FREE RADICAL INTERMEDIATES IN PHOTOSYNTHESIS*

by

N. URI

Ministry of Agriculture, Fisheries and Food Research Establishment, Aberdeen (Scotland)

The mechanism of photosynthesis is one of the problems that, despite decades of intense research, has not yet been solved. Recent claims[1,2] to the contrary are misleading. The so-called one-quantum theory by WARBURG AND BURK deals with the controversial problem of the quantum efficiency, and the authors do not commit themselves to any detailed reaction scheme in which the intermediate steps are well defined. These authors have used extensively the idea that a major part of the energy required for photosynthesis is gained in the exothermic oxidation of semi-reduced intermediates with the result that only part of the carbon dioxide assimilated is fixed in the end products of photosynthesis. This was disputed by several investigators; *e.g.* KOK[3], GAFFRON[4], FRANCK[5], ALLEN[6], and CALVIN, BASSHAM, BENSON AND MASSINI[7]. The Emerson school has consistently claimed that the total number of quanta required is at least twice as large as that postulated by WARBURG AND BURK. In another recent article[8] a solution of the photosynthesis problem is claimed to have been discovered in the fact that the ratio of chlorophyll b to the sum of chlorophyll a and chlorophyll b is similar to WARBURG's quantum ratio 1:4. In view of the well-known phenomenon of transfer of excitation energy (from chlorophyll b to chlorophyll a)[9], such a hypothesis is very unlikely and if there is any similarity of the above ratio to the quantum number required for photosynthesis it is probably accidental.

The most important advance, in recent years, in the chemistry of photosynthesis was achieved by CALVIN AND BENSON[10] who discovered phosphoglyceric acid as the first stable intermediate product in the assimilation of carbon dioxide. This finding was confirmed later by FAGER, ROSENBERG AND GAFFRON[11]. Even so, our knowledge of the chemical mechanism of photosynthesis is extremely unsatisfactory. In many systems in inorganic chemistry kineticists are able today to dissolve apparently complex reactions involving numerous species into single bond breaking and electron transfer reactions involving, at most, bimolecular collisions. The reactions of ferrous ion and ferric ion with hydrogen peroxide belong to this kind[12]. In photosynthesis research the present day position is such that there is not a single step in the overall mechanism $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$ that can be formulated with any degree of certainty. Attempts have been made to analyze certain fragments of the mechanism (*cf.* RABINOWITCH's monograph[13] and recent reviews). While it is realized by many (*e.g.* FRANCK and his school) that the occurrence of *free radicals* is almost an essential feature, no straight-

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* This work was carried out, while the author was Visiting Assistant Professor at the Institute of Radiobiology and Biophysics (Fels Fund) and Lecturer in Chemistry, at the University of Chicago Chicago 37, Ill., U.S.A.

forward experimental evidence as to their participation in the mechanism of photosynthesis has been forthcoming. The significance of this is outlined in the following paragraphs.

1. *Energetics of the primary step in photosynthesis*

It is generally assumed that the primary step in photosynthesis involves the splitting of water (*cf.*, however, ref.[14]). The bond dissociation energy H . . . OH was postulated as ~ 118 kcal by DWYER AND OLDENBERG[15]. The writer considers that ~ 120 kcal is more correct[16]. In the liquid phase a value of ~ 116 kcal was suggested for the severing of the H . . . OH bond. The photo-excitation energy of chlorophyll is only 41 kcals. Even if losses in a singlet → triplet transition are disregarded, there is a balance of 75 kcals. This implies that in the primary step compensating changes have to be assumed. H atoms and OH radicals could add on to double bonds or abstract exothermically H or OH from another compound reforming water.

