AUTOXIDATION REACTIONS IN THE PRESENCE OF LIGHT-SENSITIZED CHLOROPHYLL

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Introduction

The oxidation of substances under the influence of visible light in the presence of fluorescent pigments, especially chlorophyll, has several interesting aspects : first, the problem of photodynamic action and assimilation of carbon dioxide in the green plant ; second, the chemical and physico-chemical problem of autoxidation and chemical reaction mechanism in general ; third, the industrial utilization of the unlimited supply of solar energy through this energy-trapping natural substance, chlorophyll. The study of oxidation of fatty acid esters (the components of lipids found in all plant cells) by means of photosensitized chlorophyll was therefore taken up specially as it may help elucidating some phases of oxidation of the fatty substances, and throw light on the parameters of the general problem of oxidative metabolism.

From the work of Meyer,[1] Robinowitch[2] and Wassink,[3] the reaction of chlorophyll and oxygen may be summarized as follows :

$$(Chl)^{*} + A(O_2) \longrightarrow (Chl) + (AO_2)E$$

A being a compound with loose complex $A(O_2)$

$(AO_2)^{E} = A + O_2 + E$, dependent on temperature and *p*H

$O_2 + Chl^{*} \longrightarrow O_2^{E}$, activation of oxygen

Later findings show that

$(Chl) + O_2 \longrightarrow (Chl)O_2$, a dissociable complex.

$Chl^{*} \longrightarrow$ fluorescence

$Chl^{*} \longrightarrow$ heat

$Chl^{*} \longrightarrow A_o$ (absorbing substrate)

Furthermore, chlorophyll can act both as an oxidizing agent and a reducing agent, thus

$$Chl^{*} + Ox \longrightarrow Ochl + rOx$$

$$Chl^{*} + Red \longrightarrow rChl + O\ Red$$

$$OChl + rChl \longrightarrow 2\ Chl$$

This easily follows from the theory of Weiss[4] who has propounded that an electronically excited molecule has an increased tendency for giving away an electron, as well as a capacity for acquiring an electron to replace the one which was removed from its normal level. Ghosh et al., [5] found that chlorophyll initiates oxidation-reduction even in the absence of oxygen :

$$Chl^{*} + MeR \longrightarrow (Chl^{*}MeR)$$

(methyl red)

$$(Chl^{*}\ MeR) + Ph \longrightarrow Chl + Leuco\ MeR + oxidized\ Ph.$$

(phenyl hydrazine)

Livingstone et al.[6] confirmed these results and indicated that activated chlorophyll may form complexes with substances other than oxygen, while Franck[7] proposed a two-step process,[8] involving free radicals in the case of photo-chlorophyll oxidation, which concept is supported by the evidence in a recent investigation by Uri.[9]

In order to get a better insight into this mechanism, the stability of chlorophyll in heptane solution has been studied : (a) alone, (b) mixed with a substrate without agitation and (c) mixed with a substrate agitated by oxygen gas both in the presence and in the absence of light. A mixture of methyl oleate and linoleate (6 : 5) has been used to study some effects of temperature and solvent on chlorophyll-sensitized photo-oxidation.

A plausible mechanism involving both complex formation and oxidation reduction has been suggested for the photo-oxidation of methyl oleate and linoleate in the presence of chlorophyll. The results are substantiated by oxidation of simpler organic substances of requisite structure under similar conditions.

Experimental

Materials.—The chlorophyll was extracted from spinach (palang) leaves by means of acetone (200-250 ml./150 g. midrib-free leaves) in a waring blender containing a small amount of magnesium carbonate. Acetone solution, 500 ml., was three times diluted with water and extracted with purified petroleum ether (b.p. 30-60°C.). After

washing the petroleum ether solution with water, it was shaken with several portions of 80% methanol saturated with petroleum ether and containing 0.01 g. oxalic acid to prevent allomerization. The petroleum ether solution, freed of methanol, was dried with anhydrous sodium sulphate. Sufficient dried sugar, to absorb the chlorophyll was agitated with the petroleum ether solution of chlorophyll. The sugar containing the precipitated chlorophyll was collected on top of a powdered sugar filter in a Buchner funnel. After washing the precipitate with petroleum ether, chlorophyll was eluted with purified ether, dried over anhydrous sodium sulphate and stored below 0°C. for use. Such ether solution[13] may be evaporated or diluted to attain the desired concentration of chlorophyll 'a' and 'b'.

For isolating chlorophyll 'a', the above solution containing the mixture was chromatographed under nitrogen in the usual manner over a column of powdered sugar. The chlorophyll 'a' band (easily recognized by its bluish colour) was isolated by extruding the column and mechanically separating the chlorophyll 'a' fraction. It was redissolved in ether and recovered. The ether solution was evaporated to the desired concentration and stored at 0°C.

For crude chlorophyll solutions, the original acetone solution after dilution was directly extracted with ether, washed, dried, evaporated to the proper concentration and stored below 0°C.

