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A STUDY OF NATURALLY OCCURRING ALGICIDES PRODUCED BY FRESHWATER ALGAE

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ABSTRACT

The mode of action of the algicide produced by Pandorina morum was examined by exposing Volvox globator and isolates spinach chloroplasts to a partially purified algicide preparation. Oxygen evolution of Volvox, whole chloroplasts and broken chloroplasts (minus the Calvin cycle),was reduced indicating that the algicide inhibits the light reactions of photosynthesis. Oxygen evolution studies of other Volvocaceae confirmed the observation that Pandorina morum is not significantly influenced by its own inhibitor. Molecular weight approximation by gel filtration established that the inhibitor has a low molecular weight (probably below 100 mw). Field studies indicate that this algicide has tremendous potential as a control for the growth of nuisance algal growth.

DESCRIPTORS:

Algal toxins, aquatic algae*, algal poisoning*, aquatic weed control, Chlorophyta, nuisance algae, algal control*, water pollution sources

IDENTIFIERS:

Freshwater algae, pond scum

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INTRODUCTION

Factors influencing algal growth and particularly the formation of algal blooms are currently at the forefront of water pollution research. Much of this work is directed toward the control of nuisance algal growth. Investigations are under way concerning the possible roles of phosphates, nitrogenous compounds, carbonaceous materials, carbon dioxide levels, and other environmental factors such as pH, light intensity, and limiting micronutrients. Harder (1917) was one of the first to postulate that algal products may play a role in controlling populations of algae.

Since Harder's work with Nostoc punctiforme, many investigators have examined algal-produced substances that inhibit the growth of the producing species (auto-inhibition) and/or other species (hetero-inhibition). Lefevre (1932, 1937, 1949) demonstrated that some laboratory cultured algae produce substances that limit or check their own rates of cell division and possess bacterio-static properties. Denffer (1948) and Jorgensen (1956), Mast and Pace (1938), Levring (1945), Pratt and Fong (1940; Pratt, 1942, 1944), and Harris (1970a) have demonstrated auto-inhibitory substances for

Nitzchia, Chilomonas, Skelotonema, Chlorella vulgaris, and Platydorina caudata respectively.

Rice (1954) reported that Nitzchia, Chlorella, and Pandorina morum produce substances that inhibit the growth of other species. Proctor (1957) demonstrated that growth inhibition in Haematococcus pluvialis by Chlamydomonas reinhardtii was due to the liberation of a fatty acid upon the death of the Chlamydomonas cells. Chlorellin, the growth inhibiting substance produced by Chlorella, has been shown to be an oxidation product of unsaturated fatty acids (Spoehr et. al., 1949).

Harris (1970a) observed that Platydorina caudata produced a heat labile, auto-inhibitory substance and conducted a survey of other members of the family (Volvocaceae) to ascertain if algicidal substances were of common occurrence in this group of the Chlorophyta. Pandorina morum, of the genera examined, produced the most potent inhibitor and Volvox tertius was the most sensitive (Harris, 1970b). This combination became the basis for a model system to investigate the inhibitor (Harris, 1971a).

Thus far little is known concerning the possible mode of action of algal inhibitors. However, Swanson (1943) did demonstrate that chlorellin reduced respiration rates in Chlorella. Harris (1971b) demonstrated that the inhibitor produced by Pandorina morum reduced the rate of oxygen evolution of Volvox colonies.

The purpose of this investigation is to elucidate further the mode of action of the inhibitor produced by Pandorina morum. The two major systems involved are the Pandorina-Volvox model and isolated spinach chloroplasts.

MATERIALS AND METHODS

Strains

The strains of *Volvox globator* (LB 106) and *Pandorina morum* (18) employed in this investigation were obtained from the Culture Collection of Algae at Indiana University.

Culture techniques

Stocks were maintained in 18 x 150 mm culture tubes containing a small amount of sodium bicarbonate and enough soil to form a bottom layer approximately 1 cm in depth. The tubes were filled with glass-distilled water and heated at 98°C for 2 hr on two consecutive days prior to inoculation.

Axenic stocks were obtained by transferring individual colonies by micropipette through 10 washes of sterile, modified *Volvox* medium, hereafter referred to as V2N medium (Palmer and Starr, 1971; Provasoli and Pintner, 1959).

Axenic cultures were maintained in an incubator at 20-24°C in 18 x 150 mm culture tubes containing 10 ml V2N. A regime of 12 hr light and 12 hr dark was provided. Soil water and axenic stocks were routinely transferred after 20 days growth.

Experimental cultures of Pandorina morum were maintained in 1500 ml V2N in three liter Fernbach culture flasks. Each inoculum consisted of 10 ml of 5-6 day old cultures of P. morum. Volvox globator cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml V2N and bubbled with air containing 1% CO_2 for 10 days. The cultures were kept in continuous light to obtain dense cultures.

Extraction of inhibitor

After 21-23 days growth, P. morum cultures were harvested by centrifugation and the supernatant discarded. The cells were then resuspended in 15 ml of glass-distilled H_2O and sonicated on a Biosonik IV cell disintegrator (Bronwill Indus., Rochester, N.Y.). Excessive heat buildup was avoided by immersion of the sonication vessel in ice and by sonicating for only 30 sec at a time. A large probe of 2 cm diameter, maximum power settings, and 90 sec total sonication produced roughly 90% cell disruption. Cell debris was removed by centrifugation and the resulting dark green extract filtered through a millipore membrane (pore size of final membrane -0.22μ: Millipore Corp., Bedford, Mass.) to obtain a clear, light green, cell-free extract.

Partial purification

Sephadex gel filtration was used to remove substances of high molecular weight. Forty grams G-25

Sephadex were soaked in H_2O for a minimum of 24 hr and poured into a column with a resulting bed volume of 205 ml (Pharmacia Chemicals, Uppsala, Sweden; Column K 25/45).