Such schemes are

(a) chlorophyll (HOH, XH, YH) $\xrightarrow{h\nu}$ chlorophyll (XHOH; $\text{YH}_2^{\cdot}$)

(b) chlorophyll (HOH, XH, YH) $\xrightarrow{h\nu}$ chlorophyll (HOH, X; $\dot{\text{Y}}\text{H}_2$)

(c) chlorophyll (HOH, X–OH) $\xrightarrow{h\nu}$ chlorophyll (HOH, $\dot{\text{X}}$; $\dot{\text{O}}\text{H}$)

(d) chlorophyll (HOH, X–OH, YH) $\xrightarrow{h\nu}$ chlorophyll (2HOH, $\dot{\text{X}}$, $\dot{\text{Y}}$)

From the energetic point of view (d) is the best digestible. Bond dissociation energies vary considerably in view of the different resonance stabilization of the radicals produced[17]. Thus the difference between the bond dissociation energy $C_6H_5CH_2$ . . . H and C_6H_5 . . . H amounts to about 30 kcals. Scheme (a) which involves the splitting of water and addition of the two resulting radicals to double bonds appear to require more than 45 kcals even in favourable circumstances. The same energetic reasoning applies to (c). (b) which actually means hydrogen transfer producing the oxidizing radical $X^{\cdot}$ and the reducing radical YH_2 is energetically more favourable than (a). With the bond dissociation energy X . . . H being 75 kcals to 80 kcals the overall reaction $4X^{\cdot} + 2HOH \rightarrow 4XH + O_2$ would be about thermoneutral. In a recent publication the interesting hypothesis was put forward[14] that the primary process consists in the severing of the S–S bond leading to the formation of a biradical. However, the hypothesis encounters certain great difficulties. One of them is that the dissociation energies of an S–S bond are of the order of ~ 70 kcals and the shifts of the light absorption maxima observed by CALVIN AND BARLTROP for thioctic acid cannot account for the energy discrepancy of at least 30 kcals. Another objection to the picture that thioctic acid is the primary hydrogen acceptor was raised by FRANCK[18], *viz.* the small concentration of this compound; *i.e.* about 1000 times smaller than the chlorophyll concentration. Thus the hypothesis must be based on the assumption that a "photosynthetic unit" of 1,000 chlorophyll molecules exists, which, according to FRANCK, appears to be incompatible with aspects of the chlorophyll absorption spectrum in plants, and with its fluorescence yield.

2. *The polymerization technique*

In any of these schemes it is not feasible to conceive the evolution of one molecule of oxygen being brought about only by one quantum of light. In all of them the dominant feature is the participation of unstable free radical intermediates, which may be involved both in the oxidation of water and the reduction of carbon dioxide.

With two objects in mind (a) to put forward experimental evidence for the occurrence of free radical intermediates and (b) their identification, the author has introduced into photosynthesis research the method of tracing free radicals by their ability to induce polymerization. This method was previously applied by him to the detection of free radicals in photo-excited electron transfer[19]. The development of this method has been fully described in a recent review[12].

EXPERIMENTAL PART

Experiments with photosynthesizing algae

(a) *Oxygen evolution*

The algae cultures used were *Chlorella* and *Scenedesmus*. Experiments were usually carried out with *Chlorella* and the latter species were used for the purpose of comparison only. The vinyl monomers used were acrylonitrile and methyl methacrylate. They were purified and freed from commercially added inhibitors by distillation under reduced pressure in a nitrogen atmosphere. The algae were centrifuged from their culture medium, washed, suspended in carbonate-bicarbonate buffer solution (pH = 8.69) and the oxygen evolution in the presence and absence of added monomer was measured with conventional Warburg apparatus (one-vessel method). The algae suspensions were of a density of 5 μl/ml and the volume (including added substances) was 3 ml throughout these experiments. Table I shows the course of the oxygen evolution in the presence and absence of methyl methacrylate. Table II shows the effect of the monomer at lower light intensities. The main conclusions drawn from our experiments are: Acrylonitrile poisons the photosynthesizing algae. Their original activity is not restored by cen-

TABLE I

EFFECT OF METHYL METHACRYLATE ON THE OXYGEN EVOLUTION WITH PHOTOSYNTHESIZING *Chlorella*

(Data given present the average readings of two simultaneous experiments, the thermal barometer corrections being allowed for). Except for the initial period, the oxygen evolution is both in the absence and the presence of monomer a linear function of time.

