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THE EFFECT OF PHOTOSYNTHESIS INHIBITORS ON OXYGEN EVOLUTION AND FLUORESCENCE OF ILLUMINATED CHLORELLA

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SUMMARY

Photosynthesis inhibitors, like symmetrical triazines, substituted ureas, and anilides, are able to stop oxygen evolution from illuminated Chlorella and cause a stimulation in fluorescence. These two phenomena seem to be inter-related as shown by partial inhibition studies. The exception to the rule was found to be cyanide which inhibited oxygen evolution but had no effect on fluorescence intensity.

INTRODUCTION

The effect of symmetrical triazines and substituted ureas on $^{14}CO_2$ fixation by excised leaves of treated bean plants has been studied recently[1,2]. Photosynthetic $^{14}CO_2$-fixation was completely inhibited by Atrazine (2-chloro-4-ethylamino-6- isopropylamino-1,3,5-triazine), whereas syntheses of aspartic and glutamic acids, presumably dark reactions, were unimpeded[3]. Previously it had been shown that DCMU and the symmetrical 2-chloro-triazines[4] inhibited photosynthetic oxygen evolution by isolated chloroplasts. Some evidence had also been presented that Simazine (2-chloro-4,6-bis-(ethylamino)-1,3,5-triazine) inhibited the photosynthetic reduction of pyridine nucleotides. GOOD[5] has made a comprehensive study on the effect of 147 different photosynthesis inhibitors on the Hill reaction (using ferricyanide as oxidant) and on photosynthetic phosphorylation.

It was of interest, therefore, to test a representative number of these photosynthetic inhibitors, in addition to several other anilides, on the primary photochemical step in photosynthesis. In the studies reported here *Chlorella pyrenoidosa* was chosen as test organism, to study if the effect of these inhibitors on light-induced fluorescence was altered in any way. A similar approach has been used recently by SWEETSER *et al.*[7] who showed that Monuron (3-(*p*-chlorophenyl)-1,1-dimethylurea) did indeed have an effect on the decay of re-emitted light from algae.

EXPERIMENTAL METHODS

Culturing of algae

Chlorella pyrenoidosa was grown in pure cultures in 2-l Erlenmeyer flasks capped with cotton plugs, agitated with a New Brunswick shaker and continuously illuminated from above with fluorescent lights at about 1000 foot candles. The inorganic

Abbreviation: DCMU, 3,4-dichlorophenyl, N,N-dimethylurea.

medium[8] consisted of 0.025 M KNO_3, 0.018 M KH_2PO_4, 0.020 M $MgSO_4$, 0.001 M $CaCl_2$, $9 \cdot 10^{-6}$ M EDTA 12% Fe ("Sequestrene") and 1 ml/l of Hoagland's A-Z solution of minor elements. The cells were harvested once a week by centrifugation at 1000 × *g* and resuspended in an appropriate volume of bicarbonate buffer (pH 8.4) (0.1 M $KHCO_3$ – 0.1 M $NaHCO_3$ (65:35 v/v)) to a known cell density Cell density was determined with a hemacytometer.

Photosynthesis measurements

A refrigerated Warburg apparatus (Aminco) was fitted with a Pyrex glass bottom, and light was provided from below by 10 evenly spaced 75-W reflector lamps. Additional cooling was provided by two squirrel-cage fans and an infrared-absorbing glass plate mounted above the light bulbs. Most studies, unless specified, were carried out at 25° ± 0.05 at a light intensity of 1000 foot-candles.

To determine photosynthetic rates of an algal culture, 1 ml of Chlorella cell suspension containing about $6 \cdot 10^8$ cells and 2 ml of bicarbonate buffer were placed in the main compartment of a 15-ml Warburg manometric flask. For inhibitor studies 1 ml of buffer was replaced by 1 ml of inhibitor in buffer at appropriate concentrations. Some inhibitors, due to water-insolubility, are first dissolved in 1 ml of ethanol, diluted with bicarbonate buffer, so that the final concentration of ethanol never exceeded 1% (v/v). PARDEE's buffer[9] (0.6 ml) was pipetted into the center well in order to maintain a concentration of 1% CO_2 in the atmosphere. Dark controls were run simultaneously.