After packing the column and void volume determinations, 5 ml cell-free extract was applied to the gel surface and eluted with glass-distilled water. Fractions of 10 ml each were collected and bioassayed. The fractions routinely containing the inhibitor (121-170 ml) were combined and flash evaporated to a final volume of 5 ml.

Calibration of G-10 Sephadex column

The standard column consisted of 90 g water-soaked G-10 poured into column K 25/45. The inhibitor was applied as the whole-cell extract and as the G-25 fractions 121-170 ml flash evaporated to 5 ml. Void volumes were checked periodically with Dextran 2000. The column was calibrated by application of 20μM solutions of known amino acids that were detected in the fractions with 1 ml of 0.5% ninhydrin in a 0.2M acetate buffer (pH 5). $AgNO_3$ was used to detect Cl^- in $MgCl_2$ and NaCl solutions and PO_4 in K_2HPO_4 and KH_2PO_4 solutions. The same column was used for all experiments.

Bioassay for inhibitor

To assay for the inhibitor, 5 ml fresh V2N was added to 5 ml of the fraction to be tested and the tube

either autoclaved for 15 min or sterilized by millipore filtration. Each fraction was inoculated with a few drops of axenic Volvox and within 3-5 days the presence of the inhibitor could be detected by the reduction of growth in the tube. Controls consisted of 5 ml V2N added to 5 ml H_2O.

Oxygen evolution

Oxygen evolution provided an additional means of assaying for the inhibitor but was utilized primarily to examine the effects of the inhibitor on photosynthesis of Volvox and isolated spinach chloroplasts. For studies of the inhibitor's effects on Volvox, the fraction to be examined or the flash-evaporated concentrate was re-millipored to eliminate bacteria and added to an equal volume of a concentrated V. globator culture. Aliquots of 1.2 ml of this suspension were placed in the chamber of a Clark-type oxygen polarograph (Schwartz, 1965; Clark, et. al., 1953; Estabrook, 1967). The water-jacketed lucite chamber was maintained at 25°C by a water bath. Illumination was provided by a tungsten lamp which was focused on the front of the electrode chamber. Heat was removed from the light by use of a heat absorbing IR filter. Incubation of the preparation, when appropriate, was carried out in the dark in a water bath maintained at 25°C.

The electrode was calibrated with air-saturated water using oxygen solubility data with appropriate temperature and barometric pressure adjustments. Chlorophyll content was determined by the procedure outlined by Arnon (1949) using extinction coefficients for chlorophyll a and chlorophyll b in 80% acetone (MacKinney, 1941). One-tenth milliliter suspension was withdrawn and diluted to 10 ml with 80% acetone. The suspension was filtered (Whatman No. 1) after 5 min and the OD measured at 645 nm and 663 nm.

$$C_b = 22.9\ (OD_{645}) - 4.68\ (OD_{663})\ \mu g/ml$$

$$C_a = 12.7\ (OD_{663}) - 2.69\ (OD_{645})\ \mu g/ml$$

Spinach chloroplasts

The procedures used were those outlined by Jensen and Bassham (1966). Some modifications were necessary to insure that proper controls could be compared with inhibitor preparations. Four solutions were used for the grinding, resuspension, and assay procedures. Solution A was used to grind the spinach leaves; solution B to resuspend the isolated chloroplasts; solution C for control reaction mixtures of whole chloroplasts; and solution C2X for reaction mixtures containing the inhibitor and whole chloroplasts. Solutions A, B, and C contained the following: 0.33M sorbitol (Sigma Chemical Co.); 0.002M EDTA (Na_2); 0.001M $MnCl_2$; 0.001M $MgCl_2$; 0.002M $NaNO_3$; and 0.005M K_2HPO_4. Solution C2X

contained twice the concentrations of each of these components.

In addition, solution A contained: 0.05M MES [2-(N-morpholino) ethanesulfonic acid] (Sigma); 0.02M NaCl; and was adjusted to pH 6.1. Solution B contained 0.05M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) (Sigma); 0.02M NaCl; and was adjusted with NaOH to pH 6.7. Solution C contained 0.05M Tricine (Sigma) and was adjusted with NaOH to pH 8.1. Solution C2X contained twice the concentration of Tricine as found in Solution C and was also adjusted with NaOH to pH 8.1.

A stock solution (I) containing the EDTA, $MnCl_2$, $MgCl_2$, K_2HPO_4, and $NaNO_3$ was prepared in advance and added to the additional components and sorbitol just prior to each experiment. A separate stock solution (II) was prepared in advance with twice the concentrations of stock solution I and added to double the sorbitol and Tricine concentrations of solution C to make solution C2X just prior to each experiment.

Isolation of plastids

Commercial spinach leaves were washed and placed in a shallow pan of icewater to render the leaves turgid. The leaves were illuminated in sunlight or under a flood lamp for 10-20 min prior to grinding in order to fill intermediate pools. The midribs were removed and 7 g

of lamina were torn into small pieces and placed in a chilled semi-micro blending cup with 30 ml chilled solution A. A #8 rubber stopper held in place by a glass stirring rod effectively reduced the volume of the blending cup so that 5 sec blending on a Waring blender yielded a well-ground slurry. This slurry was rapidly poured and squeezed through 6 layers of chilled cheese-cloth into a chilled tube and centrifuged for 50 sec at 2000 x g in a refrigerated centrifuge (Sorvall RC2-B).

The supernatant was discarded and the pellet gently resuspended in a 2 ml chilled solution B. This entire resuspension procedure was carried out in the dark and the final resuspension tube wrapped in aluminum foil and placed on ice. Although this plastid preparation may be stable for some time, it was not stored but used immediately. This preparation, when examined under a microscope, appeared to be mostly unbroken chloroplasts.