Time in minutes	*μl O_2 (NTP) (a) no (b) 0.05 M methyl methacrylate*		*Rate in μl O_2 (NPT)/min (a) no (b) 0.05 M methyl methacrylate*	
→ 5	0	0		
light			1.2	0.4
10	6	2		
			1.6	0.8
15	14	6		
			2.2	0.8
20	25	10		
			2.1	0.7
30	46	17		
			1.7	0.5
40	63	22		
			2.0	0.6
50	83	28		
			1.8	0.6
60	101	34		

TABLE II

THE EFFECT OF LIGHT INTENSITY ON THE OXYGEN EVOLUTION (BELOW SATURATION)

	Methyl methacrylate	*Full intensity*	*42%*	*29%*
Amount of oxygen obtained in μl (NTP) between 15 and 55 minutes after the light has been switched on	none	76	35	25
	0.05 M	24	11	8

trifuging and washing the monomer out. Methyl methacrylate, on the other hand, reduces the rate of photosynthesis without such permanent effects. The monomer can be washed out and the original activity of the algae is completely restored. The rate of photosynthesis decreases with increasing monomer concentration. The proportional decrease in the rate of photosynthesis is independent of light intensity (at intensities below saturation). At a concentration of 0.05 M methyl methacrylate the rate of oxygen evolution is reduced by two-thirds. In view of the extraordinary effect of succinonitrile[20] (which is not yet completely understood) on the decomposition of peroxides, an experiment was carried out with a view to finding whether succinonitrile (the substance was kindly provided by Professor KHARASCH's laboratory) would have a similar effect on the evolution of oxygen with photosynthesizing algae. The results were negative. The experiments with succinonitrile and a blank test gave identical results.

(b) *Isolation of polymer*

In order to obtain weighable amounts of methyl methacrylate polymer, experiments were carried out (under otherwise similar conditions) on a larger scale. 1–1½ l of algae suspension (5 μl/ml) were irradiated in measuring cylinders. The absorbed light intensity was also increased. From the measurement of the overall pH change, it was calculated that the oxygen evolution amounted to about 50 ml per hour. It is well established that oxygen is a very effective inhibitor of chain propagation. If the polymer chain does not reach a sufficiently large molecular weight the polymer would not precipitate. Even if the monomer entered the reaction, efficient chain termination would make it practically impossible to isolate any polymer. Therefore, it was essential to carry out these experiments under a continuous stream of nitrogen gas to reduce the oxygen pressure as much as possible. The nitrogen current was passed through solutions of monomer before entering the algae suspensions so as to keep the monomer concentrations constant. In addition it proved essential to mix the nitrogen with carbon dioxide (about 5%) lest the bicarbonate-carbonate buffer should become increasingly alkaline. Under optimum conditions 50 mg of polymer were obtained in a number of experiments (lasting three hours). The yield depended considerably on the oxygen pressure in the system. The polymer was isolated by extracting the algae with warm acetone (about 50° C) and precipitating with methyl alcohol. The polymer was then redissolved and reprecipitated for the purpose of purification from absorbed impurities. The average molecular weight determined viscosimetrically was $\sim 10^5$.

It should not be ommitted that under otherwise identical conditions no polymer was detected in the dark. The same applies to experiments with irradiated dead algae and also with algae irradiated in the absence of carbon dioxide. The latter result is of special interest because it shows that photochemical processes sensitized by chlorophyll, which might occur in the algae in the absence of CO_2, either do not produce radicals, or, as is more likely, have too small rates to initiate polymerization chains in sufficient number.

(c) *Tracing of* ^{14}C

With the evidence for the occurrence of free radical intermediates described in the previous paragraphs, it occurred to us that it might be possible to gain some knowledge of the nature of these unstable intermediates by tracing them as polymer end groups. Detection of end groups in polymers of high molecular weight is very difficult; however, the tool of tracing a radio-active end group might be available. It is not certain whether the radicals giving rise to polymerization originate in the oxygen evolution or in the path of carbon dioxide assimilation; the latter is considered to be more likely in view of the negative results obtained with chloroplast suspensions. Experiments with $^{14}CO_2$ (contained in the bicarbonate-carbonate buffer) were carried out. The results of the radioactivity measurements were, however, so slightly above background radiation (by less than one count per minute) that no definite conclusions could be drawn as to whether $HCO^{\cdot}_2$ (the radical is well known to occur as an intermediate in the oxidation of formic acid[21]) or another radical derived immediately from CO_2 in a carboxylation process would be an intermediate giving rise to polymerization. When radioactive ^{14}C-glycolic acid was added, a consistent radioactivity was found in the polymer (of three to five counts per minute) above background, which was beyond the limits of experimental error. No interpretation is suggested at this stage as the experiments are not yet sufficiently complete. We hope to find out whether a free radical derived from phosphoglyceric acid is involved in the photosynthetic process and also to obtain by this method a test of the hypothesis recently put forward by CALVIN AND BARLTROP that the primary process in the utilisation of the light energy absorbed by chlorophyll is the severing of an S–S link.