The flasks were equilibrated for 10 min with the lights on, and readings were taken in 10-min intervals thereafter. Most experiments were terminated after 1 h.

Fluorescense measurements

A commercial spectrophotofluorometer (Aminco-Bowman) was used for most studies. Relative fluorescence at equilibrium was measured with a commercial photomultiplier microphotometer or for short-term time studies with an oscillograph (DuMont 304A Cathode-Ray) equipped with a polaroid camera attachment. All measurements were taken at room temperature, 24–26°.

3 ml of a Chlorella cell suspension, containing $2 \cdot 10^7$ cells/ml, were pipetted into the sample cell (quartz tube). The tube was placed into the sample housing and the cover closed. The activation and fluorescence wave lengths set respectively at 480 mμ and 689 mμ. The phototube shutter was kept open. At time "zero" the manual cell compartment shutter was opened by pushing the shaft down as quickly as possible. At the same time the camera shutter was opened and a 2-sec time-exposure of the fluorescence spectrum was taken.

For studying the effect of photosynthesis inhibitors on fluorescence by illuminated Chlorella, the above procedure was repeated, using a Chlorella suspension containing appropriate amounts of inhibitors.

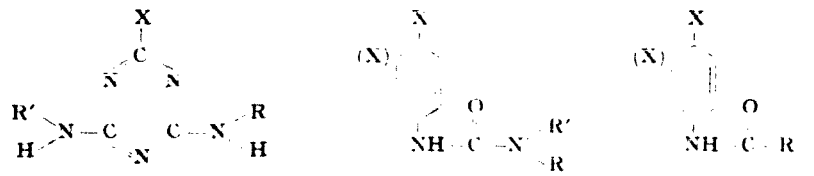
EXPERIMENTAL RESULTS

Inhibition of photosynthesis

The manometric measurement of evolved oxygen from photosynthesizing Chlorella was chosen as the most convenient method for photosynthesis rate studies with

whole cells. Since the rate of oxygen evolution is dependent on cell density, light intensity and temperature, a preliminary study showed the following: at an ambient temperature of 25° ± 0.05, cell density of $6 \cdot 10^8$ cells/3 ml and an approximate light intensity of 1000 foot candles, 45 μl O_2 was evolved in a 10-min period. This rate was relatively constant within the experimental errors of manometry for at least 60 min. During this time the pH of the Chlorella suspension did not increase by more than 0.1 pH unit. Before using PARDEE's buffer[9] in the center well, the photosynthetic rate of Chlorella decreased continuously even during a 60-min interval. This decrease was explained by the depletion of CO_2 in the buffer and the resultant rapid increase in pH.

Photosynthesis rates were next studied with several representative photosynthesis inhibitors added to the Chlorella suspension at final concentrations ranging from 10^{-5} M to 10^{-8} M. Three classes of compounds were investigated with the following structures:



Symmetrical triazines (I) | Substituted ureas (II) | Anilides (III)

X = halogen
R = alkyl

Evolved oxygen, corrected for respiration from dark controls, was measured for 1 h and the percent inhibition was calculated using the following simple equation:

$$\text{Percent inhibition } (I) = \left\{1 - \frac{\mu l\ O_{2(\text{control})} - \mu l\ O_{2(\text{treatment})}}{\mu l\ O_{2(\text{control})}}\right\} 100$$

By plotting percent inhibition against the logarithm of the concentration, straight line plots were obtained in each case (*cf.* Fig. 1). pI_{50} Values were calculated by the following equation:

