PHOTOCHEMICAL REDUCTION OF CHLOROPLAST GRANA

S. ARONOFF

(WITH SEVEN FIGURES)

Introduction

It has long been known that disintegration of green leaf tissue results in almost complete disappearance of photosynthesis. Indeed, any drastic treatment, of a type amenable to the isolation of various enzymatic systems, as intensive dehydration (above or below freezing), low temperature buffered extraction *et cetera* are of no avail. Attention has therefore been focused on some of the larger subcellular units known to function in the process, such as the chloroplasts.

ENGLEMANN (**6**) showed, by means of bacteria motile only in the presence of oxygen, that oxygen is evolved only by the chloroplasts within cells. Later, BEYERINCK (**3**) used bacteria which can luminesce only in the presence of oxygen to demonstrate the evolution of oxygen from isolated chloroplasts. The maximal sensitivity of the latter method corresponds to the determination of oxygen having a partial pressure of 10^{-8} mm. Hg. The sensitivity of the motile bacterial method is not known, except as the vague reference of PFEFFER (**23**), as a method capable of detection of one billionth of a mg. of oxygen. Using a method (quenching of a phosphorescent dye by oxygen) only slightly less sensitive than that of the luminous bacteria, but better adapted for routine quantitative measurements, FRANCK (**11**) has recently measured quantitatively the output of oxygen from isolated chloroplasts under various conditions.

Nevertheless, the activity of isolated chloroplasts, prepared by disintegration of leaf tissue, and differential centrifugation of the filtered mass, has been so low that until recently manometric methods could not be used to detect gas exchange. For contemporary routine biochemical investigations, therefore, their activity was nil. It was therefore of considerable interest that HILL (**14**) was able to show that on the addition of ferric oxalate to a solution of freshly prepared chloroplasts, considerable oxygen evolution occurred on illumination. Ferric ion was shown to be reduced to ferrous, and water was oxidized to oxygen. Quantitative measurements showed the reaction to follow the equation:

$$4\,Fe^{+3} + 2\,HOH \longleftrightarrow 4\,Fe^{+2} + 4\,H^{+} + O_2.$$

HOLT and FRENCH (**20**) showed that the reaction could be followed titrimetrically for the H^{+}.

A very much further advance was made when WARBURG and LÜTTGENS (**24**) demonstrated that an analogous reaction occurred using p-benzoquinone, not only with the chloroplasts, but with chloroplast grana. The reaction followed the equation:

$$2\ \text{quinone} + 2\,HOH \rightleftharpoons 2\ \text{hydroquinone} + O_2.$$

The advance was of a two-fold nature: first, the complicated group of reagents required in the Hill reaction was replaced by a single substance; and second, the use of an organic oxidizing agent demonstrated the generality of the reaction. ARONOFF (1) further emphasized the generality of the reaction by the use of various types of quinones; *e.g.*, naphtho- and anthraquinones, to give the reaction with the grana, though at different rates, roughly proportional to their redox potentials in strong light.

BOICHENKO (4, 5) using a solution of 0.1 per cent. fructose in almost saturated basic Mg. acetate, has reported the evolution of oxygen from dried, as well as freshly isolated, chloroplasts. Unfortunately, we have been unable to confirm his results with manometric technique (the author used the leuco-dye method).

The work of FAN *et al.* (8) is also of interest since these workers have reported the reduction of benzaldehyde to toluol and the corresponding evolution of oxygen in light from green algae in the absence of external CO_2.

The work to be described here forms the basis for the previously reported note. We have found that the reaction with quinone was given not only by the grana, but also by a clear solution containing chlorophyll in only minute amounts, and by lyophilized preparations of both. The subsequent sections therefore deal with (I) the method of preparation of the granules, solution, dried material, and experimental methods; (II) the rate of deterioration of the granules; (III) the rate of reaction of the various substances, as well as that of a leaf disc from which they were made; (IV) the action of poisons and narcotics; and (V) relation of the reaction to photosynthesis.

Methods

PREPARATION OF MATERIAL

GRANA.—In the following the use of the word "grana" is restricted to the product obtained under the conditions described, and is not intended to denote any morphological entity in normal tissue. This is a question that is, apparently, still open for further investigation. Market spinach was used as the source of granules. It had been hoped that tobacco chloroplasts, which can be prepared more abundantly (per weight of fresh tissue), and for the leaf of which more analytical data exist, could be plasmoptized and permit a comparison of chloroplast and granular reactions. Unfortunately, these chloroplasts do not disintegrate to any appreciable extent even in 48 hours of standing in distilled water, and spinach was found to be a ready source of "grana."

Spinach was purchased at a local market and was consistently of a better quality than that obtainable in a dozen or so other establishments in the immediate vicinity. It was equilibrated in a closed chamber to the temperature of the cold room (2–3° C.) within an hour, and all subsequent operations carried on therein, up to the moment of manometer manipulation.