Photosynthesis of whole chloroplasts

Photosynthetic rates were monitored by following oxygen uptake and evolution of a suspension of whole chloroplasts in an isotonic solution. The control reaction mixture consisted of 2.7 ml solution C; 0.02 ml 0.3M $NaHCO_3$; and 0.02 ml 0.25M sodium pyrophosphate. This preparation was bubbled with nitrogen for 5 min prior to the addition of 0.15 ml 0.05M PGA and 0.1 ml of the chloroplast suspension. The chloroplast suspension was added in the dark. The only modification of this

procedure for exposure of chloroplasts to the inhibitor was to add only 1.35 ml solution C2X and 1.35 ml inhibitor preparation. The volume and concentrations of components were the same in control and inhibitor preparations. Saturation curves for intermediates were made to determine how much was necessary for each preparation.

Photosynthesis of broken chloroplasts

Broken chloroplasts were prepared by placing the plastids in a hypotonic solution. The reaction mixture consisted of: 0.8 ml 0.05M Tris-HCl (pH 8); 0.1 ml 0.04M $MgCl_2$; 0.05 ml 0.1M $Na_4P_2O_7 \cdot 10\ H_2O$; and 1.4 ml distilled water or inhibitor preparation. If the inhibitor preparation had a pH that differed from that of distilled water the pH of the water was adjusted with HCl to match the pH of the inhibitor preparation. This reaction mixture was bubbled with nitrogen for 5 min prior to the addition of, in order: 0.1 ml 0.03M NADP; 0.1 ml 0.03M ADP; 0.1 ml ferredoxin (0.5 mg/ml); and 0.1 ml chloroplast suspension. All biochemicals were purchased from Sigma Chemical Company.

RESULTS

Effect of inhibitor on O_2 evolution of VOLVOX

A thick Volvox suspension was thoroughly mixed and divided into two equal parts. One portion was exposed to the inhibitor by addition of the flash-evaporated G-25 Sephadex fractions (121-170 ml, hereafter referred to as G-25FE). An equal volume of distilled water was added to the other portion as a control. Readings were taken by removing 1.2 ml of the appropriate suspension at intervals and placing them in the electrode chamber. Respiration was calculated from a 2 min dark period following the illumination interval. Results of a typical experiment are shown in Figure 1 and Table 1.

Table 1 Effect of G-25FE on O_2 evolution of Volvox
Quantitated data from Figure 1.

Minutes Exposure	Control (H_2O)	Inhibitor (G-25FE)	% Reduction	Inhibitor (1/2)**	% Reduction
10	112.23*	56.82	49	83.82	28
30	120.73	65.99	45		

*Adjusted rate of O_2 evolution μmoles $O_2/\mu g$ chl/hr x 10^{-3}

**G-25FE diluted by addition of equal volume H_2O

Figure 1. Reproduction of actual tracing of O_2 evolution of Volvox globator.

C = control (H_2O added)
I = inhibitor (concentrated G-25FE added)
Subscript = incubation time (min)

