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A. S. Holt

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THE PHOTOCHEMICAL REDUCTION AND DARK REOXIDATION OF CYTOCHROME C BY CHLOROPLAST PREPARATIONS

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INTRODUCTION

During an investigation undertaken several years ago to extend the list of oxidants capable of reduction in the "Hill Reaction" no evidence of the possibility that cytochrome c might serve as an oxidant was obtained manometrically. Nor was reduction indicated when the same illuminated mixture of chloroplasts was examined spectrophotometrically at 550 mμ. (1). This negative result was not in disagreement with the previous finding of Hill (2) who found that cytochrome c is reduced when it is included with ferric oxalate in the reaction mixture. One can account for this reduction as the result of a secondary reaction with ferrous oxalate, formed when ferric oxalate is reduced by illuminated chloroplasts. However, the fact that the total output of oxygen found by Hill, was not increased when cytochrome c was added to ferric oxalate, might indicate its reduction was due to a separate reaction not involving oxygen evolution. The finding by Warburg and Lüttgens (3) that p-benzoquinone can act as an oxidant in the "Hill Reaction" indicated that cytochrome c could play the role of an ultimate oxidant in this reaction since it can be reduced by hydroquinone. That cytochrome c could not be reduced directly by illuminated chloroplasts was explainable only in terms of specificity relationships. Further work on this question was also discouraged by the fact that negative evidence had been found for the participation of cytochrome in the respiration of Spinacea oleracea (4), which had been used primarily as the source of chloroplasts in our previous work. A reinvestigation of the question whether cytochrome can be reduced during the illumination of chloroplasts

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showed that reduction does occur. This was found with chloroplasts of both *Spinacea oleracea* and *Phytolacca americana*. The photochemical reduction is rapidly reversed in darkness by preparations from both plants due to the action of a thermolabile catalyst which is inhibited by cyanide, azide, and carbon monoxide. The presence of cytochrome oxidase and of cytochrome components in chloroplasts has also recently been reported from other laboratories (5, 6).

METHODS

Chloroplast material was obtained in the conventional manner, i.e., maceration of leaves for 1-2 min. in buffer (0-2° C., 0.05 M phosphate containing 0.01 M KCl) by means of a Waring blendor, preliminary filtration through several layers of muslin followed by centrifugation and washing. Chlorophyll concentrations were determined in methanol spectrophotometrically using the absorption coefficient given by Warburg and Lüttgens for total chlorophyll (3). Cytochrome reduction was assayed by means of a Beckmann spectrophotometer (Model DU) or with the equipment described recently, which is used for the assay of dye reduction by chloroplasts (7). Cytochrome c was obtained from Viobin Corporation, Monticello, Illinois, and from Sigma Chemical Company, St. Louis, Missouri; concentrations were determined and adjusted according to the method outlined by Potter (8). The concentration of cytochrome c chosen for measurement of photochemical reduction was such that according to the values of the absorption coefficients given by Potter (8), the ratio $\frac{d(\Delta I)}{dC}$ was maximum. In this expression ΔI signifies the difference between the intensities obtained for completely reduced and completely oxidized cytochrome c, and C signifies concentration. For determination of the rate of cytochrome oxidation the method outlined by Potter (8) was followed except that instead of using three different concentrations of suspension, an equal concentration of heated preparation was used as a control.

RESULTS

1. Photochemical Reduction of Cytochrome C by Illuminated Chloroplast Preparations

The reduction of cytochrome c by illuminated chloroplast material is readily observable within less than 1 min. of illumination by means of a hand spectroscope. Crude quantitative measurements were possible with the recording photometric apparatus described previously (7), by recording the increase of absorption in the region transmitted by Corning filters 5120, 4303, and 3484. The response of the photronic cell (Weston Photronic Cell, Model 594) to the radiation from the incandescent lamp transmitted through the above filters was very slow. This required the use of a high shunt resistance (1000 ohms) across the recorder to obtain a reasonable deflection for I_o. With such a high resistance, the response of the recorder itself is sluggish, but experiments did show the following: (1) heated chloroplast material does not catalyze the photochemical reduction of cytochrome c; (2) reduction does occur between pH 5.6 and 8.1 in phosphate buffer, with a rate approximately 50% higher at pH 6.5 than at the two extremes tested; (3) 2×10^{-4} M NH_2OH had little or no effect on the initial rate of reduction.