Leaf lamina were separated from stems and large veins in amount sufficient to fill the container of a Waring blendor without packing (ca. 100 gm. of turgid tissue); 175 ml. of phosphate buffer (pH = 6.5, 0.05 M) were poured into the blendor (just enough to cover the blades). The blendor was connected with a variable resistance and run 15 minutes at 55–57 volts in order to minimize the foaming. Below this voltage shearing action was insufficient. An additional 15 minutes were permitted for condensation of the foam to a dark green solution which was then filtered through two inches of cotton into centrifuge tubes. Although 15-minute centrifuging at 45 g. is sufficient to remove all chloroplasts, this material was run at 125 g. The resulting solution was relatively dark green, containing particles of a size at the limit of high-power microscopic resolution (ca. 0.1 μ) and having a chlorophyll concentration of ca. 0.1 mg./ml. The solution thus obtained was transferred into the celluloid tubes of a high-speed centrifuge and run at 6700 g. for 15 min. This is sufficient to precipitate almost all the grana and must be excessive, since the grana are rather tightly packed. The supernatant solution, yellow-green in color, was preserved for further work (see below). The grana themselves were washed twice while packed and then disintegrated with a stirring rod using a minimal amount of buffer. One hundred to one hundred forty ml. of initial granular material were thus concentrated to ca. 20 ml., the chlorophyll content of which normally ran ca. 0.75 mg./ml., often 1.0 mg./ml., and could be made as high as 2.5 mg./ml. The material thus obtained was used directly for experiments. The yield of material (in terms of chlorophyll) was 2–3 times as great for winter spinach (Dec., 1945) as spring (May, 1946), possibly because of the greater friability of the winter chloroplasts (whose yield, therefore, was negligible).

SOLUTION.—The yellow-green solution obtained from the high-speed centrifugation was clear, but showed a pronounced Tyndall effect. This material is referred to as "solution." It was dialyzed against distilled water for 36 hours, whereby additional chlorophyll-containing material was precipitated, and the yellow-green solution though very clear showed a very slight Tyndall effect. This material is referred to as "dialyzed solution." A hand spectroscope still showed the primary red absorption band of chlorophyll on looking through a 10-cm. path. The yellow color is due to the presence in high concentration of a yellow protein with maxima at 337 and 270 mμ (with a relative log K of 0.360 and 0.415 respectively). It is precipitable with ammonium sulphate and redissolves in saline solution. The yellow prosthetic group is so tightly bound to the protein, that no color is lost on dialysis. Although spectral indications would tend toward a flavone pigment, and a green coloration with ferric chloride shows the presence of hydroxyl groups, the characteristic orange color produced on reduction of a flavone or flavonone does not occur here. The high concentration is inferred from (a) the yellow color of a material absorbing in the far ultra-violet, and (b) the fifty-fold dilution required to obtain spectral measurement.

DRIED.—Both materials described have been dried in a lyophilizer made

by connecting in series a pump, trap in CO_2 solvent bath (in this case ethyl alcohol), and an Erlenmeyer having a male ground joint. The desiccation of 50 ml. of material, frozen before desiccation and kept frozen by the rapid evaporation of water, was effected overnight.

Reagents

1,4-benzoquinone was obtained from the Eastman Kodak Co. The material as obtained is sufficiently impure so that poor or negative results are obtained. A single sublimation is sufficient purification.

Potassium 1,4-naphthoquinone 2-sulphonate was prepared from α-naphthoquinone according to the method of Fieser (**9**). It was twice recrystallized.

Potassium 1,2-naphthoquinone was prepared in similar fashion according to Fieser's method, and twice recrystallized according to his directions.

Potassium 9,10-anthraquinone 4-sulphonate was obtained through the courtesy of Dr. W. Nudenberg of this department. It was found that the corresponding sodium salt, obtainable through the Eastman Kodak Co. is satisfactory as received.

Other compounds as poisons, narcotics, and additional substrates were used without recrystallization. Chlorophyll a + b was prepared according to the method of Mackinney (**22**) except for the drying of the product, which was performed in hi-vacuum, rather than by desiccator. It is believed that this is of value, since this material, after four years in a cork-stoppered tube at room temperature, still shows a good phase test.

Experimental method

Gas exchange was measured manometrically in the usual fashion, using either Warburg or differential manometers, as the occasion required. Purified nitrogen (Ohio Chemical) was passed through alkaline anthraquinone-sulphonate to remove oxygen (ca. 0.1%), although this precaution was found to be unnecessary when using benzoquinone, and provided the standard atmosphere for the reaction. In filling the differential manometer it was found convenient to employ a three-way stopcock attached to the two manometer arms, so that equal pressure could be maintained in both arms. Following the 15-minute gas introduction, the inlet tube *above* the stopcock was removed, the vents on the manometer vessels closed, and the entire system permitted to attain equilibrium for 5 minutes. The manometer stopcocks were then closed and the three-way stopcock removed.

A 2000-watt tungsten lamp served as light source. The beam was spread by means of a cylindrical lens, collected, and reflected up through about four inches of water into the vessels. The light intensity in the center of the system at the level of the vessel bases was 19,000 lux. Inasmuch as it diminished somewhat on both sides, corrections were made in all runs on the basis of a linear relation between the light intensity and the rate of reaction (see section III). Diminution of intensity, when required, was accomplished by means of calibrated metal screens.