Experiments with chloroplast suspensions (*Hill reagents*)

These were prepared from fresh healthy spinach leaves. Two methods were used in their preparation, which was carried out in a cold room at a temperature of 1° C: (a) the dry method, *i.e.* grinding in a mortar without grinding fluid, in accordance with the method described by CLENDENNING AND GORHAM[22] and (b) the wet method in a Waring blendor (details of method described by FRENCH AND MILNER[23]). The removal of crude particles by low speed centrifuging and the washing of the chloroplasts by high speed centrifugation and resuspension followed closely the procedure described by CLENDENNING AND GORHAM. The Hill reagents used were ferricyanide, ferricyanide + ferric oxalate and quinone. The effect of methyl methacrylate on oxygen evolution with and without deoxygenation by a stream of nitrogen gas was determined with conventional Warburg apparatus, under the same conditions in which the experiments with algae were carried out. No effect on the oxygen evolution was detected in the presence of methyl methacrylate up to a concentration of 0.1 M in contrast to the experience with photosynthesizing algae. Experiments on a larger scale (similar to those described with algae) showed that no polymer was formed. This applies not only to experiments with quinone, which inhibits polymerization, but also to the other Hill reagents which are not normally inhibitors, such as ferricyanide and ferric oxalate. It was also established that in the absence of the oxidizing agents the monomer by itself does not act as Hill reagent nor does it polymerize. Variation of experimental conditions by suspending the chloroplasts in distilled water, phosphate buffer (pH = 7), 0.5 M sucrose, in the presence and absence of chloride ions and working with crude chloroplast suspensions (not washed) did not alter the fundamental findings that the monomer did not effect the oxygen evolution and that no long chain polymers were formed. The lack of the appearance of polymers during the progress of the Hill reactions is, however, no indication that no free radicals occur in the course of these processes. In the case of quinone reduction, most certainly semi-quinones should be intermediates, but as mentioned above quinone itself inhibits polymerization. The reduction of the ferric ion to ferrous ion, on the other hand, is fundamentally a one-electron transfer process and it is not necessary to postulate free radical intermediates, as far as the reduction of oxidant is concerned. Thus the result that no polymerization is observed needs only special interpretation when the processes leading to oxygen evolution are considered. Indeed, it seems unlikely that the photochemical steps of this set of reactions should not produce radicals. Therefore, we have drawn the tentative conclusion that the radicals involved in this particular process do not induce the formation of long polymer chains because they may be efficient chain terminators. Examples of radicals which behave in this manner are SCN radicals and, equally, bromine atoms (compare with[12, 19]).

In contrast to the negative results obtained with chloroplast suspensions, it was shown that pure chlorophyll, *in vitro*, was photochemically active in the initiation of polymerization of vinyl compounds[24].

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SUMMARY

Reasonably good experimental evidence for the occurrence of free radical intermediates in the process of photosynthesis is presented. The method is based on the detection of free radicals by their ability to induce polymerization. The possibility of applying this method to establish the identity of the free radical intermediates by means of radioactive tracers is placed in focus.

RÉSUMÉ

Une preuve expérimentale assez satisfaisante de l'existence de radicaux libres intermédiaires au cours de la photosynthèse est présentée. La méthode est fondée sur la détection des radicaux libres à l'aide de leur propriété d'induire la polymérisation.

La possibilité d'appliquer cette méthode à l'identification des radicaux libres intermédiaires au moyen de traceurs radioactifs est mise en évidence.

ZUSAMMENFASSUNG

Es wird über Versuchsbeweise berichtet, denen zufolge freie Radikale als Zwischenprodukte der Photosynthese vorkommen. Die Bestimmungsmethode der freien Radikale beruht auf der Tatsache, dass letztere die Polymerisation zu induzieren vermögen. Die Möglichkeit, diese Methode an Hand von radioaktiven Isotopen auf die Identifizierung der als Zwischenprodukte auftretenden freien Radikale anzuwenden, wird erörtert.

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