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IODINATION OF CHLOROPLASTS

I. PROPERTIES OF IODINATED CHLOROPLASTS

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SUMMARY

Spinach chloroplasts exposed to iodide can be washed free of the bulk of the iodide. In the presence of lactoperoxidase and H_2O_2, iodide can be introduced into chloroplasts in high amounts and in non diffusible forms. The resultant particles, which have been named iodochloroplasts, extrude their iodide upon stimulation by light. The form and the amount of extruded iodide bears a definite relationship to the amount of incident light. A flash of marginally effective light is additive to the next such flash even after a lapse of 10 min of darkness. These and other properties of iodochloroplasts may make them of great use in the study of intermediate reactions of photosynthesis.

INTRODUCTION

Lactoperoxidase in conjunction with H_2O_2 and ^{125}I has been introduced as a means of labelling the exterior surface of cells [1, 2] as well of membrane-bound cellular components, i.e. mitochondria [3]. This reaction is based on the formation of a lactoperoxidase-iodide complex as an iodinating unit. The high molecular weight of lactoperoxidase (80 000) excludes the complex from entry into cells and consequently should limit the iodination to the exterior surface of cells.

However, detailed studies of this reaction in the presence of red blood cells have provided proof that the iodination is not necessarily limited to the membrane surface of red blood cells unless the conditions of iodination are rigorously controlled. It was shown that two primary factors were the concentration of red blood cells and the concentration of iodide. Only in the presence of concentrated solutions of red blood cells and dilute concentrations of iodide was the iodination limited to the cell surface. If these premises were not maintained, extensive iodination of inner membrane components as well as of cytoplasmic hemoglobin could occur [4, 5].

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea.

On the basis of this information we proceeded to iodinate spinach leaf chloroplasts. The original aim of this study was to define the exterior topography of the chloroplast membranes and to relate any inhibition of the normal photosynthetic pattern to iodination of particular sites. However, our results indicate that this purpose is not attainable because, although spinach chloroplasts exposed to iodide can be washed free of the bulk of the iodide, they acquire large amounts of iodide in the presence of lactoperoxidase and H_2O_2. The radioactive iodide that has been introduced into the chloroplasts does not equilibrate with inorganic iodide and is not easily diffusible out of the chloroplast; intrachloroplast concentrations higher than 10^{-1} M can be attained and the iodination is thus not necessarily limited on the exterior surface of the chloroplast membrane. Because of these considerations it is difficult to be certain that iodination is limited to the exterior surface of the membranes or that inhibition of photosynthetic activity is necessarily related to iodination of critical photosynthetic units [6, 7] and not to other factors, i.e. high localized intrachloroplast iodide concentrations.

On the other hand such iodide-containing chloroplasts, for which we propose the name iodochloroplasts, extrude their iodide upon stimulation by light while the amount and the form in which the iodide is extruded bears a definite relationship to the amount of incident light. Furthermore a flash of marginally effective light is additive to the next such flash even after a lapse of 10 min of darkness. These and other properties of the iodochloroplasts may make them of great use in the study of intermediate reactions of photosynthesis.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves (*Spinacea oleracea*) obtained from a local market. Leaves with midribs and veins removed were ground in a Sorval Omnimixer for 15 s at 35 % of the line voltage followed by 15 s at 58 % of the line voltage in a medium (8 ml/g fresh weight) containing 0.4 M sucrose, 10 mM Tris · Cl (pH 8.0) and 10 mM NaCl (sucrose buffer). The homogenate was filtered through 6 layers of cheese cloth and centrifuged for 5 min at $200 \times g$. Chloroplasts were collected by centrifuging the resultant supernatant for 12 min at $1500 \times g$. The chloroplasts were washed once, centrifuged and resuspended in the same buffer.

Chlorophyll was determined in 80 % acetone solution according to MacKinney [8]. Photosystem II activity was measured according to Vernon and Shaw [9] by determining the rate of reduction of 2,6-dichlorophenolindophenol (DCIP) in the presence or absence of 1,5-diphenylcarbazide.