Total chlorophyll was measured by means of a Klett colorimeter, using Corning filter #2403. With an abscissa of γ of chlorophyll/ml. (chph./ml.) and an ordinate of density, the equation of the straight line was $y = 8.52\,x$. This equation is valid for $x \leq 15$. In actual practice, the suspension of grana was diluted one to ten with acetone, the mixture again diluted one to ten with acetone and then centrifuged 5 minutes at 100 g. to give a clear solution.

Rate of deterioration of granules

The data of both HILL and SCARISBRICK (**17, 18**) and KUMM and FRENCH (**21**) indicate a substantial rate of loss of activity of chloroplasts although the temperature of storage is not indicated in either case. The half-lives are of the order of 1.5 and 3 hours. Granules, stored at 2–3° C., were (assuming cold storage of chloroplasts) considerably more stable (see fig. 1),

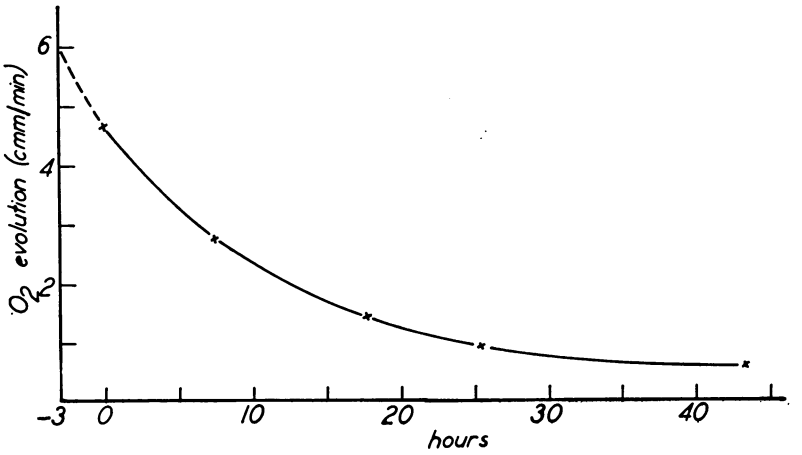


FIG. 1. The rate of deterioration of grana stored at 2° C. and illuminated 20 minutes at 19,000 lux.

the half-life (from the initial measurement) being eleven hours. As with chloroplasts, no extetrnal change in the appearance of the material was noted, though it could possibly have been noted in a change of the fluorescence yield. Because of the shape of the curve, the initial decrease is profound, there being an estimated 25–30 per cent. loss in activity of the isolated material during the time of preparation.

The loss of activity during an experiment was much greater and was presumably due to photolytic effects. Although experiments could be continued for 2–3 hours, the rate fell off markedly after 25–30 minutes.

It was noted, empirically, that the rate of deterioration was also a function of the quality of the material employed, as well as of the season. The figure shown is, therefore, of average material.

Rates of reactions of substances

The reaction had previously been shown to cease in boiled material, to decrease with time as material "deteriorated," and to diminish on narcotiza-

tion. Since each of the processes removes chlorophyll from the reaction, the first two by precipitation as colloidal, insoluble material, the latter by surface coating, it was desirable to show that the reaction did not occur with pure chlorophylls a + b in true solution. The reaction was carried out in both 95 per cent. ethyl alcohol and benzene saturated with water, with and without the addition of amino acids in both cases. No evolution of oxygen was noted. It is, of course, possible that aside from the twenty-fold reduction in amount of water present, and the replacement of hydration by alcoholation, that some essential component has been omitted.

Leaf disc

The amount of photosynthesis of a leaf disc cut from material investigated was determined at various light intensities. For this purpose the leaf was floated in 0.1 M $KHCO_3$ in an inlet atmosphere of 4 per cent. carbon

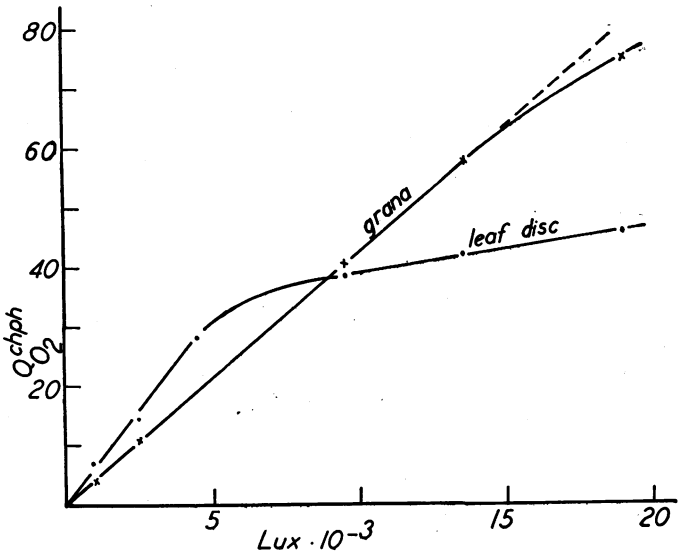


Fig. 2. Comparative rates of oxygen evolution of leaf disc with bicarbonate and grana with quinone.