$$pI_{50} = -\log \text{concn. } I_{50}.$$

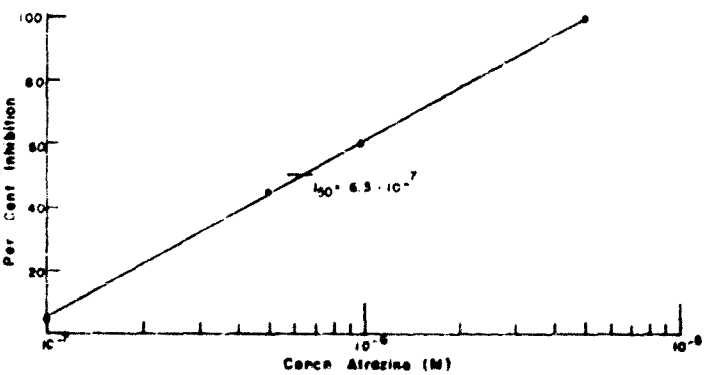
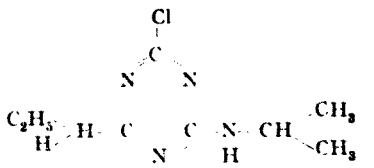
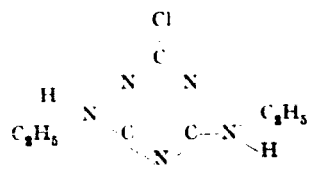
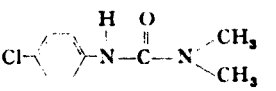
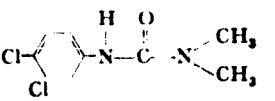
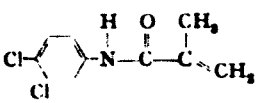


Fig. 1. Effect of different concentrations of Atrazine on oxygen evolution by Chlorella.

where conc. I_{50} is the concentration of inhibitor which inhibited oxygen evolution 50%, as compared to the control. Table I lists pI_{50} values for several compounds studied which are of the same order of magnitude as pI_{50} values from Hill reaction studies using isolated chloroplasts and ferricyanide reduction as a measure of activity[6]. These results are further discussed in connection with fluorescence studies. The compounds which were tested gave pI_{50} values ranging from 5.73 to 6.77 for members of all three classes of photosynthesis inhibitors tested. One exception was KCN which gave a pI_{50} of 3.6 making it a much poorer inhibitor than the other compounds tested (Table I). Compounds which showed no activity as photosynthesis inhibitors included the 2-hydroxy-, 2-hydro-, and 2-mercapto- derivatives of Simazine and

TABLE I

pI_{50} AND pF_{50} VALUES OF PHOTOSYNTHESIS INHIBITORS

Name of compound	*Type*	*Structure*	pI_{50}	pF_{50}	*Literature* pI_{50} *(see ref. 6)*
Atrazine	I		6.20	6.52	6.6
Simazine	I		5.73	6.10	6.4
Monuron	II		6.15	6.38	6.3
Diuron	II		6.77	7.92	7.5
Dicryl	III		6.55	7.07	6.7
KCN	IV	K^+CN^-	3.6	< 1.87	Not reported by Good[3]

Atrazine, and the 4-OH derivative of Monuron. Ethyl carbamate (urethane) and Antimycin A which disrupt the electron transport chain of cytochromes also proved inactive.

Effect on fluorescence by photosynthesis inhibitors

Determination of maximum fluorescence: It was first determined that the maximum yield of fluorescence by Chlorella in bicarbonate buffer was obtained at an excitation energy at 480 mμ and the fluorescence at 689 mμ. These values agree well with those in the literature[11]. However, it was necessary to dilute the Chlorella suspension in order to minimize quenching and self-absorption of the emitted light. This experiment is summarized in Table II, and it was found that a cell concentration of $2 \cdot 10^7$ cells/ml gave the highest fluorescence intensity. Thus, this concentration of cells was used in all fluorescence experiments reported below.

TABLE II

EFFECT OF CHLORELLA CELL CONCENTRATION ON FLUORESCENCE

Cells/ml	Relative fluorescence* (689 mμ)
$1 \cdot 10^8$	21
$9 \cdot 10^7$	22
$8 \cdot 10^7$	26.5
$7 \cdot 10^7$	34
$6 \cdot 10^7$	40
$5 \cdot 10^7$	57.5
$4 \cdot 10^7$	65
$3 \cdot 10^7$	66
$2 \cdot 10^7$	76
$1 \cdot 10^7$	56

* Excitation wave length 480 mμ.