The standard iodination technique used was the following: 0.5 ml of a chloroplast suspension, 800 μg chlorophyll/ml, were iodinated in an ice bath in the presence of 0.106 M phosphate (pH 7.4), 0.2 mg lactoperoxidase, 10^{-6} M KI (containing 0.1 mCi ^{125}I) and $7 \cdot 10^{-5}$ M Na_2SO_3; the latter was added in order to minimize the formation of I_2 [3]. The reaction was initiated and continued by the manual addition of 88 mM H_2O_2 in 0.106 M phosphate (pH 7.4) with a Hamilton syringe in 10 μl portions every 30 s, reaction time 3 min. The reaction was stopped by the addition of 1 ml sucrose buffer containing 10 mg bovine serum albumin per ml. After iodination the chloroplasts were collected by centrifugation, washed four times with sucrose buffer and resuspended in sucrose buffer for the final experiments. All steps starting from the addition of H_2O_2 were performed in the dark or in light filtered through a green filter.

Methyl viologen-mediated oxygen uptake was measured in a YSI model 53 oxygen electrode, Yellow Springs Instrument Co., according to Hall et al. [10]. The H_2O to methyl viologen assay mixture contained 3 ml of the assay buffer (0.1 M sorbitol, 0.05 M Tricine, 5 mM $MgCl_2$, 0.025 M NaCl, 0.01 M K_2HPO_4 and 2 mM EDTA, pH 7.5) as well as 2 mM methyl viologen, 2 mM sodium azide to stop the endogenous catalase action, and 20–30 μg chlorophyll/ml. The $DCIPH_2$ to methyl viologen assay mixture contained 3 ml of the above assay buffer, 2 mM methyl viologen, 2 mM sodium azide, 50 mM DCMU (3-(3',4'-dichlorophenyl)-1,1-dimethyl-urea), 0.27 mM DCIP and 10–20 μg chlorophyll/ml. The reaction was initiated by adding sodium ascorbate to a final concentration of 1.7 mM. Fluorescence was measured at 680 nm with a front-face spectrofluorometer. Excitation was performed with a broad band blue light (CS 4-96 and CS 3-73 Corning filters, and 5 cm thick of 1 % aqueous solution of $CuSO_4$), intensity 575 $\mu W/cm^2$. The chloroplasts were suspended in the sucrose buffer. Chlorophyll concentration 12 μg/ml.

The iodinated chloroplasts were extracted three times with 0.02 M phosphate buffer (pH 6.5) containing 0.02 M KCl, to remove the soluble proteins [11], followed by three extractions with 1 mM EDTA (pH 8.0) to remove the coupling factor [11] and then by two extractions with 0.2 M Tris · HCl (pH 8.0) [12]. The residual insoluble chloroplast material was then solubilized with sodium dodecyl sulfate and the chlorophyll-protein complexes I and II were separated as described below.

Gel electrophoresis of the iodinated thylakoids, which were solubilized with sodium dodecyl sulfate, was prepared by a modification of previous procedures [13]. The thylakoids were dissolved in 0.5 % to 1 % sodium dodecyl sulfate in 0.1 M Tris · HCl pH 8.0 (sodium dodecyl sulfate : protein = 2.0 mg/mg) and samples containing up to 1 mg protein were loaded on 7 % acrylamide gel columns of 1.4 cm diameter and 8 cm in length. The gels were prerun for 10 min before the samples were loaded, and were subsequently electrophoresed for 2 h at 20 mA/tube. The gel and electrophoresis buffer was 0.05 M Tris · HCl/0.25 % sodium dodecyl sulfate (pH 8.0). Subsequent to the electrophoretic separation gels were sliced, complexes I and II were isolated by re-electrophoresis into a dialysis sack. This was performed simply by centrifuging the gel slice on to the top of a fritted glass funnel, sealing off the bottom funnel tube with a small diameter dialysis bag filled with dialysis buffer and re-electrophoresing under the same conditions. The contents of the dialysis tube were then isolated and further treated.

Lactoperoxidase was purchased from Sigma Chemicals, while $^{125}I^-$ was purchased from Amersham, as a carrier-free solution, pH 8–9. The lower pH solutions are extremely dangerous because of the formation of $^{125}I_2$ which is highly volatile and is absorbed by the thyroid. It has been our experience, using a variety of commercial $^{125}I^-$ solutions, that it is preferable to subject them to a preliminary purification by paper electrophoresis in a butanol/ethanol/water (40 : 40 : 20) chromatographic system.