dioxide, 96 per cent. nitrogen, and ca. 0.1 per cent. oxygen. The results are shown in figure 2. Among points to be noted are (a) the early light saturation, normally expected at ca. 17,000 lux for this material, and indicating some physiological disarrangement (of the time involved in the cutting, transit, marketing, and measurement, only the last is known); there is also a possible deficit of oxygen; (b) the low rate of evolution of oxygen; (c) the higher efficiency of the leaf at low light intensities than that of the grana (for the same amount of chlorophyll), especially in view of the very low redox potential of the oxidant in the leaf (carbon dioxide) and the high potential in the grana (p-benzoquinone). The quantum yields in our material would therefore be quite low. The fraction of the light

absorbed by the leaf per unit area is roughly equal to that of the grana suspension in the vessel.

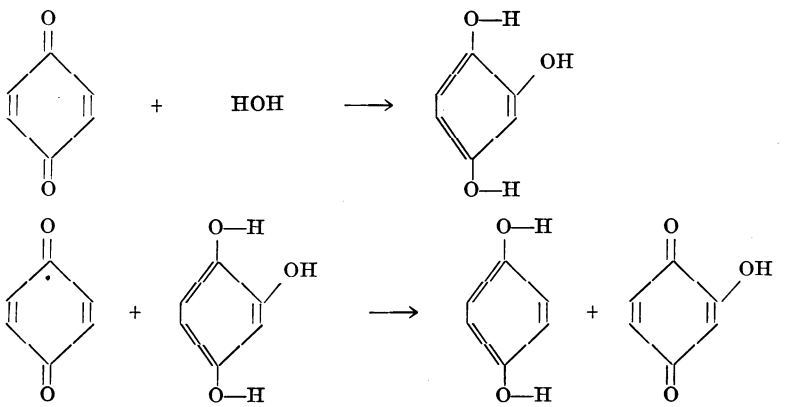
GRANA

Measurements were performed, except in the cases noted, with p-benzoquinone. An excess, ca. 10 mg./5 ml. suspension was used. Using known quantities of quinone, the writer was not able to exceed 35 per cent. yield in a number of experiments, although WARBURG and LÜTTGEN (**24**) report 85 per cent. yields. The cause of the difference is not apparent. It is, for example, necessary that the quinone be purified. Alkalinity, light, *etc.*, cause a solution of purified (sublimed) quinone in Pyrex-redistilled water to turn orange-brown, with a color similar to that of quinhydrone. The deterioration of 1,4-naphthoquinone under similar conditions results in the formation of equal amounts of hydroxy 1,4-naphthoquinone + 1,4-naphthohydroquinone (**9**) which then combine in the usual fashion similar to quinhydrone formation, giving a colored substance.[1] Were this reaction to go to completion prior to the reaction with the chloroplast grana, it would, of course automatically remove 50 per cent. of the initial quinone. If it does not, the final yield with quinone nevertheless represents a competition between the two types of reactions for the quinone.

The rate of the reaction of the grana (fig. 2) with quinone is obviously a function of light intensity. Within the region of 13,000 lux it is linear for this size of granule. There is some indication it varies with the size. Diminution of activity as a function of time at higher light intensities may be correlated to some extent (as with normal photosynthetic systems) with photo-oxidation, but thermal inactivation resulting from the dissipation of the absorbed light energy and internal oxidation-reductions are undoubtedly more prominent factors since the partial pressure of oxygen is rather low.

Rates are plotted as cm. of oxygen evolved per mg. of chlorophyll per

[1] The reaction in the case of 1,4-benzoquinone would then be following FIESER (9):



hour = $Q_{O_2}^{chph}$, and varied between 45 and 100. Inasmuch as WARBURG and LÜTTGENS (**24, 25**) did not state their light intensities, beyond the statement that the reaction was completed within 30–60 minutes, it is impossible to compare results. The low quotient of the leaf disc itself may indicate that the starting material was in part to blame for the low rates. HOLT and FRENCH (**20**) have obtained values of 300–600 with their material using lower concentrations and higher light intensities. Obviously, had we used higher light intensities, we would have obtained higher yields (at least in the case of benzoquinone, though possibly not in the other quinones). Unfortunately, in the most recently available work from this group (**11**) it is impossible to compare any data, since the manner of denoting "activity" was not stated. A more rational "quotient" would thus also require the inclusion of the light factor.

It was of interest to determine the rate where chlorophyll concentrations were identical but particle size different. Chloroplasts were removed by centrifugation at 45 g. for 10 minutes. This has been found empirically sufficient to precipitate algae and pure tobacco chloroplasts quantitatively. By centrifuging an additional fifteen minutes at 45 g., a crop of "large" granules was prepared. The "smaller" granules were precipitated by centrifuging at 125 g. The colorimeter readings on the unextracted suspensions were 430 for the larger and 362 for the smaller, a difference due to the scattering factor. Neglecting the scattering factor (assuming equal light absorption) the rates of oxygen evolution were identical for solutions of equal chlorophyll content. Actually, however, because a larger fraction of the incident light was scattered by the smaller particles, the amount of absorbed light was less, and on the basis of the light absorbed (a quantal basis) their yield would be higher.