Increase in fluorescense due to inhibitors: The most significant effect of all photosynthesis inhibitors in Classes I, II, and III on fluorescence of Chlorella is the increase in fluorescence intensity. This effect is shown in Figs. 2 and 3 which depict an oscillograph tracing of control and Atrazine-treated Chlorella. After the light is turned on, the fluorescence intensity of normal Chlorella reaches a maximum in 0.25 sec and declines slowly until a steady equilibrium intensity is reached in about 1.20 sec. All experiments were terminated after 1 min illumination. This decay has been observed by others and is termed the "KAUTSKY effect"[11].

Chlorella cells in the presence of 10^{-5} M Atrazine, which causes total photosynthesis inhibition (see Fig. 1), produced a significantly higher intensity fluorescence reaching a maximum in about the same period of time as the controls but exhibiting no decline in intensity. Similar effects were observed with other photosynthesis inhibitors (*cf.* Table I), with the exception of KCN. This seems to be contrary to the observations by WASSINK AND KATZ[12] and KAUTSKY[13]. Compounds which did not inhibit photosynthetic oxygen evolution had no effect on fluorescence intensity. Ethyl carbamate (urethane) was reported by WASSINK[12] to increase fluorescence of Chlorella, but this experiment could not be confirmed in the studies reported here.

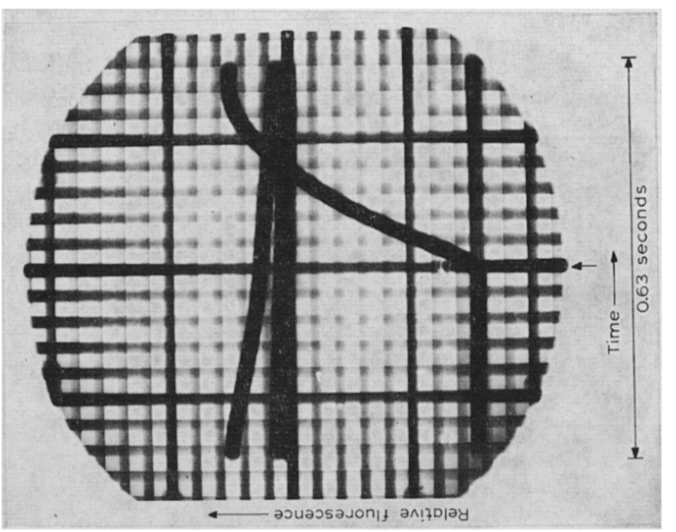


Fig. 2. Oscillograph tracing of fluorescence by illuminated Chlorella; photograph depicts four scans; → indicates start of illumination.

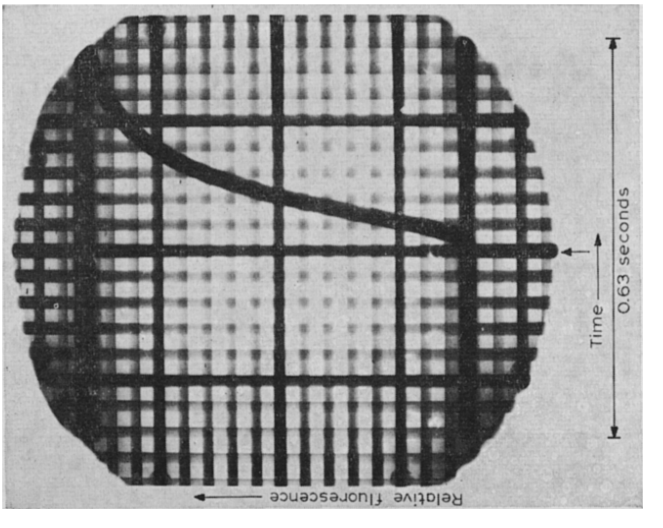


Fig. 3. Oscillograph tracing of fluorescence by illuminated Chlorella in the presence of 10^{-6} M Atrazine; → indicates start of illumination.

