THE EFFECTS OF SOME INHIBITORS OF PHOTOSYNTHESIS UPON THE PHOTOCHEMICAL REDUCTION OF A DYE BY ISOLATED CHLOROPLASTS

FERGUS D. H. MACDOWALL[1]

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Introduction

Physiological research directed upon the activity of isolated chloroplasts has been spurred by the belief that the photochemical activity which chloroplasts exhibit *in vitro* is part of photosynthesis. If this is so, then those components of the photosynthetic apparatus which are active in isolated chloroplasts can thus be brought into closer range for study. The object of the present work was to find how closely the dye-reducing system of isolated chloroplasts is related to photosynthesis, by comparing the response of the former to various poisons and narcotics whose action upon the latter is already known, and to investigate the nature of the chloroplast apparatus through its activity.

It is believed that the photochemical and enzymatic machinery of that part of the photosynthetic mechanism which brings about the oxidation of water and the liberation of oxygen can be removed from the living cell intact in the chloroplast structure in a form such that it will continue to function *in vitro* in the presence of externally supplied oxidants. It was the presence of such oxidants in aqueous leaf extracts which permitted the action of freed chloroplasts in Hill's initial experiments (**7**) wherein the evolution of oxygen was followed spectrophotometrically via the conversion of haemoglobin to oxyhaemoglobin. Such oxidants must also have been important in the original experiments by HABERLANDT (**6**) and EWART (**3**), and in those investigations by MOLISCH (**16, 17**) and INMAN (**13, 14**) which followed.

HILL (**8**) found that ferric potassium oxalate may be substituted for these unknown oxidants, and more recently WARBURG and LÜTTGENS (**20**) struck a close analogy to photosynthesis through the successful use of p-benzoquinone which accepts hydrogen in its reduction, unlike Fe^{+++} in the Hill reaction but like anabolites in photosynthesis. HOLT and FRENCH (**10**) found that chromate and ferricyanide can be reduced by the action of isolated chloroplasts with the evolution of oxygen, and developed a technique involving the visible reduction to their leuco- forms of several redox indicators (referred to here as "dyes"). The reduction of such dyes is now followed photometrically by the employment of a photronic cell, with appropriate filters, connected to a galvanometer. This method was used in the poisoning studies reported below and will be described in more detail in a forthcoming paper by HOLT, SMITH and FRENCH (**12**).

[1] Present address: Carnegie Institution of Washington, Division of Plant Biology, Stanford, California.

The following brief summary of evidence gleaned from past work on isolated chloroplasts is included here in support of the belief that the activity of isolated chloroplasts is homologous with a basic part of the photosynthetic mechanism:

1) Light is necessary; therefore, photochemical reactions are involved.

2) The quantum yield of the Hill reaction (FRENCH and RABIDEAU (**4**)) sometimes approaches the efficiency of photosynthesis in Chlorella.

3) Photochemical dye reduction occurs efficiently in red light which the dye (phenol indophenol at pH 6.5) does not absorb, so that at least certain chloroplast pigments do the sensitizing as in photosynthesis. Hill found that the most active wave lengths for his reaction are those around 600 mμ, which points to the same conclusion.

4) Etiolated chloroplasts of Swiss chard do not reduce dye under strong illumination of thirty minutes duration, which suggests the necessity of chlorophyll. Furthermore, J. H. C. Smith, C. S. French and V. Koski have recently found that the dye-reducing activity of chloroplasts from etiolated barley seedlings which were exposed to light of increasing duration, is directly proportional to the amount of chlorophyll resulting in the chloroplasts.

5) The light saturation which is characteristic of photosynthesis is also exhibited in the activity of isolated chloroplasts.

6) The reaction system of isolated chloroplasts includes one or more heat labile enzymatic reactions. Activity is completely lost in 15 minutes at 50° C.

7) The stoichiometry of the Hill reaction (wherein the evolution of oxygen is measured manometrically) and of the reactions in which quinone and the dye phenol indophenol are the oxidants respectively, shows that an oxide, like water in photosynthesis, is split by isolated chloroplasts as follows:

$$4\ Fe^{+++} + 2\ H_2O \longrightarrow 4\ Fe^{++} + 4\ H^+ + O_2$$
$$2\ Q + 2\ H_2O \longrightarrow 2\ QH_2 + O_2$$
$$2\ Ind + 2\ H_2O \longrightarrow 2\ IndH_2 + O_2$$

8) HOLT and FRENCH (**9**) found that the molar ratio of hydrogen ion (H^+) produced to oxygen (O_2) produced in the Hill reaction is 4:1 as in water ($2H_2O$).

9) In recent experiments with water with H_2O^{18}, HOLT and FRENCH (**11**) have shown that the oxygen evolved by the photochemical activity of isolated chloroplasts, in solutions of the various effective oxidants, comes from water.

10) In the absence of CO_2, intact cells of Chlorella evolve oxygen in light in the presence of Hill's solution (**10**) or p-benzoquinone (**21**) as do also Scenedesmus cells in solutions of the latter (**1**).

Further evidence for the belief that the photochemical activities of chloroplasts in the test tube and in the living cell are in fact homologous

rather than analogous phenomena is afforded here by the response of isolated chloroplasts to inhibitors of photosynthesis. Conversely, the effects of some inhibitors upon photosynthesis can be further characterized through studies on isolated chloroplasts. The fact must be borne in mind that only a part of the photosynthetic mechanism is isolated with the chloroplasts, as that part which is directly involved in the participation of carbon dioxide is inactivated in the process of isolation (with the death of the cell).

Methods

Isolation of chloroplasts

Because of the need for dependable supply and activity through the winter months, the chloroplasts used in these experiments were taken from the leaves of spinach (*Spinacia oleracea*) purchased fresh from the market, and of Swiss chard (*Beta vulgaris*) from the greenhouse. A small handful was first washed and the midribs removed. The chloroplasts were then freed by maceration for one minute in a cooled Waring Blendor with about 140 ml. of water. The mash was then strained through a cotton cloth and the resulting filtrate centrifuged at 800 g. in a small-angle centrifuge. The chloroplast portion of the precipitate was resuspended in a small volume of water (5 to 10 ml.). The whole operation was carried out as near to 0° C as possible, and resulted in a concentrated suspension of chloroplasts which was then stored in a test tube in an icebox close to 0° C.