In contrast to the experience of HOLT and FRENCH (**20**) who used ferric oxalate, but in agreement with WARBURG and LÜTTGENS (**24, 25**) who used quinone, no carbon dioxide was obtained in the reaction.

Dried granules, resuspended in redistilled water, were found to be slightly more (58 per cent.) than one half as active as the undried material (using identical initial matter). Three days later the same material, kept at 2° C., was inactive. Dehydration is therefore insufficient for preservation.

SOLUTION

The solution is remarkable for its high activity, being about ten times as great as that of the grana; ($Q_{O_2}^{chph}$ = 6–700). Almost constant yields (at 19,000 lux) were observed for periods as long as two hours. Because of the low chlorophyll content it was possible to note a certain amount of destruction of chlorophyll in the course of the reaction, although the general character of the spectrum remained unchanged. The solutions responded to the naphthoquinones, but were erratic in regard to anthraquinone.

The solution, dried in a lyophylizer and redissolved in distilled water, retains its activity for weeks if kept cold while dried. This contrasts with

the marked diminution on storage of dried granules. Using dried solution, in one experiment with benzoquinone a constant rate of oxygen evolution was maintained after the fourth hour (fifteen minute illuminations each hour—total of one hour).

Rates of reaction of substrates

Relation of redox potentials

It was reported in a previous note (**1**) that the rate of oxygen evolution was a function of the redox potential, using a series of homologous quinones. The figure therein is reproduced below (fig. 3). Because of the almost

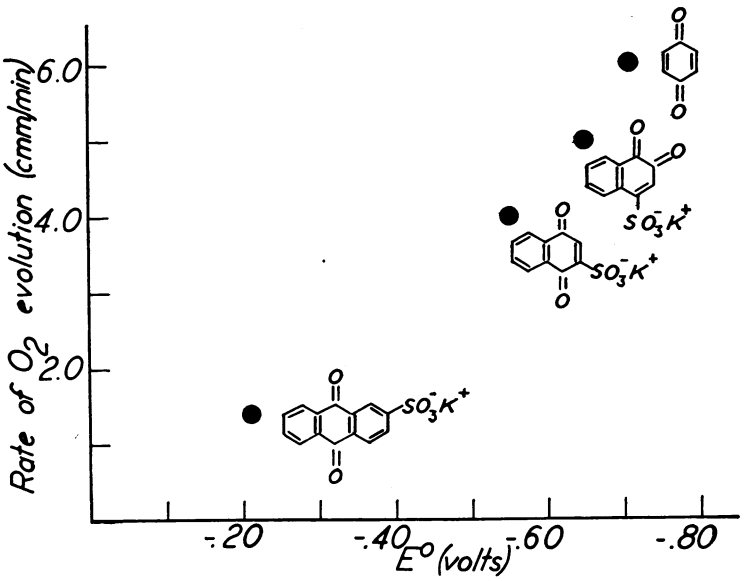


Fig. 3. Plot of rate of oxygen evolution by grana with various quinones (at 19,000 lux) against redox potentials of the quinones (E°.)

linear relationship found between the oxygen evolution and the light intensity in the case of benzoquinone, it was presumed that this relationship held for the other quinones. Furthermore, the results were interpreted in the simplest manner as due to the difference in the rate of the reverse reaction (back reaction) for the different quinones. This explanation was in part based upon the known linear relationship between the redox potentials of leuco dyes and the rate of oxidation (**2**).

Since that time measurements have been carried out with the other quinones, determining the rate of oxygen production as a function of light intensity, the results being shown in figure 4. Measurements were performed as a differential effect; that is, using the same grana preparation, adding a known amount of benzoquinone to one vessel and an equimolar amount of naphthoquinone to the other. Furthermore, the two naphthoquinones were checked against each other in a similar manner; and, of

course, a curve was obtained for benzoquinone alone in the usual manner. Vessels with identical constants were used, and because of the small differences in evolution of O_2, a cathetometer was employed. The results indicate (a) that the order of the rates at low light intensities is the reverse of that at the high (with the possible exception of anthraquinone, where the very early inflection of the curve did not permit the determination of reliable data) and (b) that the curves for the naphthoquinones were linear at only the lower light intensities.

The change in character of the slopes from a straight line to that of a curve appears to indicate that at least one dark reaction occurs in addition to that of the light reaction.