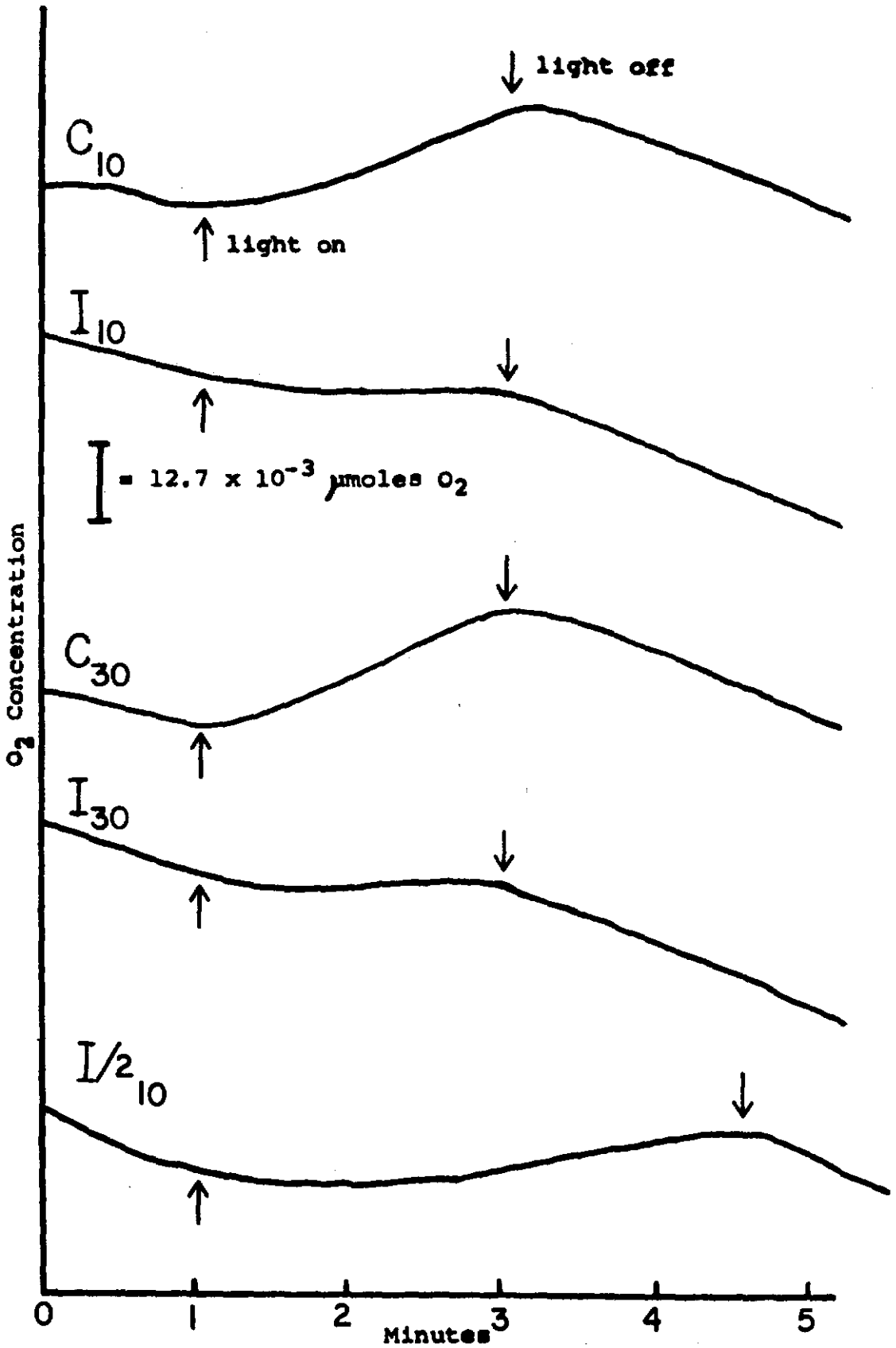

light off
C_{10}
light on
I_{10}
= 12.7 x 10^{-3} μmoles O_2
C_{30}
I_{30}
$I/2_{10}$
O_2 Concentration
0
1
2
3
4
5
Minutes

Figure 1

Effect of concentration of inhibitor (10x) and exposure time on 0_2 evolution of VOLVOX

Larger volumes of concentrated Volvox cultures were exposed to either the G-25FE or to a single 10 ml active fraction (141-150 ml). Readings were taken at intervals over periods of 4-6 hrs. Figure 2 shows the results of these experiments.

Effect of inhibitor on isolated intact spinach chloroplasts

A preparation of whole chloroplasts was exposed to the inhibitor by addition of 1.35 ml solution C2X and 1.35 ml G-25FE to the reaction mixture. Controls had 2.7 ml of solution C added. Readings of 0_2 evolution were made immediately and also after 15 min incubation. In each case the final reading was from the control preparation to insure that degradation of the chloroplast preparation was not responsible for any change in activity. Results of a typical experiment are summarized in Table 2.

Table 2 Effect of G-25FE on 0_2 evolution of intact isolated spinach chloroplasts.

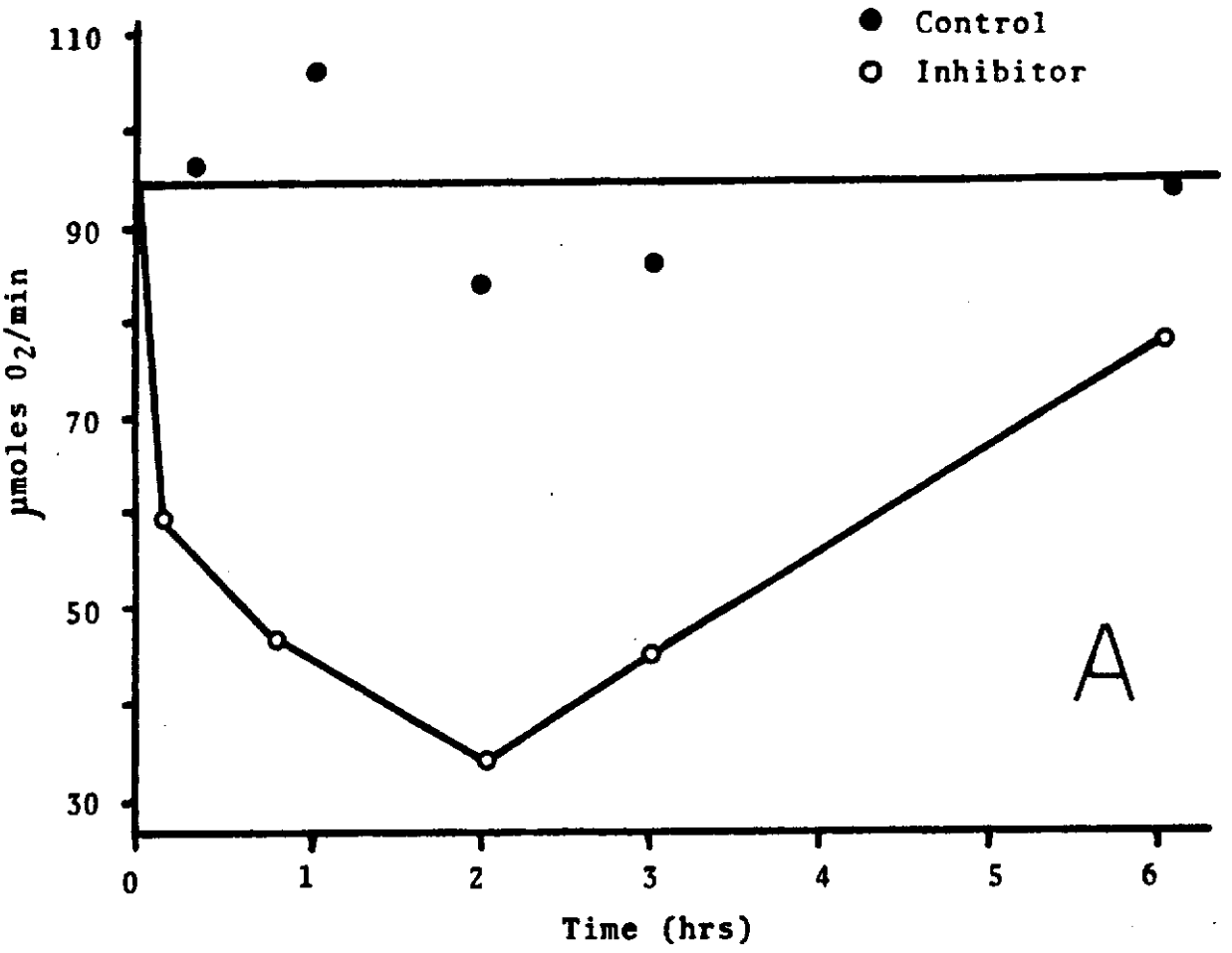
Minutes Exposure	Control (H_2O)	Inhibitor (G-25FE)	% Reduction
1	29.67*	17.29	42
15	27.09	16.77	40

*Adjusted rate of 0_2 evolution μmoles $0_2/\mu$g chl/hr x 10^{-3}

Figure 2.