Reaction mixtures and procedures

The reaction vessel was a rectangular glass cell of $1 \times 1 \times 5$ cm. inside dimensions. Three ml. of liquid in it could be fully illuminated by the light beam striking the vessel as placed in position before a photronic cell in a thermostat. As this volume was convenient to use, 1.5 ml. of the dye mixture (consisting of 10^{-4} M phenol indophenol, 0.08 M KCl, and 0.04 M phosphate buffer at pH 6.5) was run into the reaction vessel, and 1.5 ml. of the inhibitor solution to be tested—or distilled water in the case of the control—was added to it. A small volume of the chloroplast suspension (usually 0.04 to 0.1 ml. and containing between 0.03 and 0.08 mg. chlorophyll) was added to this mixture, and the chloroplasts were suspended homogeneously by gentle shaking. The chlorophyll content was found by extraction of samples with methyl alcohol and by determination with a Klett-Summerson photoelectric colorimeter.

The chloroplasts were left in the reaction mixture for five minutes in the dark at 20° C, the vessel being placed in a metal box held in the thermostat. This allowed sufficient time for the attainment of temperature equilibrium, and sufficient pretreatment for the poison or narcotic to render its effects almost complete. Readings were taken of the galvanometer deflection, which increased with the amount of light striking the pho-

tocell as the dye was reduced. The selected time for total dye reduction in the control reaction mixture during illumination ranged from two to six minutes, and readings were taken as frequently as eight times per minute when necessary.

The experiment designed to find the effects of an inhibitor consisted of a series of runs with conditions constant and with reaction mixtures identical except in the concentration of the inhibitor. Control runs were interposed between those with different concentrations of inhibitor, so that the loss of chloroplast activity with time could be determined.

In order to find whether an inhibitor affects a light (photochemical) or a dark (enzymatic) reaction, the rates of dye reduction by the control and poisoned mixtures were determined at a series of intensities of continuous illumination, obtained by using wire screens of known transmission, and the light-saturation curves were compared. When an inhibitor acts

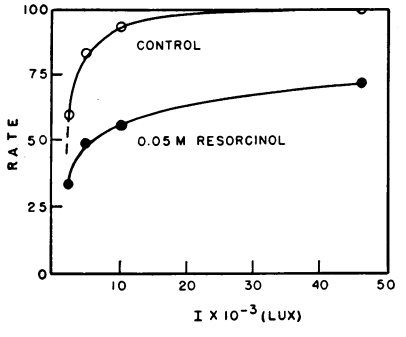

FIG. 1. Light-saturation curves showing the effect of 0.05 M resorcinol upon the rate of dye reduction by isolated chloroplasts at a series of intensities of continuous illumination.

upon a dark reaction which limits the rate at high light intensities, its effect is most evident at these high intensities (e.g. fig. 4). The inhibition of a photochemical reaction, on the other hand, shows up at low light intensities where light is limiting the rate of the overall reaction (e.g. fig. 1). These criteria have been used by workers in studies on photosynthesis.

Some physiologists have held that if an inhibitor of photosynthesis does not shift the absorption spectrum of chlorophyll in the leaf its effect is not due to an attack on chlorophyll. Some photochemists prefer to have further evidence, such as the absence of any influence upon the fluorescence of chlorophyll.

In the present work, however, tests were made only with absorption spectra, and runs were made with chloroplast suspensions and various separated pigments, as recorded in the results, in the presence of certain of the more important inhibitors. A Beckmann spectrophotometer was used for the determination of absorption spectra, and those obtained for chloroplast suspensions are surprisingly sharp.

Computations and errors

The maximum slope of the experimentally recorded curve describing the logarithm of the galvanometer deflection against time was taken as the rate of dye reduction. The galvanometer deflections were plotted logarithmically in order to obtain a linear relationship between dye concentration and time in accordance with Beer's law.

Some suspensions of chloroplasts lose their activity very rapidly after preparation. Although fresh Swiss chard chloroplasts were more active than those of spinach from the market, the former lost their activity much more rapidly—up to 50 per cent. in five hours. When necessary such an activity loss was corrected for in order to put all rates on the same basis. This was done by expressing the inhibited rate as per cent. of the control rate as taken from the curve for loss of activity with time.

The results obtained with a given sample of chloroplasts were generally reproducible within 10 per cent. However, because of some variation in

TABLE I

THE DEPENDENCE OF THE INHIBITORY ACTION OF TWO REAGENTS UPON THE CONCENTRATION OF CHLOROPHYLL IN THE REACTION MIXTURE.

Inhibitor	% Inhibition at different molar concentrations of chlorophyll (contained in the chloroplasts) in the reaction vessel		
	3.6×10^{-5} M	1.8×10^{-5} M	3.6×10^{-6} M
2×10^{-5} M o-phenanthroline	38	41	43
2.5×10^{-3} M thymol	71	73	85

responses to inhibitors between different samples of chloroplasts, even from the same species of plant, it is not safe to state the results of this work in rigidly quantitative terms. As already mentioned the amount of chlorophyll contained in the chloroplasts used in the reaction vessel throughout this work varied with experiments between 0.03 and 0.08 mg., which is between 10^{-5} and 3×10^{-5} molar concentrations relative to the volume of fluid in the reaction vessel. At the most then, if the results obtained with o-phenanthroline and thymol (table I) can be generalized, such differences would not cause more than 5 per cent. variation in a measurement of inhibited activity. Nevertheless, caution should be observed in comparing the results of the present inhibition experiments with those on photosynthesis obtained by other workers who have not measured or recorded chlorophyll concentrations.

Results

In those figures below which describe the effects of inhibitors upon the rate of dye reduction by isolated chloroplasts, the molar concentration of the inhibiting reagent is plotted logarithmically on the abcissa against the per cent. of the control (unpoisoned) rate on the ordinate.