Radioactivity was measured by small volume liquid scintillation counting which we have developed [14] in a Packard Scintillation Counter. Paper chromatography was performed on 2-cm wide strips, cut up in 4 mm widths and counted in the same small volume scintillation system. The location of inorganic iodide was determined by spraying the paper with 1 M lead acetate. Protein was determined by the Lowry procedure [15].

RESULTS

Uptake of ^{125}I by chloroplasts in the presence of lactoperoxidase

Chloroplasts have a high endogenous ability to degrade H_2O_2, which is probably due to their content of catalase [16]. When H_2O_2 and $^{125}I^-$ is added to a suspension of chloroplasts, the H_2O_2 is rapidly decomposed but very little ^{125}I is taken up by the chloroplasts. However, when lactoperoxidase is added to such a system the iodide is taken up from the medium and is found associated with the particulate chloroplast fraction (Table III, compare line 1 to line 2). It may therefore be assumed that the uptake of iodide by the chloroplasts in the presence of lactoperoxidase is due to the ability of lactoperoxidase to form a lactoperoxidase-iodide complex [17], a property which is not necessarily shared by other peroxidases.

In the experiments reported here we have taken into account the high endogenous rate of degradation of H_2O_2 by the chloroplasts and have added enough lactoperoxidase so that the rate of decomposition of H_2O_2 by the lactoperoxidase alone is twice that of the endogenous rate of decomposition of H_2O_2 as measured by the O_2 electrode.

In experiments involving labelling of surfaces of animal cells with $^{125}I^-$, many laboratories have preferred to generate the H_2O_2 enzymatically, i.e. in the presence of glucose and glucose oxidase [18, 19]. In the chloroplast system, however, because of the high endogenous degradation of H_2O_2, such enzymatic systems which generate H_2O_2 are of value only when performed in the presence of large concentrations of glucose oxidase. Under our conditions we have found the addition of glucose oxidase to be unnecessary; working at 0 °C, addition of 0.8 μmol of H_2O_2 every 30 s is adequate for optimal iodination; optimal conditions include a chloroplast concentration of 1 mg chlorophyll/ml. The reason for the high chloroplast concentration is derived from the work on red blood cells, which indicated that iodination by the lactoperoxidase-iodide system is not necessarily limited to the surface membrane but

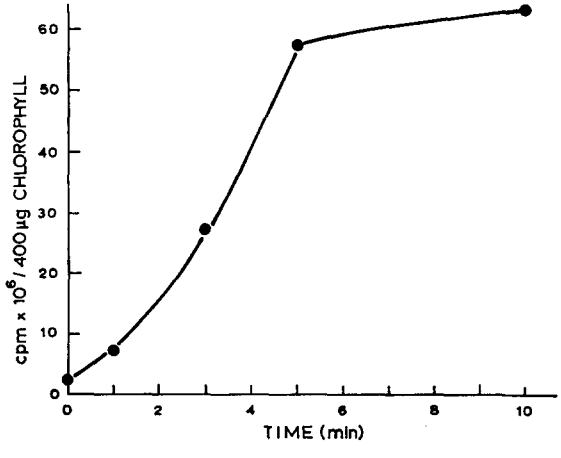

Fig. 1. Uptake of $^{125}I^-$ by chloroplasts with time of iodination. The iodide concentration ($1 \cdot 10^{-3}$ M) was held constant. Other conditions were as described in the text. At 30-s intervals 0.8 μmol of 10^{-3} M H_2O_2 were added and samples removed to measure the total uptake of $^{125}I^-$.

that intracellular iodination of red blood cells occurred when the cell concentration fell below $1 \cdot 10^8$ cells/ml. Chloroplasts, as visualized under the phase contrast microscope, have a smaller diameter than red blood cells, so we have selected the optimal value of $1 \cdot 10^9$ chloroplasts/ml, which corresponds roughly to 1 mg chlorophyll/ml. The rationale for this is further detailed in the Discussion.

Fig. 1 shows the time curve for the uptake of $^{125}I^-$ by chloroplasts. It should be appreciated that 0.8 μmol H_2O_2 was added every 30 s and the additions were interrupted at the indicated time; consequently, the continued uptake of iodide by the chloroplasts is also a function of the amount of added H_2O_2. Because of the mechanics of addition of H_2O_2, including the mixing of the samples, etc., we cannot be certain of the significance of the apparent lag in the curve. Under these conditions of experimentation, the curve in Fig. 1 shows that the chloroplasts can take up iodide for several minutes.