A. Effect of single active fraction from G-25 gel filtration (141-150 ml) on O_2 evolution of Volvox globator.

B. Effect of concentrated inhibitor (G-25FE) on O_2 evolution of Volvox.


Control
Inhibitor
110
90
70
50
30
µmoles O_2/min
0
1
2
3
4
5
6
Time (hrs)
A

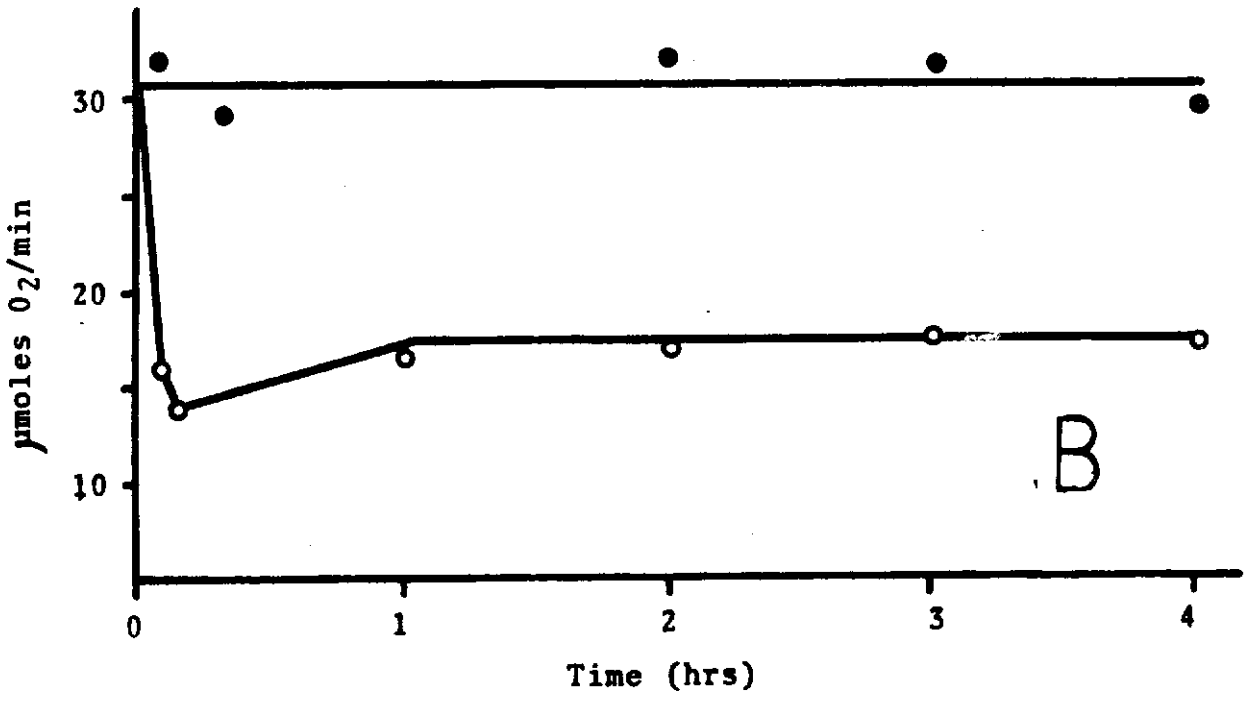

30
20
10
µmoles O_2/min
0
1
2
3
4
Time (hrs)
B

Figure 2

Effect of the inhibitor on broken chloroplasts

The dependence of O_2 evolution on the Calvin cycle associated with whole chloroplasts, was removed by breaking the chloroplast with a hypotonic solution and adding intermediates. Portions were exposed to the inhibitor by addition of G-25FE. Addition of distilled water constituted the controls. Oxygen evolution readings were taken at intervals ranging from immediate reading to 10 min exposure. Results are summarized in Table 3.

Table 3 Effect of G-25FE on O_2 evolution of broken isolated spinach chloroplasts.

Minutes Exposure	Control (H_2O)	Inhibitor (G-25FE)	% Reduction
1	26.03*	13.27	50
5	19.90	11.73	40
10	14.29	7.35	50

*Adjusted rate of O_2 evolution μmoles O_2/μg chl/hr x 10^{-3}

Effect of inhibitor on O_2 evolution of other members of the Volvocaceae

The procedures outlined for Volvox were followed for other members of the family. Gonium pectorale, Eudorina cylindrica, and Pandorina morum were exposed to the inhibitor. Results of these exposures are summarized in Figure 3.

Figure 3. Effect of inhibitor (G-25FE) on 0_2 evolution of some other Volvocaceae including the producer, Pandorina morum.