Poisons for metallo-catalysts

Cyanide—It was found that the rate of dye reduction by isolated chloroplasts is retarded 15 to 20 per cent. by 10^{-2} M KCN. Cyanide therefore does not poison this system specifically.

Azide—Azide also is not a specific poison for the present reaction system as 50 per cent. inhibition is brought about by 0.08 M sodium azide. Tests at two intensities of light with Swiss chard chloroplasts showed that the inhibition by 10^{-3} M azide was less at the lower intensity, thus indicating that a dark reaction is affected. The results were as follows:

Light intensity (lux):	46,300	4,900
Per cent. of control rate:	68%	83%

Pyrophosphate—This substance like cyanide is considered to be an inhibitor of certain iron-catalyzed reactions. Difficult solubility limited the concentration of the poison stock solution to 0.2 M, and it was necessary to use sodium pyrophosphate and sodium acid pyrophosphate in the appropriate proportions to give a poison solution of approximately pH 6.5.

The results, corrected for the loss of chloroplast activity with time, show that pyrophosphate up to 0.05 M concentration does not affect the rate of dye reduction by isolated chloroplasts.

Hydroxylamine—It was found here that a mean value, between samples of chloroplasts from spinach and Swiss chard leaves, of 3.1×10^{-4} M hydroxylamine hydrochloride caused 50 per cent. inhibition in the rate of dye reduction. The mean amount of chlorophyll in the chloroplasts used in the reaction vessel through these tests was 0.042 mg. which is a concentration of approximately 1.6×10^{-5} M. There was wider variation in the concentrations of poison causing complete inhibition and causing no inhibition between the different samples; for example, 9×10^{-4} to 4×10^{-3} in the former instance. Hydroxylamine can reduce the dye autonomously, but at concentrations of the poison which are critical for chloroplasts the rate of this reduction is not significant relative to the action of the chloroplasts. Results can readily be corrected for such a factor if necessary.

Somewhat irregular results were obtained in experiments on the effects of hydroxylamine at different intensities of light with either one of the dyes phenol indophenol or 2,6-dichlorophenol indophenol. For example, the following results were obtained with 10^{-3} M hydroxylamine:

Light intensity (lux):	32,400	15,900	7,650	3,800	1,800
Per cent. of control rate:	53%	48%	50%	44%	46%

However, the data all show that this poison retards the activity of chloroplasts by a constant fraction at the different intensities, and/or, because of a slight trend toward a greater effect at lower intensities, that it acts upon a light reaction.

It was found that hydroxylamine does not shift the absorption spectra of leaf xanthophylls in ether nor of chlorophyll *a* in ether, nor the red absorption maximum of chlorophyll in active chloroplasts (at poisonous concentrations). The interpretation of this evidence, which stands or falls on the validity of the spectrum-shift criterion, makes it unlikely that the effect of hydroxylamine is due to a direct combination of the poison with one of the above substances.

o-PHENANTHROLINE—Samples of chloroplasts containing a mean value of 0.034 mgm. chlorophyll (1.3×10^{-5} M) were completely inhibited by a mean concentration of 1.3×10^{-4} M o-phenanthroline (a 1:10 ratio of chlorophyll: o-phenanthroline), were retarded 50 per cent. by a mean concentration of 2.7×10^{-5} M o-phenanthroline, and were not affected by about 3.8×10^{-6} M o-phenanthroline at the highest.

The action of zinc sulphate in preventing the inhibition of the activity of isolated chloroplasts by o-phenanthroline, as shown by WARBURG and LÜTTGENS (**22**), was confirmed here. Tests were made at several concen-

TABLE II

THE PREVENTION, BY SOME METALS, OF THE INHIBITION OF DYE REDUCTION BY O-PHENANTHROLINE.

REAGENTS IN MIXTURE	PER CENT. OF CONTROL RATE OF DYE REDUCTION BY CHLOROPLASTS
10^{-4} M o-phenanthroline	37%
" + 4.7×10^{-5} M $CuSO_4$	115%
" " $CoSO_4$	104%
" " $NiSO_4$	92%
" " $FeSO_4(NH_4)_2SO_4$	66%

trations of $ZnSO_4$ such that it could be shown that approximately 2×10^{-5} M $ZnSO_4$ completely prevented the inhibition caused by 10^{-4} M o-phenanthroline upon a sample of spinach chloroplasts, whereas 3.8×10^{-6} M was the highest concentration of $ZnSO_4$ having no effect. Cupric sulphate, cobaltous sulphate, nickelous sulphate, and ferrous ammonium sulphate were also tried and found to prevent the inhibition by o-phenanthroline in that order (table II), but $ZnSO_4$ was the most effective. This prevention of inhibition is not the same thing as reversal.

The rate of dye reduction was measured for control and poisoned mixtures at a series of intensities of continuous light (table III). The results show that o-phenanthroline inhibits a light reaction. It was also found that this poison does not shift the red absorption maximum of chlorophyll in poisoned chloroplasts.

RESORCINOL—In the light of WARBURG and LÜTTGENS' (**22**) conclusion that o-phenanthroline inhibits by combining with catalytically active zinc, it was decided to try here another means of tying up zinc. Of the cations occurring in the chloroplasts only zinc should react with resorcinol, at least under analytical conditions. As shown in figure 2, it was found that

resorcinol inhibits dye reduction by isolated chloroplasts (from Swiss chard) only at high concentrations, so that unlike o-phenanthroline, it is not a specific poison. Whether or not resorcinol can combine with zinc in phosphate buffered media at pH 6.5 is not certain.

Trials at different light intensities (fig. 1) show that both light and dark reactions are inhibited by 0.05 M resorcinol.

THIOUREA—Dye reduction by Swiss chard chloroplasts containing 0.052 mg. chlorophyll was retarded 50 per cent. by 0.15 M thiourea. Inhibition started at about 10^{-2} M and was complete at about 2 M (by extrapolation). This poison can reduce the dye, but again this rate is negligible relative to the action of chloroplasts at effective concentrations of poison.