Distribution of ^{125}I within the chloroplasts

We have selected a butanol/ethanol/water (40 : 40 : 20) chromatographic system as a means of quickly surveying the distribution of ^{125}I within any given fraction. Three main radioactive bands are evident in this system: with R_F values of 0.0, 0.4 and 1.0; detailed survey of the material corresponding to these bands indicates this is primarily due to (1) high molecular weight bound iodide material $R_F = 0$, (2) inorganic iodide, $R_F = 0.4$ and (3) iodide bound to low molecular weight material, $R_F = 1.0$. This last material is soluble in organic solvents, is distinct from chlorophyll and other plant pigments and may be due to phospholipid bound iodide; it has been found in substantial amounts (Table III).

Using these three fractions as markers we can see from Table I, where iodinated and washed chloroplasts are extracted with a variety of high and low ionic strength buffers, EDTA and Tris · HCl, that in most cases a large fraction of the radioactivity can be accounted for by inorganic iodide and very little by low molecular weight

TABLE I

EXTRACTION OF IODINATED CHLOROPLASTS WITH PHOSPHATE BUFFER, EDTA AND TRIS · HCl

The iodinated and washed chloroplasts were extracted as indicated below for 30 min at 4 °C in each extraction buffer. Each supernatant fluid was cleared by centrifugation at 140 000 × *g* for 1 h, counted and the distribution of radioactivity in each fraction after chromatography was determined. The amounts of radioactivity found at $R_F = 1.0$ were insignificant; however, they were appreciable in the results presented in Table III.

Buffer	Extraction No.	cpm/mg chlorophyll ($\times 10^{-6}$)	Percent of total counts	
			$R_F = 0.0$	$R_F = 0.4$
Phosphate	1	22.8	4.0	96.0
	2	3.74	5.4	94.6
	3	2.16	6.5	93.5
EDTA	4	2.94	11.0	89.0
	5	1.67	16.0	84.0
0.2 M Tris · HCl	6	6.65	15.0	85.0
	7	4.65	11.0	89.0

TABLE II

SPECIFIC ACTIVITIES OF THE VARIOUS PROTEIN COMPONENTS OF THE IODINATED CHLOROPLASTS

The experimental conditions were as described in Table I. The extracts from the same buffer were pooled, the proteins precipitated with ammonium sulfate (90% saturation), the residual thylakoids dissolved in sodium dodecyl sulfate and complexes I and II were isolated by electrophoresis. All isolated protein fractions were resuspended and washed with ammonium sulfate (90% saturation), and protein [14] and radioactivity determined in each fraction.

Sample	Protein bound ^{125}I (% of total extract*)	Specific activity (cpm/mg protein) $\times 10^{-6}$
Phosphate buffer	6.0	1.0
EDTA extract	–	–
Tris extract	10.0	0.25
Thylakoid residue soluble in sodium dodecyl sulfate	30.0	0.25
Complex I	80.0	0.22
Complex II	80.0	0.22

* Each fraction, under the conditions of extraction, had a given number of total e/m; of these, varying proportions were associated with proteins.

bound organic iodide. These results emphasize the difficulty in equilibration of inorganic iodide with various cell compartments, as well as the difficulty in removing iodide from compartments even with hypotonic solutions; in other words a certain degree of compartmentalization seems to have occurred and the iodide that is thus compartmentalized is not easily extractable or available for extraction. It is interesting to mention here that treatment of the chloroplasts with 0.2 M Tris · HCl buffer (pH 8.0), which is known to remove all oxygen-evolving capacity [12], causes the extraction of a number of proteins, as shown by gel electrophoresis (unpublished results).

Table II shows that the specific activities of the bulk proteins extractable by a variety of means from chloroplasts are remarkably similar. Of course this does not include specific activities of individual purified proteins. Results with autoradiography (not presented) obtained with analytical acrylamide gel electrophoresis have established that some bands which stain only lightly with Coomassie Blue are heavily labelled, indicating that some proteins may become preferentially labelled. However, because the specific activity of a protein will depend on the number of tyrosine groups accessible for iodination as well as its location on the membrane surface, it is difficult to derive any definitive conclusions from these facts at this time. The relatively little label associated with the proteins of each fraction in the presence of the high non-protein label becomes disconcerting because it raises the question as to the mechanism by which this label was attached to the protein. This is especially true of the various fractions obtained during the earlier extractions (Table II).

