The Behavior of Alloxan in the Hill Reaction

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Summary. Hill activity induced by alloxan is characterized by non-stoichiometric oxygen evolution and cyclic electron flow due to autoxidation of dialuric acid and alloxantin, the reduction products of alloxan.

Although a new observation of induced Hill activity by a compound is regarded as less significant than earlier, novel behavior and resulting implications are of current interest. In this connection, during investigations to evaluate the Hill activity of various oxidizing agents as possible materials to be used in solar energy conversion studies, alloxan (I) was observed to function as a Hill oxidant in an interesting way.

Our experiments have indicated that less than the expected stoichiometric quantity of oxygen was evolved from illuminated chloroplasts in the presence of (I). Results from a typical experiment are presented in Figure 1. A much earlier report stated that (I) will not function as a Hill oxidant[2]. Although the quantity of oxygen produced is smaller than expected, a definite light induced evolution is observed. We interpret these results as being a consequence of the nature of the reduction product of (I), namely dialuric acid (II). The sensitivity of (II) to spontaneous oxidation by oxygen is well documented[3]. In addition to this consideration, (II) is also known to react with (I) to form alloxantin (III)[4]. Several experiments were performed that characterize the nature of the behavior of alloxan as a Hill oxidant.

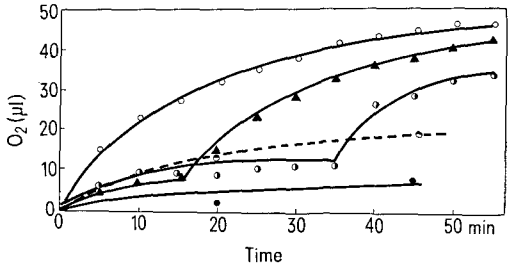


Fig. 1. Preincubation in the dark of reaction mixtures prior to illumination. The assay conditions are described in the methods section. Concentration of alloxan, 0.01 *M*. ○, no preincubation; ▲, preincubation of 15 min in the dark; ◑, preincubation of 30 min in the dark; ●, boiled chloroplasts with no preincubation; ◓, no oxidant with no preincubation.

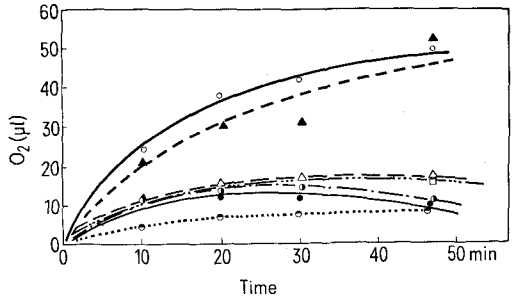
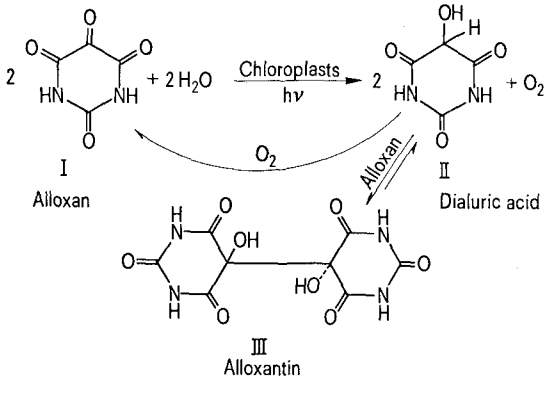


Fig. 2. Oxygen inhibition of the Hill reaction with alloxan. Conditions for the experiments were identical to those described for Figure 1. Concentration of alloxan, 0.01 *M*. ◑, contained air atmosphere; ●, contained air atmosphere; ○, contained N_2 atmosphere; ▲, contained N_2 atmosphere; △, contained N_2 atmosphere with boiled chloroplasts; ◓, contained N_2 atmosphere with no illumination; □, contained N_2 atmosphere but no oxidant (alloxan).

Material and methods. Chemicals used in preparing the buffer and reaction medium were of highest purity available and used without purification. Disintegrated chloroplasts were prepared from fresh spinach (*Spinacea oleracea*) as needed according to Gorham[5] from 150 g of leaf material for each preparation. After the final centrifugation step the disintegrated chloroplasts were suspended in 5 ml of cold 0.02 *M* potassium phosphate buffer, pH 6.5. Alloxan was dissolved in 0.002 *M* potassium phosphate buffer, pH 6.5 containing 0.0067 *M* potassium chloride and 0.2 *M* sucrose. Distilled water was used throughout in all preparations. Oxygen evolution was measured with a model WBP4 circular Warburg bath (Gilson Medical Electronics, Inc.) which was equipped with a transparent plastic bottom and illuminating capability. Temperature was controlled by a thermostat on the Warburg bath and a cold finger, model PBC-4 bath coolers (Neslab Instruments, Inc.), inserted into the bath. Standard manometric techniques were used throughout this study at 15 °C with a nitrogen gas phase. 2 ml of alloxan solution were added to the main compartment