8-HYDROXYQUINOLINE—This substance is not very soluble in water

TABLE III

THE EFFECT OF O-PHENANTHROLINE UPON THE RATE OF DYE REDUCTION BY CHLOROPLASTS AT A SERIES OF LIGHT INTENSITIES.

CONCN. O-PHENANTHROLINE	% CONTROL RATE AT DIFFERENT LIGHT INTENSITIES (LUX)					
	46,300		10,200	4,900		2,100
	1	2	1	1	2	1
Samples 1, 2: 10^{-4} M	22%	18%	17%	12%	17%	10%
	6,700		4,400	2,200		1,000
Sample 3*: 10^{-5} M	70%		60%	48%		47%

* 2,6-dichlorophenol indophenol used as the dye (redox indicator) with different apparatus and at 15° C.

and gives a yellow solution. However, it did not reduce the dye and was apparently not affected itself by the activity of the chloroplasts. Because of solubility difficulties it was not used above 2.2×10^{-3} M in the reaction mixture. This concentration gave 22 per cent. inhibition. It can combine with many metals including zinc.

HEAVY METALS

COPPER SULPHATE—The rate of dye reduction by isolated Swiss chard chloroplasts containing 0.066 mg. chlorophyll (2.5×10^{-5} M) was retarded 50 per cent. by approximately 10^{-5} M $CuSO_4$. The results of rate measurements at a series of intensities of light show, as follows, that $CuSO_4$ (10^{-4} M) poisons a light reaction:

Light intensity (lux):	46,300	10,200	4,900	2,200
Per cent. of control rate:	39%	29%	16%	7%

The results obtained by GREENFIELD (**5**) with $CuSO_4$ and the photosyn-

thesis of Chlorella at a series of light intensities are shown for comparison as follows: (5×10^{-6} M $CuSO_4$ was used)

Light intensity (lux):	22,000	4,000	1,600	700	350
Per cent. of control rate:	54%	64%	72%	79%	69%

It was found that $CuSO_4$ does not shift the absorption spectrum of the chloroplast pigments in the chloroplast.

MERCURIC CHLORIDE—$HgCl_2$ poisoned the reaction mechanism of Swiss chard chloroplasts very specifically (fig. 2). For a concentration of chloro-

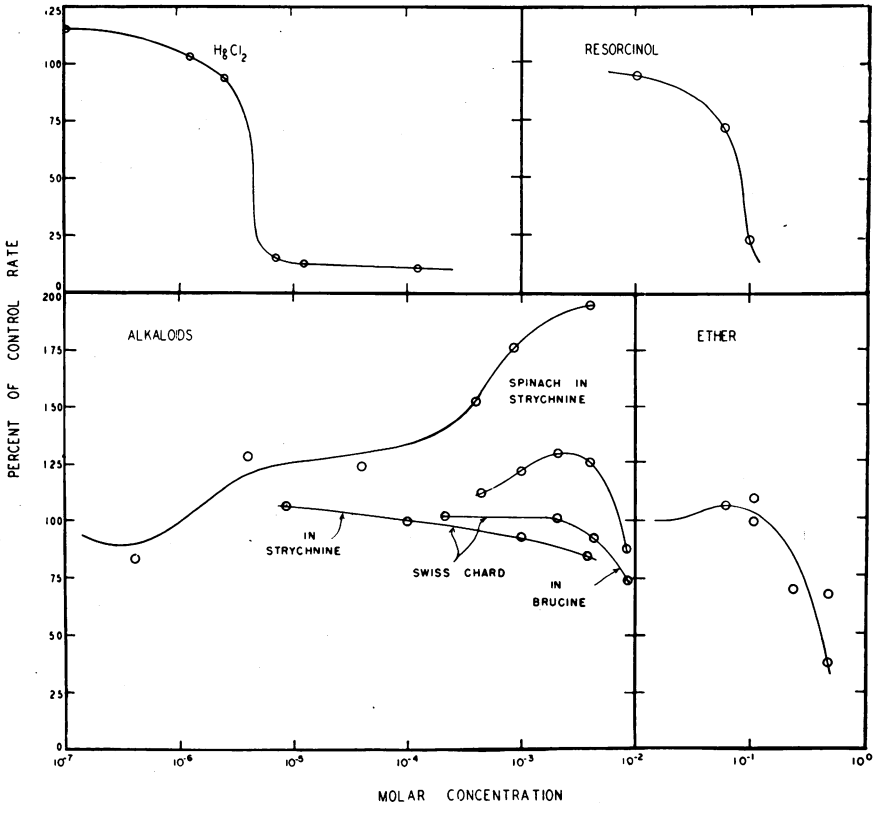

FIG. 2. The effects of a number of inhibitors upon the rate of dye reduction by isolated chloroplasts.

phyll in the reaction vessel of 7.4×10^{-5} M, concentrations of $HgCl_2$ below 10^{-6} M apparently stimulated the system, and 50 per cent. inhibition was brought about by 4×10^{-6} M poison. The absorption spectrum of the pigments in the chloroplast was not affected by even 10^{-3} M $HgCl_2$. Tests at different intensities of light gave the following results, showing that a dark reaction is inhibited by 6×10^{-6} M $HgCl_2$:

Light intensity (lux):	46,300	10,200	4,900	2,200
Per cent. of control rate:	14%	15%	17%	25%

OTHERS—Nickel, cobalt and zinc as their respective sulphate salts were tried without success because, within the concentration ranges in which they are known to affect photosynthesis, precipitation occurred in the presence of buffer (phosphate or borate) at pH 6.5.

OTHER SPECIFIC POISONS

DINITROPHENOL—Sodium, 2,4-dinitrophenol was found to have a specific effect upon the reduction of dye by isolated chloroplasts. Samples of chloroplasts from spinach and Swiss chard leaves, containing an average concentration of 1.3×10^{-5} M (0.035 mg.) chlorophyll, were inhibited 50 per cent. by a mean concentration of 6.3×10^{-4} M dinitrophenol. Variation between samples in concentrations of poison giving 50 per cent. inhibition was not great (4.5×10^{-4} M to 7.5×10^{-4} m), but incipient inhibition was brought about by concentrations ranging from 6×10^{-6} M to 10^{-4} M dinitrophenol, and complete inhibition occurred in solutions of 5×10^{-3} to 5×10^{-2} M poison.