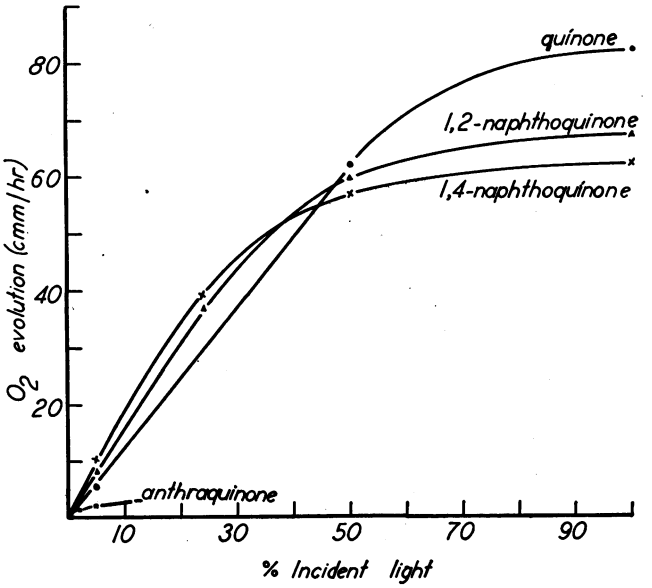


FIG. 4. The rate of oxygen evolution by grana with various quinones as a function of light intensity.

No explanation will be attempted at the present concerning the order of the rates at low light intensity, since this involves further experimentation (as a function of time, *etc.*)

At the higher light intensities there is a choice of two possible explanations. The first, which would be valid if true saturation occurred, would be that of the limitation of the rate by an enzyme with a finite turnover number, worked with insufficient rapidity to keep up with the light reaction. This enzyme would in this instance have different turnover rates for the different substrates and the relation to the potentials indicated would, in a sense, be fortuitous, although in the long run possibly ascribable to the same structural properties which determine potential difference in compounds. The second possible explanation for a diminution in the rate is the increased rate of the reversal of the light reaction with increasing concentration of the

products of the forward reaction. In this instance, saturation, though approached, would never be reached, on the basis of a bimolecular reaction. Unfortunately, the instability of the material at high light intensities does not lend itself to a ready choice between the mechanisms.

Non-quinones

Various other substances have been tried as oxidants; *e.g.*, using salicylaldehyde, a small amount of oxygen is evolved initially, ceasing within a short time, and bleaching of chlorophyll is considerable. Benzaldehyde, although much more susceptible to oxidation, evolves a greater amount of oxygen prior to cessation, than does salicylaldehyde and the chlorophyll bleaching, though considerable, is less severe. Salicylic acid was most effective as a bleaching agent, and no evolution of oxygen was detected. A number of other substances gave very little response: fructose, dehydroascorbic acid, methyl alcohol.

Attempts to obtain a wider indication of the possible effect of potential using a series of dyes (2,6-dichlorophenol indophenol) were abandoned because of the large fraction of light absorbed by the amount of dye required to produce a nominal manometric effect.

The grana decompose urea peroxide in the dark, as does catalase. This probably denotes a high dissociation of the substrate into urea and hydrogen peroxide. Butadiene monoxide (3,4-epoxide, l-butene) also gave no oxygen.

It is interesting to note that benzoyl peroxide evolved oxygen to some extent, with no observable liberation of gas in the dark.

Algae

Fan *et al.* (**8**) have reported that green algae are able to evolve O_2 in the light and explained their results as the reduction of benzaldehyde to the corresponding alcohol. Their experiments were complicated by (a) the relatively large amount of metabolic carbon dioxide present in the dense suspensions, permitting a release of oxygen due to normal photosynthesis as great as that due to the reduction of benzaldehyde; (b) the apparently usual presence of air during the experiment, resulting in the oxidation of the aldehyde; and (c) the slow rate of entry of the aldehyde into the algal cells.

We have attempted to repeat our experiments on the alga, Scenedesmus. As with the grana, the algae were in an atmosphere of nitrogen (+ 0.1% O_2), and bathed in a phosphate buffer identical with that used for grana. The sole carbon dioxide available was that produced by anaerobic metabolism. The density of the suspension was far from that resulting in total light absorption. Since an excess of quinone above that of a saturating solution did not increase the rate of oxygen evolution, it was presumed that the rate of quinone entry into the cells limited the oxygen evolution. Under these conditions, oxygen was evolved only in the light. The same algae, under the same conditions, without quinone did not evolve oxygen.

Poisons and narcotics

One of the anomalies of the Hill reaction is the inaction of the usual poisons. Thus, cyanide is of no effect, nor is azide. The former is believed to affect the carboxylating enzyme in normal photosynthesis, and since there is evidence (**14**) to indicate that this enzyme may be located outside the chloroplast, the inaction of cyanide may thus be understood. The same cannot be said of azide[2] which would presumably act on the oxygen-liberating enzyme. Even more pronounced is the lack of activity of hydroxylamine.[1] We had, indeed, hoped for some activity, since, from the point of view of potentials, hydroxylamine should be a good oxidizing agent. Actually, it is a very poor one and is, in fact, used as a reducing agent in normal practice. We have found no evolution of oxygen with this substance, except for a slight initial activity which soon ceased. This activity produced may be due to reaction of the amine with naturally occurring ketones or aldehydes. Thus, the primary action of hydroxylamine in the presence of quinone is to form the dioxime. This is accompanied, however, by considerable evolution of gas, apparently nitrogen (and all, of course, without relation to light or enzymes).