These results emphasize the ubiquitous presence of iodide in a variety of chloroplast fractions as well as the general distribution of radioactivity associated with proteins of various fractions.

Inhibition of photosynthesis by iodination

One of the questions that could be raised would be the consequence of iodination of a particular protein or complex of proteins upon the photosynthetic process. Such a direct correlation could be of paramount importance in locating the architecture of the labelled site as well as the three dimensional association of the various labelled fractions. We have approached this problem by labelling the chloroplasts in the presence of a different iodide concentration and associating the degree of inhibition of the photosynthetic process with the extent of iodination; we have also correlated these factors with various concentrations of chloroplasts. These results, which are tabulated in Table III, indicate that inhibition of Photosystem II is a predominant result of iodination and that the degree of inhibition of Photosystem

TABLE III

EFFECT OF I^- CONCENTRATION ON THE INHIBITION OF PHOTOSYSTEM II DURING IODINATION OF CHLOROPLASTS IN THE PRESENCE OF LACTOPEROXIDASE

Experimental conditions as described in the text. The chloroplasts (800 μg chlorophyll/ml) were washed three times with sucrose buffer, twice with sucrose buffer containing 4 mM KI and once with 20 mM phosphate/20 mM KCl (pH 6.5). The chloroplasts were dissolved in sodium dodecyl sulfate, centrifuged and further treated as described in Table II.

I^- concentration (M)	Photosystem II (% inhibition)	^{125}I uptake (% of total*)	Radioactivity in sodium dodecyl sulfate-soluble thylakoids (% of total uptake)		
			Rf = 0.0	Rf = 0.4	Rf = 1.0
$4 \cdot 10^{-3}$ (no lactoperoxidase)	0	0.54	0.09	0.31	0.14
$4 \cdot 10^{-3}$	90	39	10.7	18.7	9.6
$4 \cdot 10^{-4}$	80	41	16.4	10.2	14.4
$4 \cdot 10^{-5}$	20	41	8.2	17.2	15.6
$4 \cdot 10^{-6}$	0	32	9.3	11.6	11.1
$4 \cdot 10^{-7}$	0	32	12.0	9.6	10.4
Carrier-free ^{125}I	0	30	12.0	9.6	8.4

* Total refers to 0.1 mCi ^{125}I added to each sample and available for uptake.

TABLE IV

EFFECT OF CHLOROPLAST CONCENTRATION ON THE INHIBITION OF PHOTOSYNTHESIS DURING IODINATION IN THE PRESENCE OF LACTOPEROXIDASE

The reaction mixture contained in a final volume of 4 ml 0.106 M phosphate (pH 7.4), 0.2 mg lactoperoxidase, $4 \cdot 10^{-4}$ M KI, $7 \cdot 10^{-5}$ M Na_2SO_3, and chloroplast. The reaction was initiated and continued by the manual addition of 8.8 mM H_2O in 10 μl portions every 20 s. Time of the reaction, 2 min. Other conditions as described in the text. Results are given as % inhibition. PSI, Photosystem I; PSII, Photosystem II; MV, methyl viologen.

Chloroplast concentration (μg chlorophyll/ml)	PSII (H_2O DCIP)	PSII+PSI (H_2O MV)	PSI ($DCIPH_2$ MV)	Variable fluorescence
100	56	57	12	64
800	0	0	0	0

TABLE V

THE EFFECT OF KI (± LACTOPEROXIDASE) ON THE EXTRACTION OF ^{125}I FROM IODOCHLOROPLASTS

Chloroplasts, 0.8 mg chlorophyll/ml, in three tubes were iodinated in the dark for 3 min, as described in the text. After centrifugation, the samples were pooled, washed three times with an excess of sucrose buffer, and divided into three tubes. Each tube was extracted with 2 ml buffer as indicated above for 20 min at 4 °C, centrifuged at 140 000 × *g* for 1 h and the radioactivity of the supernatant determined. The release of ^{125}I in the various tubes represents less than 1 % of the total radioactivity available on the chloroplasts.