Experiments showed that dinitrophenol inhibits more as the light intensity is decreased, i.e., that it inhibits a light reaction. For example, as follows with 10^{-3} M dinitrophenol:

Light intensity (lux):	6,700	4,350	2,200	1,000
Per cent. of control rate:	45%	37%	35%	28%

Because of the comparatively low light intensities used it is not certain whether or not the poison also affects a dark reaction.

This poison does not affect the spectral absorption maxima of pure chlorophyll *a* in ether solution, nor of the chlorophyll in poisoned chloroplasts. As was the case with most inhibitors tested in this work, the dye was not reduced by dinitrophenol.

IODOACETIC ACID—The dye-reducing activity of isolated Swiss chard chloroplasts was inhibited by iodoacetate over a wider inhibitory transition-range than was the photosynthesis of Chlorella as found by KOHN (**15**). The results from two of the present experiments are shown in figure 3. The rate of dye reduction was retarded 50 per cent. by approximately 10^{-2} M iodoacetic acid. Penetration of the poison was not the problem it was for live cells (Kohn), as a thirty-minute pretreatment to 10^{-2} M iodoacetate in the dark gave only 6 per cent. greater inhibition than did the regular five-minute pretreatment.

The following results show that a dark reaction is inhibited by 5×10^{-3} M iodoacetate, thus agreeing with Kohn's findings in photosynthesis:

Light intensity (lux):	46,300	4,900
Per cent. of control rate:	43%	56%

NARCOTICS

PHENYLURETHAN—The position of the inhibitory transition-range of phenylurethan in dye reduction by isolated spinach chloroplasts, fell at a

tenfold higher concentration range than that found by WARBURG (**20**) for the photosynthesis of Chlorella (fig. 3). In the present work, approximately 2×10^{-3} M phenylurethan brought about 50 per cent. inhibition, the concentration of chlorophyll within chloroplasts in the reaction vessel being 3.2×10^{-5} M (0.088 mg.).

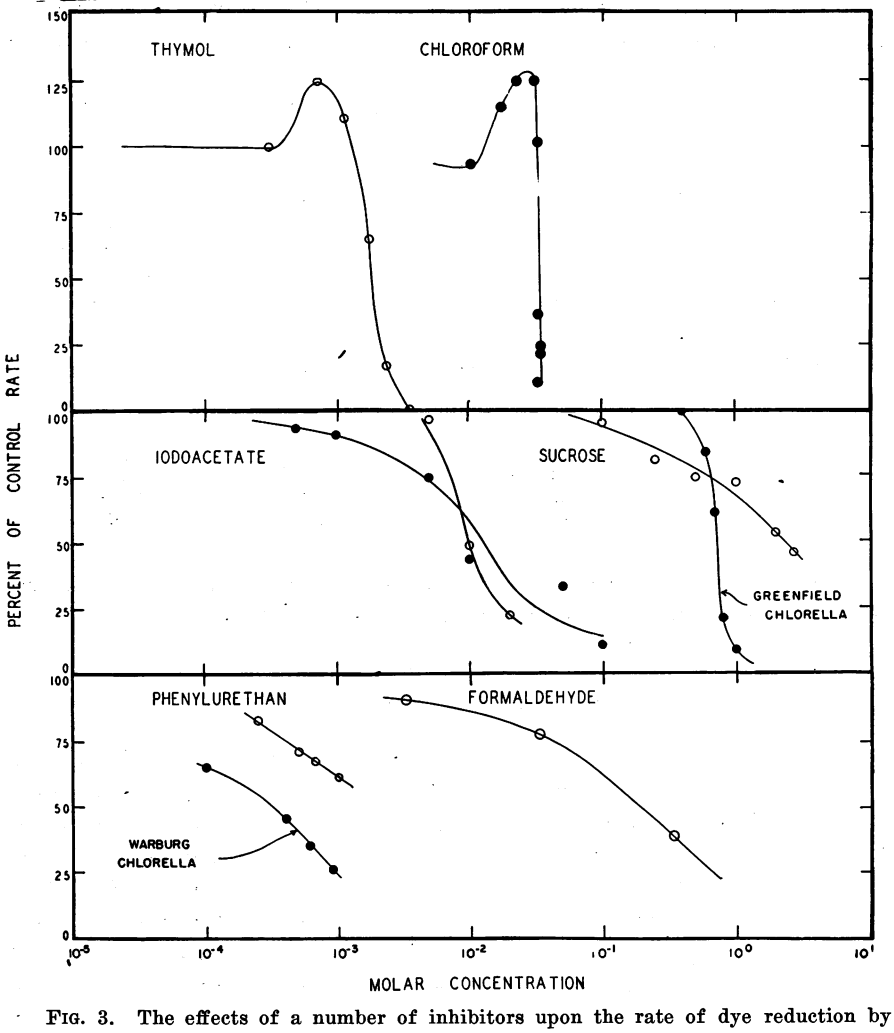

FIG. 3. The effects of a number of inhibitors upon the rate of dye reduction by isolated chloroplasts.

The stronger inhibition observed at the lower light intensity (table IV) means that phenylurethan inhibits a light reaction.

In accordance with the suggestion made by RABINOWITCH (**18**, p. 322), the absorption spectrum of active spinach chloroplasts (but *in vitro* here) was followed before and after treatment with strongly poisonous concentrations of phenylurethan. There was no shift in the absorption peaks of

chlorophyll in the chloroplasts so that the possibility of direct action of the narcotic upon chlorophyll is not favored by this evidence.