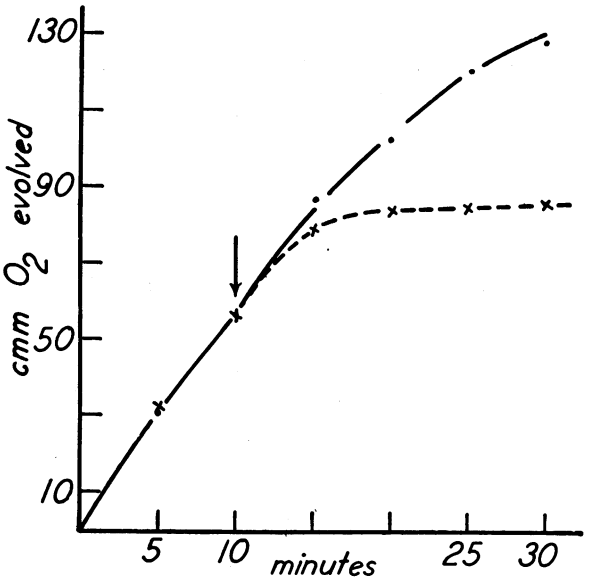


FIG. 5. Inhibition of the evolution of oxygen by grana and quinone by o-phenanthroline. ———— control; - - - - - - o-phenanthroline.

The action of o-phenanthroline was found, in agreement with WARBURG and LÜTTGENS, to be an effective narcotic (fig. 5). The almost complete sup-

[2] FRENCH, *et al.* (12) have noted the action of hydroxylamine and azide as that of poisons. This is at variance with the results of both HILL and SCARISBRICK (**16**) on chloroplasts, and with ours on granules. They also mention the poisonous effect of fluoride, which HILL and SCARISBRICK found was not a poison. Furthermore they class Duponol (presumably sodium lauryl sulphate) as a poison. Undoubtedly this detergent acts as a surface agent and should therefore be classed as a narcotic.

pression of photoreduction makes it a very powerful inhibitor (although this may be due to its relatively large concentration compared to other narcotics). The substance is termed a narcotic despite its ability to be a poison by virtue of its chelation with ferrous ion, since WARBURG and LÜTTGENS (**24, 25**) found that its inhibiting action can be removed merely by washing and resuspending the grana.

The most common photosynthetic narcotic, phenylurethane, was found, in agreement with HILL's data on chloroplasts to be effective with grana (fig. 6). One notes, however, that with chloroplasts, by using sufficiently high concentrations, almost complete suppression of activity can be obtained with both ethyl- and phenylurethane. In the case of grana, using a saturated solution of phenylurethane, only two-thirds inhibition results. An

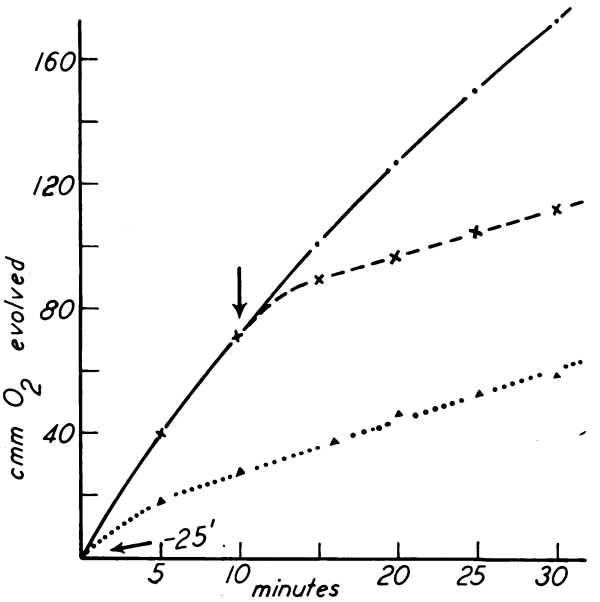


FIG. 6. Inhibition of the evolution of oxygen by grana and quinone by phenylurethane. ———— control; - - - - - phenylurethane (2.0×10^{-3} M); . . . . . phenylurethane (excess; crystals).

additional difference, one which occurs with grana and apparently not with chloroplasts, is the constancy of the inhibited rate. The time effect with plastids is considerable, indicating a considerable diffusion factor through the membrane to the effective loci. The lack of an increased effect of excess phenylurethane (including a previous twenty-five minute incubation) indicates that the two-thirds rate diminution is the limit with this substance.

The principle of narcotic action is well illustrated by comparison of thymol with phenylurethane. Using identical concentrations (1.2×10^{-2} M) in which phenylurethane diminished the control rate by 55 per cent., thymol diminished the rate by 80 per cent. (fig. 7). The distribution coefficient between olive oil and water for phenylurethane is 150, and for thymol, 600

(**19**, p. 357). On the assumption that the effectiveness of a sub-limiting concentration of inhibitor is then determined by its solubility in the (oily) matrix of the grana, one would have inferred that thymol should be about four times as effective as phenylurethane.

It is to be noted, though possibly at present it is considered coincidental, that the limit of effective narcotic action is attained when the ratio of phenylurethane is one. This implies that with less concentrated grana solutions, less concentrated phenylurethane will be required to produce a given effect. This experiment has not been performed.