Figure 1 shows the effect of 0.001 M HCN on the subsequent rate of reoxidation of the reduced cytochrome formed during illumination. The chloroplasts were illuminated at 20° C. in the presence of cytochrome c by light from a 1000 watt incandescent lamp filtered through ferrous sulfate and Corning filter No. 2404. The readings given are those obtained at 550 mμ. with the Beckmann spectrophotometer. Although reduction can be demonstrated it has not yet been possible in four separate experiments to demonstrate oxygen evolution manometrically.

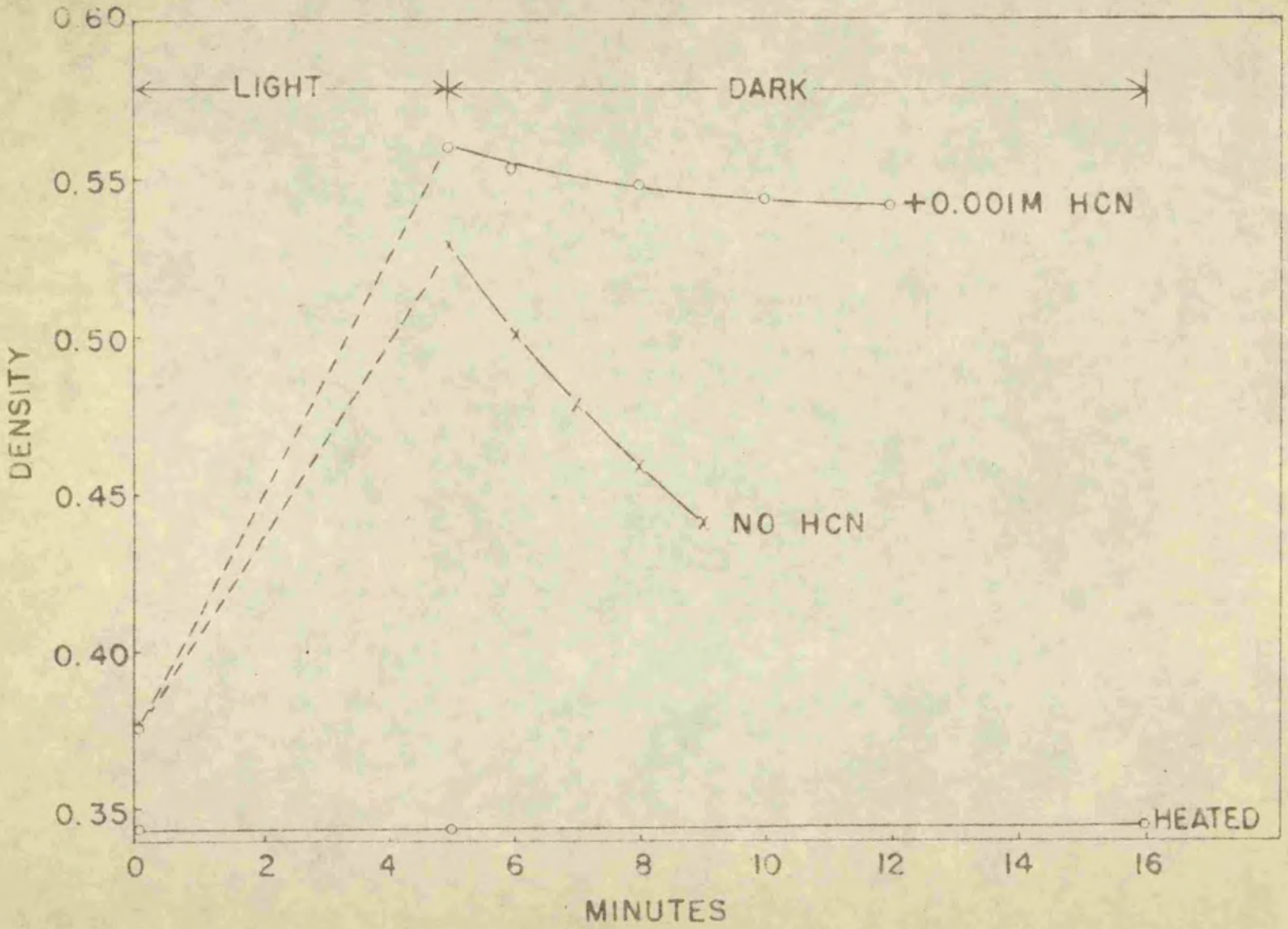
2. Enzymatic Oxidation of Reduced Cytochrome by Chloroplast Preparations