Effect of inhibitor concentration of fluorescense: Fluorescence increase by the addition of photosynthesis inhibitors is dependent on the concentration of these inhibitors. Results of such concentration studies are summarized in Table III. The concentration effect is further illustrated in Fig. 4 which shows a straight-line relationship when plotting percent fluorescence intensity increase against logarithm of the concentration. Concentrations at which 50% of the fluorescence intensity increase above equilibrium intensity is achieved has been termed F_{50}, and the corresponding pF_{50} values are calculated from the negative logarithm of F_{50}. pF_{50} and pI_{50} values for a number of photosynthesis inhibitors are compared in Table I and fair to excellent agreement is seen by comparing these two independently obtained values. The same agreement may be seen from pI_{50} values calculated by Goop[6] from Hill reaction studies.

TABLE III

EFFECT OF INHIBITOR CONCENTRATION ON FLUORESCENCE INCREASE

Compound	*Class*	*Concentration (M)*	*Fluorescence (arbitrary units)*	
			Total	*Net increase*
Atrazine	I	$1 \cdot 10^{-4}$	144	63
		$1 \cdot 10^{-5}$	144	63
		$5 \cdot 10^{-6}$	144	63
		$5 \cdot 10^{-7}$	120	39
		$1 \cdot 10^{-7}$	96	15
		0	81	0
Simazine	I	$1 \cdot 10^{-5}$	136	53
		$5 \cdot 10^{-6}$	128	45
		$1 \cdot 10^{-6}$	113	30
		$1 \cdot 10^{-7}$	87	4
		0	83	0
Monuron	II	$1 \cdot 10^{-4}$	138	60
		$1 \cdot 10^{-6}$	120	42
		$5 \cdot 10^{-7}$	108	30
		$1 \cdot 10^{-7}$	93	15
		0	78	0

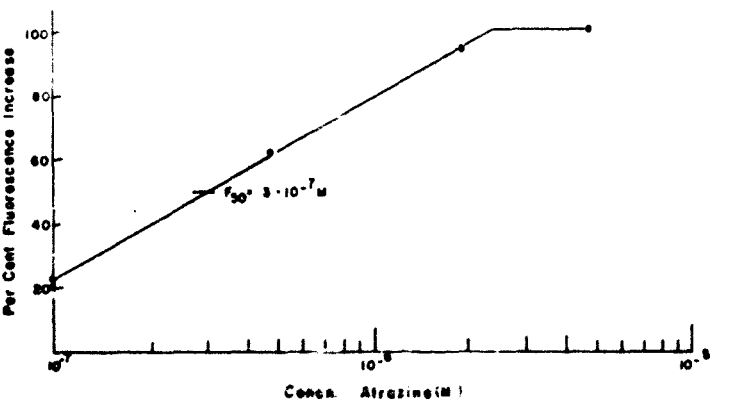


Fig. 4. Effect of different concentrations of Atrazine on fluorescence increase by illuminated Chlorella.

Maximum fluorescence intensity is achieved at concentrations ranging from 10^{-5} M to 10^{-6} M but are limited sometimes by the water solubility of these compounds. However, once maximum fluorescence is achieved, *e.g.* $3 \cdot 10^{-6}$ M for Atrazine (*cf.* Fig. 4), greater concentrations have no further effect on fluorescence increase. Atrazine at $5 \cdot 10^{-6}$ M concentration inhibits photosynthetic oxygen evolution completely (*cf.* Fig. 1).

Fluorescence of "dead" Chlorella

In order to determine if the fluorescence phenomenon was related to the inhibition of physiological processes in the living plant, a suspension of Chlorella was grown for 27 days in a nutrient medium containing 10^{-4} M Atrazine. At the end of this period the cells were harvested, suspended in bicarbonate buffer and tested for photosynthetic activity. No oxygen evolution could be observed, but the cells gave the fluorescence when illuminated at 480 mμ (relative fluorescence 128). Addition of 1 ml of $3 \cdot 10^{-4}$ M Atrazine to 2 ml of cell suspension resulted in a fluorescence reading of 110; the decrease was due to a dilution of the cell suspension.

The cells were now centrifuged and resuspended in fresh buffer, and this washing procedure was repeated twice. After the final washing, Atrazine was added to give a final concentration of 10^{-4} M but no increase in fluorescence was observed.

