with an increase in the time of ¹⁴CO₂ fixation resulted in a decrease in the proportion of ¹³C which leaked out. The decrease in ¹³C-leakage may also be attributed to the fact that the proportion of aspartic and glutamic acids which leaked out decreased with an increase in the time of ¹⁴CO₂ fixation. The behavior of these two dicarboxylic amino acids may be explained by the hypothesis that in the later stages of CO₂ fixation, a portion of these compounds may become localized in certain cell sites which are not accessible to the action of dichlone. However, it seems apparent that the action of dichlone causes the efflux of relatively small molecules.
creased leakage by loss of aerobic conditions, cell greater be of a attention of with the energy weight, as well as by p-chloromercuribenzoate, p-chloromercuribenzenesulfonate, iodoacetate, and N-ethylmaleimide have been found to affect the permeability of cells (14, 15). Sutherland et al. (15) suggested that these reagents react with the sulphydryl groups of the membrane resulting in a change in its configuration. Recently, Gimmler and Avron (3) reported that treatment with benzoquinone caused changes in the cell membrane of Porphyridium cruentum as indicated by increased permeability of cells to electron acceptors and donors. These authors suggested that benzoquinone caused an alteration of the cell membrane by formation of addition products of relatively low molecular weight, as well as by cross-linking of proteins in the cell membrane.

Another mechanism which may be suggested to explain the effect of quinones on cell permeability is the interference with the energy metabolism of the cell. Dichlone and other quinones have been observed to inhibit photophosphorylation (13) and interfere with electron transport and oxidative phosphorylation (11, 15). The free pool of the intracellular material in Chlorella suspended in mineral medium is maintained against a concentration gradient, requiring the expenditure of energy. Thus, the interference by dichlone with energy-generating reactions may create a shortage of energy available for the retention of intracellular material.

Quinones have been shown to be photoreduced by illuminated isolated chloroplasts to hydroquinone via the formation of a semiquinone free radical intermediate (2). The possibility that free radicals are formed in the cells, and may damage the cell membrane, is concluded by the experiments in which the loss of cellular material from the treated cells was found to be greater under anaerobic conditions than in the presence of air. If the damage of the cell membrane had depended on the generation of free radicals, one would have expected an increased leakage under aerobic conditions since oxygen is known to increase the rate of free radical formation (5). Under anaerobic conditions, a higher concentration of reduced thiols could be expected to be present and react with quinones. If we hypothesize that dichlone alters the membrane permeability by reacting with SH groups of the membrane, one might expect a greater effect in the absence of oxygen; this was actually observed.

The evidence presented here strongly suggests that important membrane changes in Chlorella are caused by quinones. Cell permeability is altered, and the membrane could be the primary site of action of the quinones. It is, therefore, reasonable to assume that the metabolic changes previously observed in Chlorella (12, 17, 18) in the presence of quinones are partly due to changes in the cell membranes.

**LITERATURE CITED**