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Tests for alloxantin in the Hill reaction

Data reference	Reaction vessel[a]						
Figure	1	2	3	4	5	6	7
1	+(○)	+(▲)	+(◑)	−(●)	−(◓)		
2	+(▲)	+(○)	−(△)	−(◑)	−(●)	−(◓)	−(□)

[a]Color test for alloxantin with $Ba(OH)_2$: +, positive, blue color observed; −, negative, no blue color observed.

of a Warburg flask. The center well contained 0.1 ml of 2 *N* KOH solution and a fluted filter paper. The side arm contained 0.2 ml of chloroplast suspension. A control flask containing boiled chloroplast suspension was always prepared in each experiment. The reaction was initiated by tipping in the chloroplast suspension into the main compartment and turning on the illuminating lamps.

Results. As indicated in Figure 1, significant oxygen evolution was delayed until illumination was begun. Alloxantin (III) was determined to be present, by a qualitative test, in runs where significant oxygen evolution was observed. This test involves the formation of a characteristic blue-colored complex when (III) is treated with $Ba(OH)_2$[6]. The results for the sets of runs presented in Figures 1 and 2 are presented in the Table. Hill activity was not inhibited by (II) or (III) as determined by experiments where (III) was placed in the reaction medium prior to reaction initiation. An equilibrium quantity of (II) would become available from dissociation of (III) in this case.

We observed inhibition of the Hill reaction by oxygen in high concentrations as determined by experiments performed in an air atmosphere and also a slight volume decrease near the end of the experiment (Figure 2). Oxygen inhibition is a characteristic of the Hill reaction[7] and a volume decrease is similar to observations made previously by MEHLER[7a] using other oxidants.

Our observations are consistent with a system as depicted in the Scheme. Oxygen and dialuric acid (II) are produced by a light-dependent reaction involving the chloroplasts. The amount of alloxantin (III) formed and present at any given time will be described by the equilibrium constant for the reaction between (I) and (II)[8]. An equilibrium quantity of (II) will always be available for reaction with oxygen to spontaneously re-form (I). If (II) were the sole product, then the theoretical quantity of oxygen produced under the conditions of the experiments presented in Figures 1 and 2 would be 224 µl. However, if all of (II) reacted with (I) to form (III), then the theoretical volume of oxygen would be 112 µl. In all experiments the observed oxygen volumes were considerably less than 112 µl (see Figures 1 and 2). As the light-dependent production (II) and oxygen increases, their rate of reaction with each other to produce (I) will also increase. The reaction scheme then begins to approach a steady-state where oxygen will react as fast as it is produced. This will appear as a decrease in the rate of oxygen production with an eventual cessation when in reality oxygen continues to be produced.

We regard this behavior in the Hill reaction to be novel and interesting with respect to the light induced cyclic electron flow and implications relevant to solar energy conversion.

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Effect of Fungal Staling Growth Products on Growth Behaviour of Rhizosphere Microfungi

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Summary. Strong mycostatic property of *Trichoderma harzianum*, *Aspergillus flavus* and *Penicillium rubrum*, which have very high competitive ability in staled agar disc from rhizosphere soil inocula of Lentil (*Lens esculantum* Moench.), was observed corresponding to similar growth-behaviour in staled culture filtrates of dominant microfungi.

Effect of staling growth products of microfungi on growth behaviour of rhizosphere mycoflora has received little attention. DWIVEDI and GARRETT[2] reported that tolerance of microfungi to mycostatic substances was an important factor in their colonization on nutrient agar plates. PARK[3] demonstrated that mycostatic substances of microbial origin might be analogous with staling products of fungal cultures. In the present investigation, fungal flora from rhizosphere of Lentil (*Lens esculantum* Moench) was studied in relation to competitive colonization on staled agar plates at different time intervals. This study was supplemented with the effect of staled culture filtrates on growth behaviour of microfungi.

Materials and methods. Rhizosphere soil samples were collected and thoroughly mixed in aseptic conditions. 15 ml sterilized nutrient Czapek agar (acidified to pH 4.5 by orthophosphoric acid) were poured into Petri dishes and soil impression was given over the whole agar surface by using a flat bottomed glass beaker. 5 series of plates, each with 5 replicates were incubated at 25 ± 1 °C for 24, 48, 72, 96 and 120 h and thereafter the entire circle of agar in each series of plates was placed upside down and

[1] Acknowledgments. Thanks are due to the Head, Botany Department, Banaras Hindu University, for the facilities. One of us (D.K.A.) whishes to thank the Council of Scientific and Industrial Research, Govt. of India, for financial assistance.

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