THYMOL—Thymol completely inhibited the photochemical activity of spinach chloroplasts at 2.6×10^{-3} M concentration and caused no change in activity at 1.2×10^{-3} M concentration (fig. 3). This sharpness of action might indicate a type of high specificity. The enhancing of chloroplast activity by concentrations of thymol immediately preceding those causing inhibition is of theoretical interest.

Tests run at series of intensities of continuous light showed that thymol

TABLE IV

THE EFFECTS OF SOME NARCOTICS UPON THE RATE OF DYE REDUCTION BY CHLOROPLASTS AT TWO LIGHT INTENSITIES.

INHIBITOR	CONCENTRATION	PER CENT. OF CONTROL RATE	
		46,300 LUX	4,900 LUX
Phenylurethan	5×10^{-4} M	71%	44%
	1×10^{-3} M	62%	47%
Chloroform	2.3×10^{-2} M	71%	57%
	2.6×10^{-2} M	43%	36%
Strychnine	4.8×10^{-3} M	194%	87%
	1.1×10^{-3} M	178%	78%
	4.8×10^{-4} M	153%	87%
	4.8×10^{-5} M	123%	80%

inhibits the rate of reaction equally well at all light intensities. The results of one experiment with 10^{-3} M thymol were as follows:

Light intensity (lux):	18,500	2,200	440
Per cent. of control rate:	51%	50%	52%

It was also found that thymol does not bring about any shift in the red absorption peaks of chromatographically separated chlorophylls *a* and *b* in ether. This evidence is contrary to that which might be expected if thymol combines with chlorophyll. Nor does this narcotic shift the red absorption peak of chlorophyll in active chloroplast fragments after their treatment by supersonic vibration. Hence, as is also the case with the other reagents which do not cause a shift in this spectrum, it also does not bring about a dissociation of the chlorophyll-protein complex. The absorption spectrum of pure carotene pigments in ether is not affected by thymol.

CHLOROFORM—The activity of spinach chloroplasts containing 0.065 mg. chlorophyll (2.4×10^{-5} M in the reaction vessel) was retarded 50 per cent. by 3.4×10^{-2} M chloroform and completely arrested by 3.5×10^{-2} M chloroform, which shows that this narcotic is about ten times less "specific" than thymol. The shape of the curve describing the inhibitory transition-range for chloroform (fig. 3) is very like that for thymol and shows a 25 per cent. stimulation of chloroplast activity at concentrations of the

narcotic just preceding those causing inhibition. Reversal was attempted by centrifuging and washing the chloroplasts followed by a second centrifuging and the resuspension of the chloroplasts in control reaction mixtures. Single treatments were partly successful (up to 50%).

According to the results obtained at two intensities of continuous illumination (table IV) chloroform inhibits a light reaction.

ETHER—The results obtained in the present experiments were fairly irregular, possibly because of the volatility of ether. To minimize this, ether solutions were made to desired concentration immediately in the reaction vessel and the chloroplasts were suspended by a single slow inversion of the vessel. The curve representing the inhibition by ether (fig. 2) is drawn as a mean curve through the values which were corrected for loss of chloroplast activity over the duration of the experiment. It can be seen that the inhibitory transition-range of ether is similar in form (with evidences of stimulation) to that of thymol and of chloroform, and that ether is the least specific narcotic. 0.45 M ether inhibited the rate of dye reduction of isolated Swiss chard chloroplasts by 50 per cent.

The rates of dye reduction in the presence of 3.5 per cent. ether (0.47 M) at two intensities of continuous illumination indicate that a dark reaction is inhibited by ether, as the rate is inhibited by 30 per cent. and 23 per cent. at light intensities of 20,600 and 2,200 lux respectively.

STRYCHNINE AND BRUCINE—The results obtained for the action of these alkaloids upon the activity of isolated chloroplasts, from several samples of leaves, are shown graphically in figure 2. Stimulation was again apparent for most samples. One sample of spinach chloroplasts showed a stimulation by 0.25 per cent. strychnine (3×10^{-3} M) which doubled its normal rate of dye reduction. Rates for this sample at two intensities of continuous illumination and with different concentrations of strychnine (table IV) show clearly that a dark reaction is stimulated by strychnine. One sample of Swiss chard (not in figure 4) was stimulated 17 per cent. by 0.17 per cent. strychnine (2×10^{-3} M).

The alkaloids, like the other narcotics and many poisons, did not reduce the dye autonomously.

OTHER INHIBITORS

SUCROSE—A study was made on the effect of the concentration of chemically inactive molecules, using solutions of sucrose of increasing concentration, upon the rate of dye reduction by isolated chloroplasts. The results obtained with a single sample of Swiss chard chloroplasts (fig. 3) show that the influence starts at a concentration of sucrose of about 0.08 M which corresponds to about 2.1 atmospheres of osmotic pressure, weight molar solutions having been used. GREENFIELD (**5**) studied the effect of dehydration by osmosis with sucrose solutions upon the rate of photosynthesis of Chlorella, and his results are reproduced in figure 3 as a matter of interest; however, they may not be directly comparable with the present results, as broken chloroplasts may not have semi-permeable surfaces.

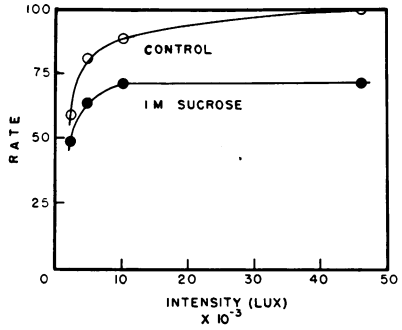

FIG. 4. Light-saturation curves showing the effect of 1 M sucrose upon the rate of dye reduction by isolated chloroplasts at a series of intensities of continuous illumination.

Curves describing the light saturation of the system (fig. 4) show that dark reactions are hindered by sucrose at a high concentration.

FORMALDEHYDE—BOSE (**2**) found that 10^{-7} per cent. formaldehyde increased the rate of photosynthesis of Hydrilla by 80 per cent.

The photochemical reduction of indophenol by freed chloroplasts of Swiss chard would be completely inhibited by nearly 10 per cent. (3.3 M) formaldehyde (fig. 3). No stimulation occurs down to 10^{-10} per cent.