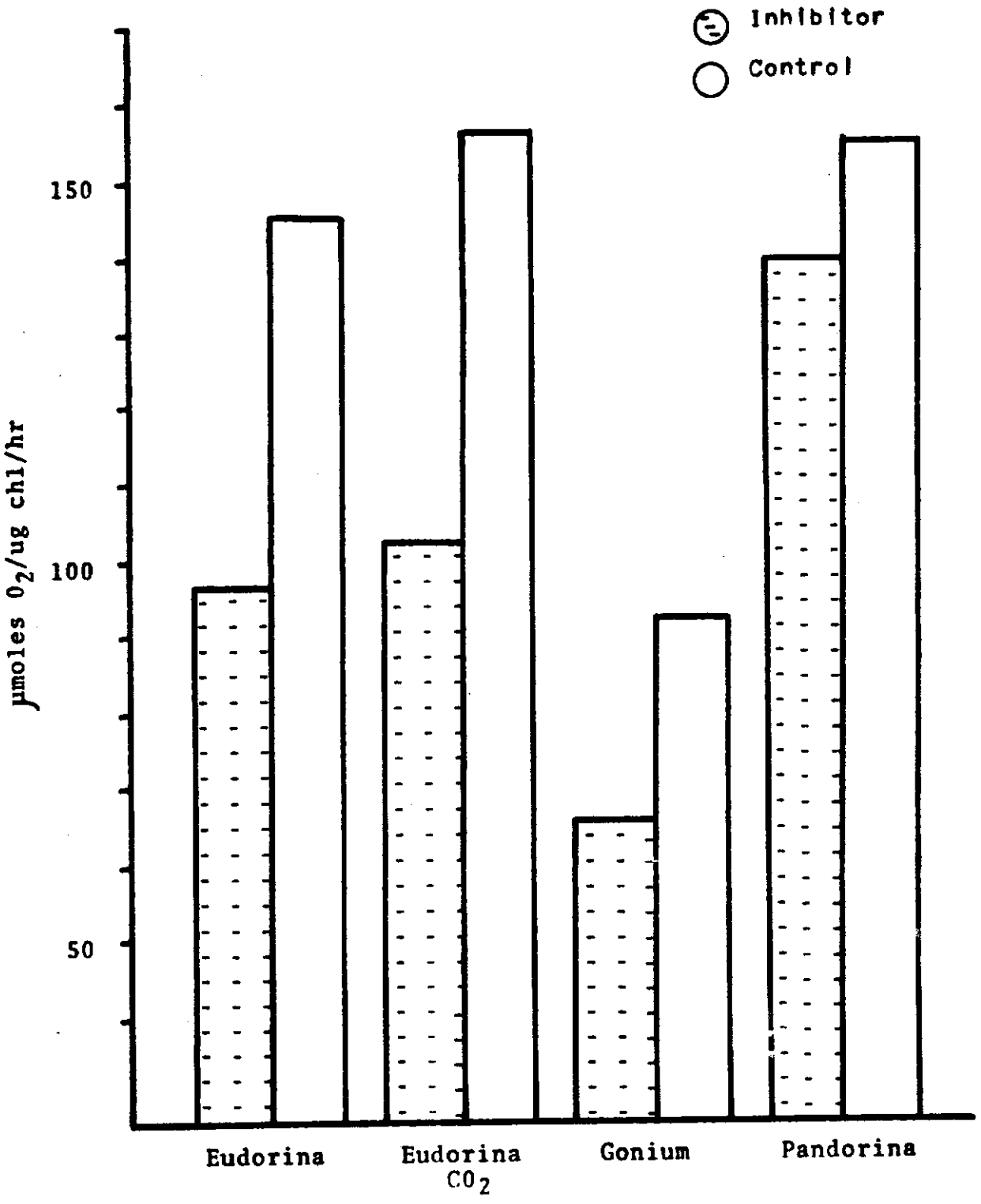

Inhibitor
Control
150
100
50
µmoles O_2/ug chl/hr
Eudorina
Eudorina CO_2
Gonium
Pandorina

Figure 3

Molecular weight approximation

G-25 Sephadex - The standard column of 40 g gel routinely had a void volume of 70 ml. Bioassay of the 10 ml fractions indicated that the inhibitor was present in fractions ranging from 121-150 ml. Variation was never greater than a single 10 ml fraction. The column was not calibrated with standards. However, the active fraction was always ninhydrin-positive, indicating the presence of amino acids. Sephadex G-25 does not resolve molecular weights below 1500; therefore, G-10 was chosen to do a molecular weight approximation.

G-10 Sephadex - The standard column of 90 g gel routinely had a void volume of 68 ml. Bioassay of 2 ml and 4 ml fractions indicated that the inhibitor was present in fractions ranging from 123-160 ml. The column was calibrated with amino acids and other detectable compounds. The results of the calibration are shown in Figure 4.

Figure 4. Calibration of G-10 Sephadex column for molecular weight determination of inhibitor.