In another experiment, normal Chlorella cells after the addition of Atrazine were immediately centrifuged and washed three times as before. This time a fluorescence increase was observed upon the addition of Atrazine.

Another experiment was designed in which chloroplast suspension of Chlorella obtained by a French press treatment exhibited fluorescence at 689 mμ but no increase upon the addition of Atrazine. The chloroplast suspension was also inactive when tested by the ferricyanide reduction for the Hill reaction[6].

DISCUSSION

The increase in fluorescence in excised leaves and Chlorella by the action of general respiratory inhibitors at high light intensities was first discovered by KAUTSKY[13] and WASSINK[12]. However, WASSINK could only demonstrate this stimulation with cyanide when photosynthesis was completely inhibited but not when oxygen evolution was only partially blocked[14]. The effect on fluorescence by the photosynthesis inhibitors studied in the work reported here seems to be quite different than KAUTSKY's and WASSINK's observation. (a) The effect on fluorescence stimulation could be demonstrated by us at various concentrations and was well correlated with partial inhibition of photosynthesis (oxygen evolution). (b) The effect on fluorescence increase could be observed within half a second after illumination, whereas WASSINK's fluorescence increase due to cyanide commenced only after the initial induction rise, *i.e.* about 5 sec after illumination and did not reach a plateau until after about thirty seconds (Fig. 4 in ref. 12). (c) No increase in the inductive fluorescence rise was observed by WASSINK[12] with CN^-, although the photosynthesis inhibitors reported here seemed to have a singular effect just on the inductive fluorescence rise.

Intensity measurements of incident light were not made in these studies. Thus, it is not possible to relate our inability to achieve fluorescence stimulation by cyanide or urethane to WASSINK's report that the effect could only be observed at high light

intensities above the compensation point. Thus, the explanation for our failure to repeat WASSINK's work with cyanide or urethane might be that the activation is below the critical intensity. On the other hand, with equal light intensity we are able to achieve almost double the fluorescence of control Chlorella (Fig. 3) with the inhibitors studied here.

It seems, therefore, that the photosynthesis inhibitors of Classes I, II, and III have the effect of "stabilizing" the momentary induction fluorescence increase which usually has a life-time of only 1.26 sec (Fig. 2). The correlation between pI_{50}, related to oxygen evolution, and pF_{50}, related to fluorescence increase suggests a close relationship between energy requirements for oxygen evolution (oxidation) and fluorescent energy. A measurement of the fluorescent light energy has not been possible as yet, but such a value should clarify this relationship. In other words, the energy required to photolyze x molecules of water in y minutes, if completely inhibited, should be converted quantitatively to fluorescent energy.

The fact that cyanide is known to inhibit cytochrome reductase[10] but does not stimulate fluorescence under our experimental conditions suggests that cyanide does not interrupt all of the steps in the electron transport system after the initial excitation of chlorophyll. It further suggests that the photosynthesis inhibitors studied here may inhibit the primary energy transfer and not only the oxidation of OH^- as suggested by others[15,16].

From an approximate cell volume and ^{14}C-Atrazine studies a gradient between cell and medium (3.62) was determined[17]. From this gradient it was calculated that for each 100–1000 chlorophyll molecules only one Atrazine molecule was necessary for maximum fluorescence. This ratio agrees well with that found for Monuron[7]. Thus FRANCK's view, as discussed by RABINOWITCH[18] that the change in fluorescence intensity is due to the formation of chlorophyll complexes ("narcotization") is doubtful in the case of the triazines, ureas, and anilides.

Further evidence that the fluorescence phenomenon which we have observed is related to physiological processes of a living system is provided by the studies in which no fluorescence stimulation was observed when plants had been killed by prolonged contact with an inhibitor. By [^{14}C]Atrazine studies[17] it has been shown that Atrazine could easily diffuse in and out of Chlorella cells just by dilution of the medium. Thus after a thorough washing of the treated cells, no residual Atrazine should have been present within the cell. Thus, the failure to produce a fluorescence increase by the addition of fresh inhibitor to these cells suggests a close relationship with the physiological processes of the living plant. The same kind of evidence was provided by the study of isolated Chlorella chloroplasts which were inactive in the Hill reaction.

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