The fact that the cytochrome reduced photochemically is reoxidized in darkness suggests the presence of cytochrome oxidase in such preparations. Manometric

Figure 1

Photochemical reduction of cytochrome c by chloroplast fragments. Plant: *Phytolacca americana*; chlorophyll conc., 0.04 mg., 2.6×10^{-5} M cytochrome c. pH 6.5, 0.05 M phosphate, 20° C. Illuminated with light transmitted by Corning filter #2404. Density after reduction with hydrosulfite: 0.862.

FIG. I


0.60
0.55
0.50
0.45
0.40
0.35
DENSITY
LIGHT
DARK
+0.001M HCN
NO HCN
HEATED
0
2
4
6
8
10
12
14
16
MINUTES

experiments to characterize the component responsible for the reoxidation were therefore undertaken. In Table I are presented data which demonstrate the increased oxygen uptake by chloroplast fragments of Phytolacca in the presence of reduced cytochrome c.* An experiment with spinach chloroplast fragments presented in Fig. 2 shows similar results. Figure 3 presents the data obtained with the inhibitors used to characterize cytochrome oxidase but fails to confirm one test for the identity of this enzyme with the enzyme usually considered, since the inhibition by CO was not reversed by light. It is possible, however, that photochemical reversal of the inhibition by CO might be demonstrated with thin suspensions, since most of the light in the region close to 430 mμ might have been absorbed by chlorophyll. An indication of such a possibility is the increased rate of oxygen uptake during illumination in the case of the azide and cyanide experiments. Such an effect might be explained as being due to photooxidation of the ascorbic acid sensitized by chlorophyll protein, an oxidation which can be readily demonstrated. In Table II data are presented showing the increase in the rate of oxygen consumption with increases in temperature. No apparent damage to the system was found during the first 40 min. of measurements at 35° C., but deleterious effects were immediately evident at 45° C. The temperature optimum for this system is therefore close to that found for cytochrome oxidase in animal tissues.

It has been recommended that $AlCl_3$ be added to the reaction medium used for the assay of cytochrome oxidase in animal tissue homogenates (8). No decrease of cytochrome oxidation was observed when this reagent was omitted during the activity measurements with the chloroplast preparations.

Of further interest was the comparison of the activities for cytochrome oxidation and photochemical dye reduction in such fractions as could be obtained with centrifugation procedures. No large difference in the activity of any single

*No evidence of polyphenol oxidase was found in preparations from leaves of Phytolacca americana when tested with mixtures of catechol and hydroquinone.

TABLE I

Oxygen Uptake by Chloroplast Fragments

Plant: *Phytolacca americana*; 0.8 mg. of chlorophyll; 20° C. pH 7.2

	mm^3 O_2
Suspension + cytochrome c + ascorbate	178
Suspension + ascorbate	9
Ascorbate + cytochrome c	16

Figure 2

Oxygen uptake by chloroplast fragments. Plant: Spinacea oleracea; chlorophyll conc., 0.53 mg. 35° C. 1 -- suspension + cytochrome c + ascorbate; 2 -- heated suspension + cytochrome c + ascorbate; 3 -- suspension + ascorbate; 4 -- suspension only.