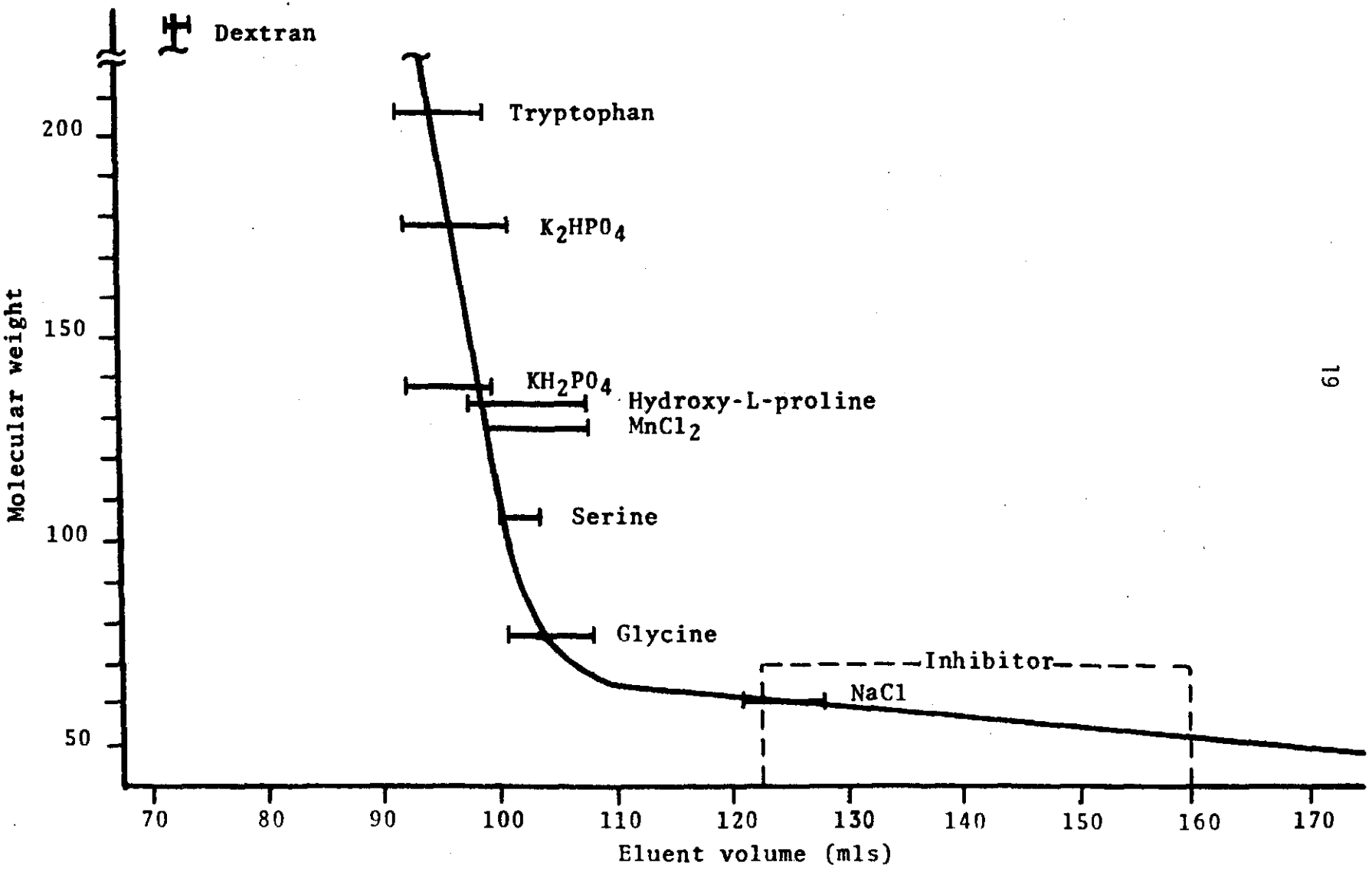

Dextran
Tryptophan
K_2HPO_4
KH_2PO_4
Hydroxy-L-proline
$MnCl_2$
Serine
Glycine
Inhibitor
NaCl
Molecular weight
200
150
100
50
70
80
90
100
110
120
130
140
150
160
170
Eluent volume (mls)

Figure 4

DISCUSSION

Since the first observations of inhibition caused by Pandorina morum (Lefevre et. al., 1949; Rice, 1954), work has continued to elucidate the role, mode of action, and identity of the inhibitor. Preliminary investigations of the Pandorina-Volvox model system proposed by Harris (1971a) have yielded the following information: (1) in cultures grown under the described conditions, the inhibitor appears on the 12th day and persists for the next 14 days (2) the inhibitor is relatively heat stable (10 min autoclaving slightly reduces activity) (3) activity is not affected by proteolytic enzymes (trypsin, chymotrypsin, pronase) (4) the substance passes through a dialysis membrane (5) the substance reduces photosynthetic 0_2 evolution rates in Volvox globator. The results of the present study provide additional information about the mode of action of the inhibitor and an approximation of its molecular weight.

Figure 1 is a reproduction of an actual tracing from an experiment involving exposure of Volvox to the concentrated G-25FE. Table 1 is a quantitation of this data.

The apparent photosynthesis (0_2 evolution during the light period) is the result of both 0_2 evolution and uptake occurring simultaneously. To correct for this, the rate of uptake during the dark period (negative slope) is subtracted from the rate of evolution (positive slope) during the light period. This adjusted rate of photosynthesis on a μmole 0_2/μg chl/hr basis is used for comparisons throughout this work.

For this experiment and others that follow the amount of total chlorophyll and the ratio of chl a/chl b was compared for cultures with and without the inhibitor present. After two hours the total chlorophyll and the chl a/chl b ratio remained the same. The observations, therefore, were not due to any measurable change in chlorophyll content.

Figure 1 and Table 1 show that the inhibitor does reduce the overall rate of 0_2 evolution in Volvox. However, Figure 1 is atypical in one respect. It suggests that the rate of 0_2 evolution is reduced to zero during the light period when the inhibitor is present. This indicates that the effect of the inhibitor is to increase respiration rather than decrease 0_2 evolution. Other experiments with Volvox and spinach chloroplasts discussed later show that this is not the case. The rates of photosynthesis and respiration just happened to be the same. In addition, Figure 1 and Table 1 show the same

G-25FE inhibitor preparation diluted to one-half concentration with distilled water. This suggests that the reduction of 0_2 evolution is proportional to the amount of inhibitor present. The exposure times were too short for positive conclusions, but both concentration and exposure time are examined and compared in Figure 2.

Graph A in Figure 2 is the result of exposure of Volvox to a single 10 ml fraction (141-150 ml) from the G-25 column. Although the control culture did not give a straight line, the initial and final readings differ by only 12×10^{-3} μmoles 0_2/min. Note also that the final reading after 6 hr is actually higher than the initial reading. Within 1 min exposure to the active fraction, 0_2 evolution dropped dramatically. The maximum inhibition occurred after 2 hr exposure (59% reduction over 2 hr control). However, the 0_2 evolution rate increased during the next 4 hr until at 6 hr exposure the inhibitor preparation was within 17% of the control preparation. A possible explanation is that at lower concentrations, without a constant source of the inhibitor, enough of the inhibitor is metabolized or inactivated to allow the cells to recover. Graph B in Figure 2 shows the result of exposure to the more concentrated G-25FE. In this case the rate of 0_2 evolution drops 50% within the first minute and remains at or near 50% inhibition for the entire 4 hr period.