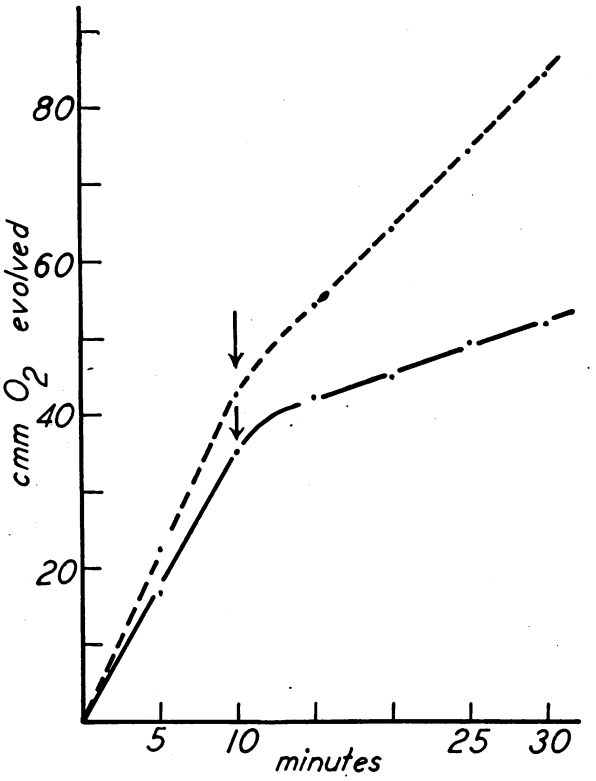


FIG. 7. Inhibition of the evolution of oxygen by grana by submaximal amounts of phenylurethane and by thymol; - - - - - phenylurethane (1.2×10^{-2} M); ———— thymol (1.2×10^{-2} M).

Relation of the reaction to photosynthesis

HILL (**14**) correlated the activity of iron in photoreduction in his isolated chloroplasts with a specific material reacting as a catalase-like enzyme. That various types of quinones (**1, 24, 25**), aldehydes, *etc.*, react in a similar manner with products of the chloroplasts, the grana, indicates a generality of the reaction greater than previously thought. It is, of course, possible to write a general equation describing all of the above reactions in a manner

identical to the generalized equation for photosynthesis suggested by van Niel, *viz.*

$$2B + 2HOH \xrightarrow[chph]{light} 2BH_2 + O_2$$

In particular the oxidant B is represented by relatively strong oxidizing agents, as ferric ion, or quinone, whereas in normal photosynthesis, the oxidizing agent is the very weak carbon dioxide. In both cases the oxidant is water, although this has not been shown for photoreductions where a reducing agent, in addition to that of water, is required.

It is not possible to say at present, however, that this reaction is the "last half of photosynthesis." Certainly it is not the "first half" since this occurs in large measure outside the chloroplast. The lack of effect of the poisons (with the possible exception of o-phenanthroline) indicates that the normal enzymatic system responsible for the release of oxygen is not present, or at least is inoperative under the conditions used in the present work. Indeed, as indicated previously, we cannot even be certain that the enzyme(s) involved in the stabilization of the primary photolytic products is operative. The only mechanism of which we may be certain is the fission of water in the light reaction; and it is this step, the "middle" of photosynthesis, which the reactions may have in common.

The nature of the transfer of light to chemical energy is, however, of the utmost importance and significance, and therefore worth continued study.

Summary

1. Methods of preparation of grana and of a clear solution which can be used for the evolution of oxygen by photochemical reduction of quinones have been described.

2. The rate of deterioration of the grana, stored at 2° C. is such that a half-life of about eleven hours is indicated. Approximately 25–30 per cent. of the activity is lost in time of preparation.

3. The rate of oxygen evolution with grana and using quinone is only a tenth that of the solution (at maximal light intensity) based on the amount of chlorophyll. The solution is much more stable in light than grana. Both lyophilized grana and solution are active on rehydration. The lyophilized solution is more stable than the grana.

4. The rate of oxygen evolution varies with different quinones (benzoquinone, 1,2-naphthoquinone, 4-sulphonate, 1,4-naphthoquinone 2-sulphonate, β-anthraquinone sulphonate). At lower light intensities the order of rates is the reverse (with the possible exception of anthraquinone) of that at the high light intensities. Saturation, or an approach to it is indicated at high light intensities. Various other substances give small effects: benzaldehyde, benzoyl peroxide, and salicylaldehyde. Others give very little effect: fructose, and dehydroascorbic acid. Others which might have been

expected to be active give none: butadiene monoxide and salicylic acid. Urea peroxide is decomposed in the dark by both the grana and a solution of pure catalase, indicating dissociation of the urea peroxide to urea and hydrogen peroxide.

5. The photochemical reduction of quinone is inhibited strongly by o-phenanthroline, by phenylurethane, and by thymol. Complete inhibition by phenylurethane does not occur with saturated solution; the degree of inhibition is not increased by incubation or with excess material. Thymol is more fat-soluble than phenylurethane and is more effective as an inhibitor at the same molar concentration.

6. A brief discussion of the relation of photochemical reduction with various substances by grana to photosynthesis is given.

RADIATION LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA

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