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FIG. 2

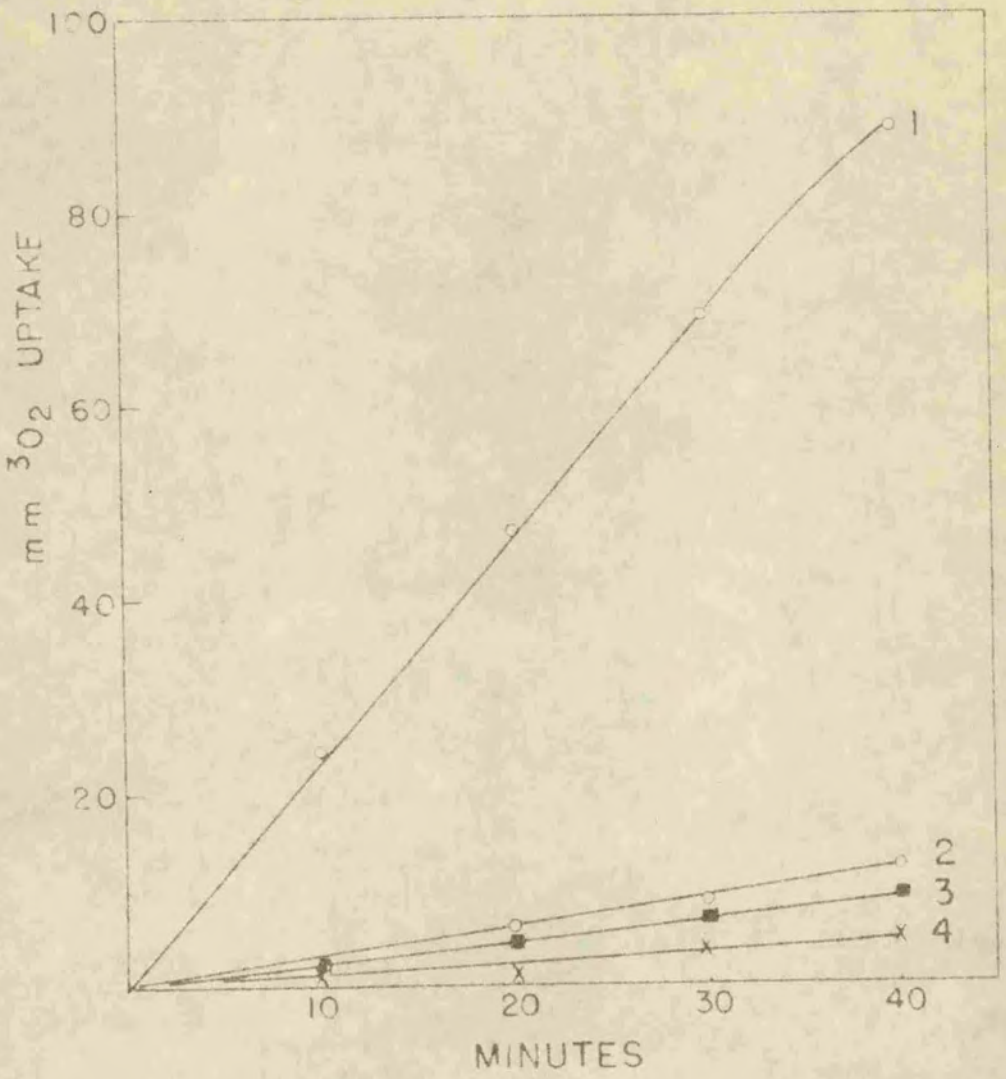

Figure 3

Effect of inhibitors on oxygen uptake by chloroplast fragments in presence of reduced cytochrome c. Plant: Phytolacca americana; chlorophyll conc., 1.13 mg. NaN_3; 1×10^{-4} M; HCN; 1.3×10^{-2} M, 1 M NaCN in center cup; 80% CO + 20% O_2. 20° C. Light sources: (A): 1 mercury discharge tube; (B): 2 neon tubes.