This data suggests that reduction of O_2 evolution caused by low concentrations of the inhibitor and without a constant source may be partially overcome by the cells, but that higher concentrations prevent recovery for longer periods.

Isolated spinach chloroplasts provided a means for obtaining a system that was less complicated than whole Volvox colonies that may consist of thousands of individual cells. Since the green algae (Chlorophyta) and higher plants have the same photosynthetic pigments and chloroplast structure, spinach chloroplasts constituted a valid, simplified system. Whole chloroplasts were, therefore, prepared and exposed to the inhibitor. The data in Table 2 shows that the inhibitor reduces O_2 evolution by 40-42% in whole chloroplasts.

Incubation time, temperature, light exposure, and pH were the same for both control and inhibitor preparations. Since whole chloroplasts contain all the cell machinery for the Calvin cycle and photoreactions, the system was further simplified by breaking chloroplasts as outlined in the materials and methods section.

When the Calvin cycle enzymes are diluted into the reaction mixture, the reduction of O_2 evolution would suggest that the effect of the inhibitor is upon the light reactions. The results of a typical experiment that are summarized in Table 3 show this to be the case.

Reduction in O_2 evolution ranges from 40-50% over a 10 min period in broken chloroplasts.

In his original survey Harris (1970b) showed that although Pandorina morum inhibited other members of the Volvocaceae, it did not show auto-inhibition. In view of this observation it was decided to observe the inhibitor's effects on O_2 evolution of other Volvocaceae and upon Pandorina itself. Cultures of Eudorina cylindrica, Gonium pectorale, and Pandorina morum were exposed to the G-25FE. Figure 3 shows that the rates of O_2 evolution of Gonium and Eudorina were markedly reduced by the inhibitor while Pandorina was relatively insensitive. Although this is expected, it remains unexplained. An additional observation was made during these experiments. When a 2 month old culture of Pandorina was exposed to the inhibitor, the rates of O_2 evolution were reduced 31% after 5 min exposure. If the inhibitor provides an advantage over other organisms during early growth stages, perhaps it functions later in the final growth stages as a population stabilizer. An alternative explanation is that the older cells are simply weaker in general and subsequently more easily affected. Since the kinetics of inhibitor production show that the concentration of inhibitor drops after 23 days under culture conditions, the second alternative seems more likely. However, culture conditions do not duplicate natural

conditions, and at this point the importance of the inhibitor in a particular environment is as yet unknown.

Figure 4 also includes two readings for the Eudorina culture where 0.1 ml 0.1M Na_2HCO_3 was added to 2 ml of the suspension. The overall rates of O_2 evolution are not significantly different from cultures without added CO_2 indicating that the cultures are not CO_2 limited and that addition of CO_2 does not negate the effects of the inhibitor.

Along with experiments concerned with O_2 evolution of whole cells and isolated spinach chloroplasts, the behavior of the inhibitor during gel filtration suggested additional possibilities and questions.

Although the use of G-25 gel filtration was primarily intended to reduce the molecular size range of substances appearing in the inhibitor preparation, some characteristics of the substance were suggested by a positive ninhydrin response of the active fractions. This reaction was always observed in the active fraction and was successfully used as a preliminary assay for the inhibitor. There are two obvious possibilities: (1) the substance is an amino acid or ninhydrin positive (2) the substance is not an amino acid or ninhydrin positive, but its molecular size is close to that of amino acids, and it subsequently accompanies them through the gel column. Amino acid analysis by descending paper chromatography and thin layer chromatography on cellulose-coated plates

did not rule out the first possibility. Five ninhydrin-positive spots appeared but could not be positively identified by simultaneously running known amino acids. Automatic amino acid analysis of the hydrolyzed G-25FE revealed the definite presence of 16 amino acids and one unidentified peak less basic than phenylolanine. The second possibility was tested by eluting the G-25FE or the whole-cell extract on G-10 Sephadex. This gel theoretically fractionates compounds from 0-700 molecular weight. The ninhydrin-positive spots appeared before the fractions containing the inhibitor but were very faint in some fractions indicating that this assay may not be sensitive enough to rule out the possibility of the inhibitor being ninhydrin positive. Calibration of the column with known amino acids, Cl^- containing compounds (white ppt with $AgNO_3$), and PO_4 containing compounds (yellow ppt with $AgNO_3$) suggested that the inhibitor was a low molecular weight substance (probably below 100 mw; Figure 4). What this substance might be or exactly how and where it has its effect is still highly speculative.

Since isolated spinach chloroplasts are suitable for study, it would be possible to further pinpoint the action site of the inhibitor by isolating photosystem I and II. This can be accomplished by utilizing selective inhibitors such as DCMU or separating System I and System II particles by digitonin incubation and differential

ultracentrifugation (Anderson and Boardman, 1966). Another method would be utilization of the Scenedesmus photosynthetic mutants 8 and 11 that lack System I and System II activity respectively (Bishop, 1971).

Active fractions from G-10 gel filtration should contain the inhibitor in a relatively pure state and may make the identification of the substance possible.

The greatest problem to date is that there is as yet no reliable means of assaying for the inhibitor other than bioassay. The G-25 active fraction does have an absorption peak at 275 nm, but there has not been sufficient testing for a positive correlation between the appearance of this peak and the presence of the inhibitor.

SUMMARY

This investigation has yielded the following additional information concerning the inhibitor:

(1) The substance inhibits O_2 evolution of isolated intact spinach chloroplasts.

(2) Inhibition of O_2 evolution also occurs in isolated spinach chloroplasts that have been broken to remove Calvin cycle enzymes. This suggests that the site of inhibition is in the light reactions of photosynthesis.

(3) Auto-inhibition of Pandorina morum was not observed with culture filtrates, and O_2 evolution studies indicated that P. morum was relatively insensitive to its own inhibitor.

(4) The inhibitor has a low molecular weight (probably below 100).

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