TABLE V

THE EFFECTS OF OTHER SUBSTANCES UPON THE RATE OF DYE REDUCTION BY ISOLATED CHLOROPLASTS.

SUBSTANCE	CONCENTRATION	% OF CONTROL RATE
Methyl alcohol	5%	103
Ethyl alcohol	5%	91
	10%	39
	19%	17
Carbitol	5%	0
Isopropanol	5%	50
n-propyl alcohol	5%	50
Propylene glycol	5%	113
	10%	89
	20%	59
	30%	31
	40%	10
Acetone	5%	50
	10%	34
Diethylene glycol-monobutyl ether	5%	30
Glycerine	10%	80
Toluene	1%	70
Dioxane	10%	0
$MgSO_4$	2% sat'd.	25
	5% "	15
Na_2SO_4	1% "	96
	2% "	87
	4% "	70
	6% "	51
	10% "	43
$(NH_4)_2SO_4$	10% "	33
$CaCl_2$	0.2 M	0

(3×10^{-11} M) formaldehyde. Tests at two intensities of light showed that 0.3 M (1%) formaldehyde exercises its toxicity upon a light reaction: tion:

Light intensity (lux):	46,300	4,900
Per cent. of control rate:	39%	20%

OTHERS—Previous work in this laboratory included some studies designed to find a stabilizing agent for the storage of isolated chloroplasts

TABLE VI

A SUMMARY OF THE EFFECTS OF VARIOUS INHIBITORS UPON THE PHOTOCHEMICAL REDUCTION OF INDOPHENOL BY ISOLATED CHLOROPLASTS.

INHIBITOR	MOLAR CONCN. GIVING 50% INHIBITION	MOLAR CONCN. RANGE GIVING 10–90% INHIBTN.	REACTION INHIBITED
Poisons for metallo-cats.:			
Cyanide	(>0.01)		
Azide	0.08	0.02 – 0.1	Dark
Pyrophosphate	(>0.1)		
Hydroxylamine	0.00031	0.00002 – 0.0006 0.0007 – 0.0014	(Light) Constant Fraction
o-Phenanthroline	0.000027	0.0000014 – 0.00001 0.000007 – 0.00013	Light
Resorcinol	0.09	0.012 – 0.1	Dark & Light
Thiourea	0.15	0.02 – 1.0	
8-Hydroxyquinoline	(0.08?)		
Heavy metals:			
Copper sulphate	0.00001		Light
Mercuric chloride	0.000004	0.000002 – 0.000005	Dark
Other specific poisons:			
Dinitrophenol	0.00063	0.00012 – 0.02 0.000015 – 0.0022	Light
Iodoacetate	0.01	0.003 – 0.03	Dark
Narcotics:			
Phenylurethane	0.002	0.00015 – 0.02	Light
Thymol	0.0019	0.0014 – 0.0029	Constant Fraction
Chloroform	0.034	0.034 – 0.035	Light
Ether	0.45	0.2 – 0.8	Dark
Strychnine	(>1.0)		Dark stimulated
Other inhibitors:			
Sucrose	2.3	0.12 – 5.0	Dark
Formaldehyde	0.18	0.02 – 1.2	Dark
Sodium sulphate	0.42		
Acetone	0.68		
Ethyl alcohol	1.36		

(**12**). The substances listed in table V were found by Holt and French to retard the rate of dye reduction as shown. These authors have kindly permitted the inclusion of this survey here. The visibly gauged time for decolorization of phenol indophenol was the measurement employed.

The effective concentrations of the various inhibiting reagents which have been tested in this work are summarized in table VI.

Discussion and summary

This paper reports a survey of the actions of some of the known inhibitors of photosynthesis upon the dye-reducing activity of chloroplasts freed *in vitro*. Related work has already been adequately reviewed (**18**). The dye used was phenol indophenol, and 2,6-dichlorophenol indophenol in several instances, and the chloroplasts were obtained from the leaves of spinach and Swiss chard. The object of the paper is to substantiate the belief that the photochemical activity of isolated chloroplasts (which includes the oxidation of water, the liberation of oxygen, and the reduction of added oxidants) is actually a part of photosynthesis; and to report on the basis of this fact the effects of various reagents with an eye toward the elucidation of the reaction system which operates in chloroplasts.

The specificity of the various inhibitors relative to one another may be judged from the summary of experimental results in table VI.

The following inhibitors act specifically upon isolated chloroplasts, as they do on photosynthesis: Mercuric chloride; copper sulphate; o-phenanthroline; hydroxylamine; dinitrophenol. The action of the narcotics is of course not specific in terms of concentration, but that on isolated chloroplasts parallels that on photosynthesis.

Among the poisons, cyanide, azide, pyrophosphate, thiourea, possibly 8-hydroxyquinoline, and iodoacetate, are not specific inhibitors of dye reduction by isolated chloroplasts. This indicates that neither iron nor copper is involved in the reaction system of isolated chloroplasts. The effects of these reagents upon photosynthesis are probably connected with reactions which are directly concerned with the participation of carbon dioxide in this process.

Contrary to the results obtained here (see the comparison in the preceding section) GREENFIELD (**5**) found that $CuSO_4$ primarily inhibits a dark reaction in the photosynthesis of Chlorella. It is therefore suggested that Greenfield's dark reaction is not involved in the dye-reducing mechanism of isolated chloroplasts but is concerned with the utilization of carbon dioxide in photosynthesis. In several ways the results with $HgCl_2$ contradict GREENFIELD'S (**5**) conclusion that this poison attacks and destroys chlorophyll. The fact that copper inhibits a photochemical reaction in isolated chloroplasts whereas mercury inhibits a dark reaction suggests either different specificities or a difference in degree of effect, toward or upon presumably sulfhydryl groups.