Extraction buffer	Release of ^{125}I (cpm/ml) × 10^{-3}
Sucrose	655
Sucrose+KI ($1 \cdot 10^{-6}$ M)	675
Sucrose+KI ($1 \cdot 10^{-6}$ M)+lactoperoxidase+H_2O_2	670

II is greatest in the presence of high iodide concentrations. Table IV shows the effect of chloroplast concentration on the inhibition of photosynthesis during iodination in the presence of lactoperoxidase. When the chloroplast concentration is 800 μg chlorophyll/ml, the iodination is noninhibitory; however, it becomes inhibitory if the chloroplast concentration is lowered to 100 μg chlorophyll/ml.

When the uptake of iodide by the chloroplasts in the presence of different iodide concentrations is evaluated, considering the volume of the chloroplasts, it can be calculated that the iodide concentration associated with the chloroplasts can become as high as 10^{-1} M. From this value and the known content of chlorophyll it can be calculated that such chloroplasts contain as much as 6 mol iodide/mol chlorophyll.

At this point it should be emphasized that these conditions of iodination which lead to inhibition of Photosystem II, i.e. low chloroplast concentration and high iodide concentration are the exact conditions which have been found to be required for intracellular iodination of red blood cells and for extensive iodination of non-exposed intramembrane components [4]. In other words, it has been shown that the iodinating species cannot be the lactoperoxidase-iodide complex but must be some form of free radical iodide. The consequence of this finding is that under such conditions iodination will not be limited to the sites accessible to lactoperoxidase and therefore will not be limited to iodination of the surface sites of the chloroplast membranes by the high molecular weight of the lactoperoxidase.

The intrachloroplast ^{125}I does not equilibrate with the added nonradioactive iodide (Table V); it also does not equilibrate with the added nonradioactive iodide in the presence of lactoperoxidase and H_2O_2 (Table V). In other words the conditions necessary to introduce the ^{125}I into the chloroplast are not adequate to permit its movement out of the chloroplast, i.e. the $^{125}I^-$ which has been introduced into the chloroplast in the presence of lactoperoxidase is not present at sites available for reassociations with the high molecular weight lactoperoxidase.

Release of iodide upon illumination of iodochloroplasts

When chloroplasts, iodinated according to the standard method and resus-

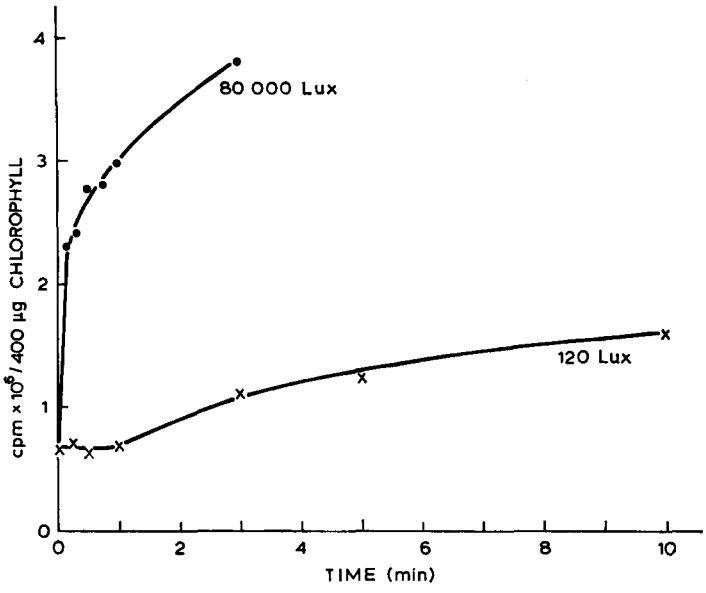

Fig. 2. The light dependent secretion of iodide from iodochloroplasts. Chloroplasts, 0.8 mg chlorophyll/ml, in three tubes were iodinated in the dark for 3 min as described in Materials and Methods. After centrifugation, the samples were pooled, washed three times with an excess of sucrose buffer, suspended in 6 ml sucrose buffer and divided into six tubes. Each tube was illuminated at 4 °C for different intervals of time, centrifuged at 140 000 × *g* for 1 h and the radioactivity in the supernatant determined.