FIG. 3

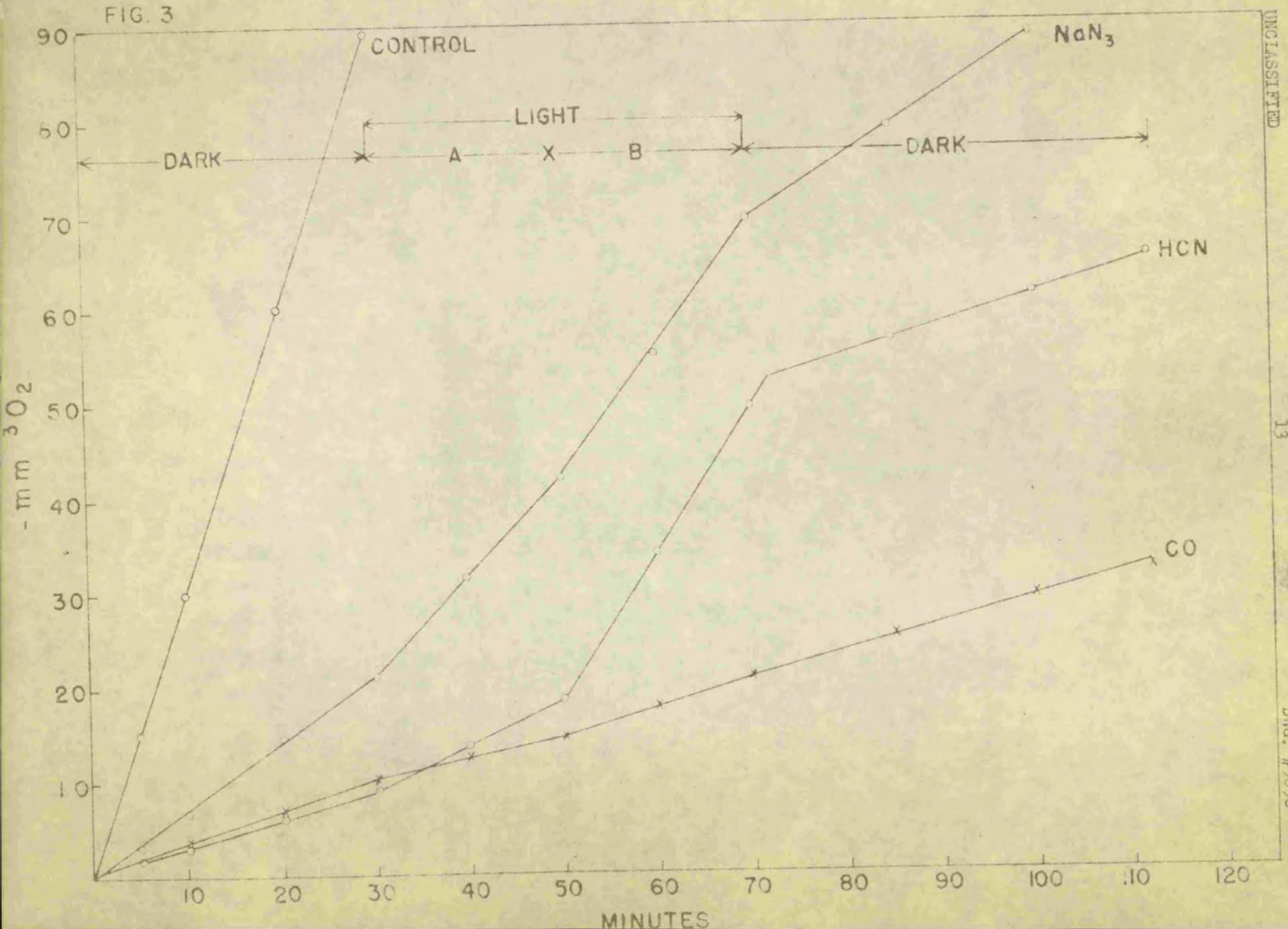

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TABLE II

Temperature Effect on Rate of Cytochrome Oxidation by Chloroplast Fragments

Plant: *Phytolacca americana*; chlorophyll concentration 0.16 mg.

Temperature in degrees C.	mm^3 O_2 uptake per 40 min. corrected for blank run with heated suspension
15	25
25	50
35	97
45	36

fraction relative to another fraction was found for either reaction. Table III presents a summary of one of several fractionation experiments. It will be noticed that assays were not made with the residual supernatant fraction. This final fraction was omitted because no disintegration procedure other than the initial maceration was used, with the result that in 15 min. at 25,000 g. almost all the green material was sedimented. It was also found that this fraction contained a considerable amount of soluble, heat-stable, reducing material in such concentration that from 50 g. of fresh leaves of Phytolacca macerated in 300 ml. of buffer, 5 ml. were equivalent to 4.3 ml. of 9×10^{-4} M indophenol dye used for the photochemical assay. Consequently, several washes to remove this material, presumed to be ascorbic acid, were required in order that accurate assays of the "Hill Reaction" could be made. Other experiments similar to that outlined in Table III showed also that lower activities for the two reactions are given by the precipitates from F_3. Although the stability of the cytochrome oxidizing system is much greater at 35° C. than that of the dye reducing system, it was found that after storage for 72 hrs., 0° C., a preparation similar to F_2 (Table III) retained 50% of the initial cytochrome oxidase activity and still retained 30% of its photochemical activity. This might indicate that both systems are inactivated by similar factors during storage at low temperatures.

DISCUSSION