Mercuric chloride, iodoacetate, azide, resorcinol, ether and sucrose inhibit dark reactions (probably enzymatic). Strychnine stimulates a dark reaction, and the other narcotics also stimulate the system at concentrations which are just below those which inhibit. Copper sulphate, o-phenanthroline, dinitrophenol, phenylurethan, chloroform, resorcinol, formal-

dehyde, and possibly hydroxylamine inhibit a light (photochemical) reaction. Thymol inhibits equally well at all intensities of light, and it is safer to say the same for hydroxylamine, which indicates according to WELLER and FRANCK (**23**) that a light-activated enzyme is attacked.

SCHIAU and FRANCK (**19**) attributed the influence of phenylurethan upon the fluorescence of isolated chloroplasts to adsorption of the narcotic on the chlorophyll, thus suppressing the photochemical activity of the latter. It may be contradictory that this narcotic does not influence the absorption spectrum of chlorophyll in the chloroplast.

It is interesting to note that among the narcotics all three types of effect are found upon the usual response to light saturation, thus implying that despite the probable common action of these inhibitors in an "indiscriminate blocking of surfaces," each may nevertheless have specific effects in blocking certain surfaces more or less effectively.

The evidence obtained here shows that WARBURG and LÜTTGENS' postulated zinc enzyme, which is specifically poisoned by o-phenanthroline, is concerned in a photochemical reaction and is thus perhaps a chemical photocatalyst. The same is true of one or both of the hypothetical oxygen-liberating enzymes, which are specifically poisoned by hydroxylamine, and of the hydrogen transport agent which is specifically poisoned by dinitrophenol. Chlorophyll does not seem, by spectral analysis, to be affected by these poisons (and others). The simplest picture in alignment with known evidence and theory is that these three poisons concern themselves with only two catalysts, one or both of which are directly sensitized by chlorophyll. The evidence also indicates that there is at least one enzymatic dark reaction included in the reaction system of isolated chloroplasts, and that there are enzymatically active or essential sulfhydryl groups in the system.

It is a pleasure to express profound gratitude for his kindness and help throughout to Dr. French who recommended this project and in whose laboratories this work was carried out. It is also a pleasure to thank members of the Botany Department at the University of Minnesota and particularly members of the Division of Plant Biology of the Carnegie Institution of Washington at Stanford for their interest and for suggestions for the presentation of the data.

DEPARTMENT OF BOTANY
UNIVERSITY OF MINNESOTA
MINNEAPOLIS 14, MINNESOTA

LITERATURE CITED

1. ARONOFF, S. Photochemical reduction of chloroplast grana. Plant Physiol. **21**: 393–409. 1946.
2. BOSE, J. C. Effect of infinitesimal traces of chemical substances on photosynthesis. Nature **112**: 95–96. 1923.

3. EWART, A. J. On assimilatory inhibition in plants. Jour. Linnean Soc. London Jour. Bot. **31**: 439–443. 1896.
4. FRENCH, C. S., and RABIDEAU, G. S. The quantum yield of oxygen production by chloroplasts suspended in solutions containing ferric oxalate. Jour. General Physiol. **28**: 329–342. 1945.
5. GREENFIELD, S. S. Inhibitory effects of inorganic compounds on photosynthesis in Chlorella. Amer. Jour. Bot. **29**: 121–131. 1942.
6. HABERLANDT, G. Die Chlorophyllkörper der Selaginellen. Flora. **71**: 291–308. 1888.
7. HILL, R. Oxygen evolved by isolated chloroplasts. Nature **139**: 881–882. 1937.
8. ————. Oxygen produced by isolated chloroplasts. Proc. Roy. Soc. Ser. B: Biol. Sci. **12**: 192–210. 1939.
9. HOLT, A. S., and FRENCH, C. S. The photochemical production of oxygen and hydrogen ion by isolated chloroplasts. Arch. Biochem. **9**: 25–43. 1946.
10. ————, and ————. Oxygen production by illuminated chloroplasts suspended in solutions of oxidants. Arch. Biochem. **19**: 368–378. 1948.
11. ————, and ————. Isotopic analysis of the oxygen evolved by illuminated chloroplasts in normal water and in water enriched with O^{18}. Arch. Biochem. **19**: 429–435. 1948.
12. ————, SMITH, R. F., and FRENCH, C. S. The photochemical reduction of phenol indophenol and the evolution of oxygen by chloroplast preparations. (Unpublished.)
13. INMAN, O. L. The evolution of oxygen in the process of photosynthesis. Cold Spring Harbor Symposia Quant. Biol. **3**: 184–190. 1935.
14. ————. The Mirsky-Pauling theory of the structure of native, denatured, and coagulated proteins, and some theoretical aspects of the evolution of oxygen from the irradiated green plant. Plant Physiol. **13**: 859–862. 1938.
15. KOHN, H. I. Inhibition of photosynthesis in Chlorella pyrenoidosa by the iodoacetyl radical. Jour. General Physiol. **19**: 23–34. 1936.
16. MOLISCH, H. Ueber Kohlensäure-Assimilations-Versuche mittelst der Leuchtbacterienmethode. Botan. Zeit. Abt. 1 **62**: 1–10. 1904.
17. ————. Über Kohlensäure-Assimilation toter Blätter. Zeit. für Botan. **17**: 577–593. 1925.
18. RABINOWITCH, E. I. Photosynthesis and related processes. Vol. I Interscience Publishers. 599 pp. 1945.
19. SCHIAU, Y. G., and FRANCK, J. Chlorophyll fluorescence and photosynthesis in algae, leaves and chloroplasts. Arch. Biochem. **14**: 253–295. 1947.
20. WARBURG, O. Über die Geschwindigkeit der photochemischen Kohlensäurezersetzung in lebenden Zellen. Biochem. Zeit. **100**: 230–270. 1919.

21. ————, und LÜTTGENS, W. Experiment zur Assimilation der Kohlensäure. Naturwissenschaften **32**: 161. 1944.
22. ————, und ————. The photochemical reduction of quinone in green granules. (in Russian) Biochimia **11**: 303–321. 1946.
23. WELLER, S., and FRANCK, J. Photosynthesis in flashing light. Jour. Physical Chem. **45**: 1359–1373. 1941.