TABLE VI

THE EFFECT OF INTERMITTENT ILLUMINATION ON THE RELEASE OF ^{125}I BY IODOCHLOROPLASTS

Iodochloroplasts, washed in sucrose buffer and resuspended in equal portions in the dark, were illuminated for the indicated times at 120 lux, except for the last sample which was used as an indication of the maximum possible release under intense light. The abbreviations L and D refer to light and dark. Note that the total amount of iodide released depends on the total amount of illumination and is independent of the interspersed 1-min periods of darkness. Illumination was performed in an ice bath at 0 °C. The cpm present in the supernatant solution of the control tube kept dark for 10 min, is considered to be the control background value.

Condition of illumination at 120 lux (min)	Release of ^{125}I (cpm/ml) × 10^{-3}	Change (% of control)
10 dark control	554	0
1	520	−6
3	668	21
5	807	46
10	950	72
1 L, 1D, 1 L	637	15
1 L, 1 D, 1 L, 1 D, 1 L	768	39
(1 L, 1 D) five times	872	57
4 at 80 000 lux	2900	423

pended in sucrose buffer, are exposed to varying intensities of light for varying periods of time they extrude or secrete iodide.

Fig. 2 shows the characteristic of this iodide release at two levels of light intensity. The lower curve shows that at low light intensity (120 lux) there is a lag of at least 1 min before iodide is released into the medium from the iodochloroplasts; subsequent to this a progressive release of iodide occurs with time of illumination at the same light intensity. However, at very high light intensity, in this case 80 000 lux, there occurs a very rapid initial release of iodide within 10 s; this also progressively increases with time of illumination at this light intensity. Consequently we can conclude from these experiments that the release of the intrachloroplast iodide is in some way associated with photosynthetic or photodissociation reactions.

Cumulative effect of illumination

The results of Tables VI and VII emphasize two important characteristics of the iodochloroplasts. Table VI shows that two 1-min periods of illumination interrupted by 1 min of darkness release an amount of iodide which is intermediate between that released after 1 min and that released after 3 min of continuous illumination. Furthermore it shows that three 1-min periods of illumination or five 1-min periods of illumination release amounts of iodide equivalent to those released after continuous illumination for 3 or 5 min, respectively. In other words the interposed 1 min dark periods do not reverse the effect of the previous illumination; the amounts of illumination summate.

Table VII shows that the additive nature of the 1-min periods of illumination continue even though these light periods are interrupted by dark periods as long as 5 or 10 min. The long periods of time after which the initial light effect can be ex-

TABLE VII

RELEASE OF ^{125}I BY IODOCHLOROPLASTS: CUMULATIVE EFFECT OF ILLUMINATION AND ITS INDEPENDENCE OF THE LENGTH OF THE INTERPOSED PERIODS OF DARKNESS

The experiment was performed in the same way as described in the legend to Table VII, except that a constant 3-min period of total illumination was used, in 1-min intervals interspersed with varying lengths of dark periods, to establish the cumulative nature of the illumination on the release of iodide. D and L refer to dark and light. Control values are considered to be the values obtained from the zero and 23 min dark control tubes.

Conditions of illumination (min)	Release of ^{125}I (cpm/ml) $\times 10^{-3}$	Change (% of control)
0 dark control	572	0
23 dark control	570	0
3 L, 20 D	755	32
20 D, 3 L	760	34
1 L, 1 D, 1 L, 1 D, 1 L	745	31
1 L, 2 D, 1 L, 2 D, 1 L	775	36
1 L, 3 D, 1 L, 3 D, 1 L	765	34
1 L, 5 D, 1 L, 5 D, 1 L	870	55
1 L, 10 D, 1 L, 10 D, 1 L	725	28

pressed suggest that a threshold is attained and maintained in a compartment which does not equilibrate easily with its surroundings.

DISCUSSION

Previous experiments have indicated that the conditions of iodination are critical; iodination in the presence of low cell concentrations or high iodide concentrations result in the iodination of intracellular components; i.e. sites located at some distance from the lactoperoxidase-iodide complex. This was convincingly proven in the case of red blood cells because both hemoglobin and intramembrane components could be shown to be iodinated under such conditions [4, 5]. This phenomenon was attributed to the half-life of the lactoperoxidase-iodide complex versus the time required for this complex to collide with an iodinatable site on the membrane surface; if the latter time is longer, a condition favored by a low concentration of red blood cells, the lactoperoxidase-iodide complex would decompose, freeing an iodide radical which could then iodinate sites not available to lactoperoxidase, i.e. intramembrane and intracellular sites.