At the present time it is premature to speculate concerning the possible participation of cytochrome c or a related iron porphyrin in photosynthesis, especially in view of the fact that oxygen production has not yet been demonstrated. However, it has been difficult to reconcile the fact that methemoglobin could serve as an oxidant for oxygen evolution, (9), and not cytochrome c, since both systems have approximately the same oxidation-reduction potential (10). If cytochrome c

TABLE III

Relative Activities for Cytochrome Oxidation and for Hill Reaction by Fractions from Chloroplast Suspensions

Procedure	Description	$Q_{O_2}^{ch}$	
		Cytochrome oxidation (35° C)	Dye reduction (20° C)
1 min., 2500 xg (A)	Almost completely starch; some green material		
F_1: Supernatant from A 5 min., 3000 xg	Intact plastids and little starch	540	2000
F_2: Supernatant from F_1 15 min., 3000 xg	Intact plastids and fragments	585	2840
F_3: Supernatant from F_2 15 min., 25,000 xg	Fragments	393	2000

were a component of the photosynthetic apparatus some mechanism would be needed to keep a continued supply of the oxidized form available, a mechanism which if operative, remains obscure at the present time. However, if cytochrome c is not utilized during CO_2 reduction, there remains the possibility that its photochemical reduction might siphon off some of the energy absorbed by chlorophyll.

SUMMARY

1. Illuminated chloroplasts can reduce oxidized cytochrome c *in vitro*.

2. In darkness, it is found that reduced cytochrome is reoxidized by a thermolabile component in chloroplast preparations.

3. The rate of reoxidation of reduced cytochrome c is inhibited by cyanide, azide, and carbon monoxide. Photochemical reversal of the inhibition by CO was not demonstrated.

4. Between the ranges 15-35° C. the rate of oxidation is increased with a temperature coefficient of 2. At 45° C. immediate deleterious effects were evident.

5. Centrifugation procedures did not yield any sharply defined separation of oxidase and photochemical activity.

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