The mechanism by which iodide enters the chloroplast is based on the formation of the iodide-lactoperoxidase complex as well as on the impermeability of the chloroplast membrane to iodide. One of many possible schemes is that the lactoperoxidase-iodide complex iodinates an unstable carrier molecule on the surface of the membrane (possibly water) which decomposes randomly to yield inorganic iodide. If the decomposition of the unstable intermediate occurs so as to permit the iodide to move towards an intrachloroplast or cryptic site of the chloroplast, the iodide becomes trapped; alternatively, if the decomposition occurs so as to permit the iodide to move towards the surrounding solution, it will mix with the free iodide of the solution and become available for recapture by lactoperoxidase.

Based on the results obtained with red blood cells, the present work emphasizes that when conditions are chosen to favor the iodination of exposed chloroplast sites by the lactoperoxidase-iodide complex, photosynthesis is not inhibited; however, photosynthesis is inhibited when the conditions are changed to favor iodination of interior chloroplast sites. We should therefore conclude from these results that either iodination of exposed chloroplast sites does not inhibit photosynthesis or that photosynthetic units sensitive to iodination are not accessible to iodination by lactoperoxidase. In either case the topographical location of these sites cannot be defined by these experiments.

The results presented in this paper further indicate that chloroplasts into which iodide has been introduced by this method secrete or extrude iodide upon illumination, that the iodide is secreted in association with a photodissociation reaction and that the amount of iodide secreted depends on the degree of illumination. Furthermore, at low intensities of illumination there is a lag in secretion of iodide, subsequent to which iodide secretion is in proportion to the time of illumination.

An intriguing aspect of this lag period is that small amounts of light will produce an effect which is cumulative; even after 5–10 min of darkness the effect of a new small amount of light will add on to the previous light effect and eventually summate into the secretion of an amount of iodide corresponding to the total dose of illumination. The primary site upon which the light acts does not appear to be in rapid equilib-

TABLE VIII

LIGHT-DEPENDENT SECRETION OF BOUND ^{125}I AND INORGANIC ^{125}I FROM IODOCHLOROPLASTS

Experimental conditions were as described in Fig. 2. Intensity of light, 120 lux; high intensity, 80 000 lux. D and L refer to dark and light.

Condition of illumination (min)	Percent of total counts $R_F = 0.0$	$R_F = 0.4$
10 min dark control	8.0	92.0
9 D+1 L	8.5	91.5
5 D+5 L	10.0	90.0
10 L	12.7	87.3
3 high intensity light	18.0	82.0

rium with its environment in a manner which reverses the previous light effect; rather the light effect summates to a certain threshold, subsequent to which all ensuing illumination will be mirrored by a corresponding secretion of iodide.

The nature of this primary site cannot be defined as yet; however, it has the following characteristics. It is very sensitive to low intensity illumination and can be saturated with low amounts of light energy; subsequent to its saturation, further low intensity illumination produces an effect which can be reflected in the secretion or extrusion of iodide and furthermore the iodide secreted is mostly accounted for as inorganic iodide; however, a significant amount of "high molecular weight bound iodide" is correspondingly secreted so that the ratio of bound iodide ($R_F = 0.0$) to inorganic iodide ($R_F = 0.4$) is increased (Table VIII).

The bound iodide that is released cannot be due to the removal of proteins from "membrane pores" which now permit iodide to escape; the data indicate that except for the slow background leakage of iodide, iodide release is only associated with illumination, no release occurs in the dark following illumination. It is characteristic of these reactions that illumination does not result in the release of the iodide bound to low molecular weight chloroform-soluble compounds, $R_F = 1.0$. Should these prove to be phospholipids our results indicate that they are not mobilized in response to these phenomena. An additional property of this primary site is that it responds to illumination at 0 °C. The most probable explanation that suggests itself as being in conformance with all these properties is that a conformational change occurs on the membrane, with illumination.

Because these chloroplasts contain iodide as free iodide, as protein-bound iodide and as iodide bound to low molecular weight compounds, presumably lipids or phospholipids, and because they extrude these iodide pools in response to light, it is obvious that they shall be used extensively as a tool in photosynthesis. For these reasons we believe that they should be differentiated from normal chloroplasts and we believe the name iodochloroplasts to be descriptive enough.

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