The powdered sugar was dried at 90°C. for 1 hour in a vaccum oven. The ethyl ether was purified by distilling first over anyhdrous calcium chloride and then over metallic sodium. Petroleum ether, cyclohexane, *n*-heptane, *n*-decane, decalin and mineral oil were purified by agitation twice with concentrated sulphuric acid, twice with fuming sulphuric acid and then with acidified permanganate solution, to free them from unsaturates. After this treatment they were thoroughly washed with distilled water, dried over anhydrous calcium chloride and finally distilled (petroleum ether, cyclohexane, *n*-heptane, and *n*-decane at atmospheric pressure and mineral oil under vacuum). The middle fractions were used in all cases. Cyclohexene and tetralin were left for several days over potassium hydroxide pellets and then fractionally distilled over potassium hydroxide by means of the Podbielniak column (cyclohexene at atomospheric pressure and tetralin under vacuum). The constant boiling fraction was employed for the experiments. Naphthalene was purified according to the standard methods.[10] Methyl and ethyl alcohols were distilled over a mixture of zinc powder and potassium hydroxide pellets. Cottonseed oil methyl ester was prepared through methanolysis in the presence of sodium methoxide followed by vacuum distillation of the methyl esters. Methyl oleate (iodine value, 84.3) and methyl linoleate (iodine value, 171.7, diene 0.15%) were obtained through the courtesy of the Hormel Foundation, U.S.A. The peroxide concentrates were prepared by means of contercurrent extraction, using the immiscible pair of solvents, petroleum ether and alcohol. Ethyl stearate was prepared by esterification of stearic acid[11] in the usual manner (benzene and ethyl aclohol and 2% concentrated sulphuric acid). In order to completely free the ethyl stearate from the oleate, the completely neutral stearate was subjected to hydrogenation in the presence of platinum catalyst [12] and then crystallized twice from ethyl alcohol solution.

Apparatus and Methods.—The low temperature bath consisted of an 18″ diameter jar fitted with a double-walled perforated annular tin vessel (Fig. 1) having an annular space

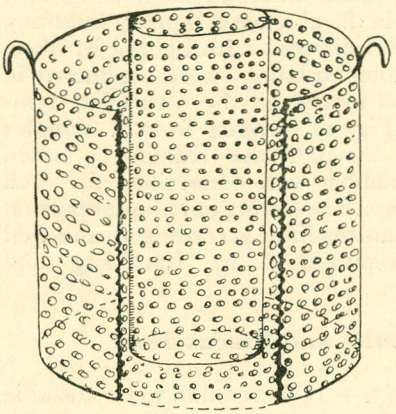

Fig. 1.—Perforated metal support for ice chips.

for holding ice chips and a clear 8″ diameter space in the centre. The perforations allow the brine solution to come in contact with ice, while leaving the central region undisturbed by ice. The bath medium in the middle is stirred by an efficient motor. By proper manipulation and 8-hourly addition of ice this bath could be maintained at a temperature between -4°C. and 0°C., while iced water could be used for temperatures between 10° and 18°C. The light from a 300 W. photoflood bulb (cooled in its housing by forced ventilation) passed through a plate glass window, the Pyrex glass bottom of the bath, and a 6″ layer of water before it reached the half litre three-necked Pyrex reaction flask. The flask was fitted with an inlet for dry oxygen or air (Corning fitted glass dispersion tube), a stirrer and an outlet provided with an anhydrous calcium chloride

absorption tube. The oxidation was carried out on 30% solutions of the substances in heptane, while hexane, cyclohexene, cyclohexane, tetralin, decalin, decane and the other solvents were employed as such (*i.e.*, 100% pure) when they were used alone during oxidation reactions. The oxidized material was freed of chlorophyll products by passing through a mixed column of charcoal and sugar[13] at 10-15°C. (ice-water jacket).

The solutions of chlorophyll (a + b) in different solvents, petroleum ether (b.p. 30°-60°C.), heptane, acetone, alcohol, ethyl ether and iso-octane (2.5 mg. per ml.) were left in stoppered Erlenmeyer flasks (cap. 250 ml. with 100 ml. solution, each) at 0°C., or close to it. The solutions were checked at intervals through observations on degree of changes in green colour and fluorescence of chlorophyll. The degree of stability of the chlorophyll led to the oxidation studies on heptane in the presence of chlorophyll at 0°C.

The cotton-seed oil mixed esters in heptane solution (30%) with chlorophyll (2.5 mg./ml.) was autoxidized in the absence of light by bubbling oxygen in a vessel covered with light-tight black paper. The photo-oxidations were conducted in a reaction vessel placed in the controlled temperature bath (Fig. 1), oxygen from a cylinder being bubbled through the reaction vessel. The peroxide number was determined for each substance at intervals by taking out aliquot portion of the substance autoxidized according to the well known methods published by Lundberg and Chipault.[16]

Results and Discussion

Chlorophyll (a + b) was found to be unstable in petroleum ether, heptane and acetone and alcohols, even at 0°C. It was found to be very stable in ether, with its green colour persisting for a long time, but the activity of chlorophyll in causing photo-oxidation falls off after six months or so at temperatures below 0°C. Ether used as solvent for storing chlorophyll at 0°C. was found to help the experimentations of shorter duration. Chlorophyll (2.5 mg./ml.) did not catalyze the oxidationoj heptane even in two days at 0°C., but the chlorophyll changed colour to olive green. At 17-18°C., chlorophyll became bleached without much effect on the heptane. Consequently, it was thereafter used as solvent for oxidations involving other substances, fatty acid esters in particular. Purified iso-octane preserved the green colour of chlorophyll for over 3 days at 0°C. Iso-octane too is inactive towards oxidation by photo-sensitized chlorophyll at 0°C., indicating lack of reactivity by secondary H-atom during such reactions in contrast to ordinary autoxidation.

Without chlorophyll, the photo-oxidation of cottonseed oil mixed esters in heptane solution at 0°C. was not appreciable. At 17-18°C., it was also quite slow. In the absence of light, chlorophyll increased the stability of mixed esters three times. This may be attributed to the possible consumption of available oxygen in the formation of chlorophyll-oxygen complexes without involving energy transfer from the light that initiates oxidation. Thus the resulting lack of oxygen indirectly protects mixed esters.

Photochlorophyll oxidation of the unsaturated fatty acid esters proceeds at a rapid rate, much higher than that for ordinary photo-oxidation. This is brought out in Table 1 and Fig. 2, which

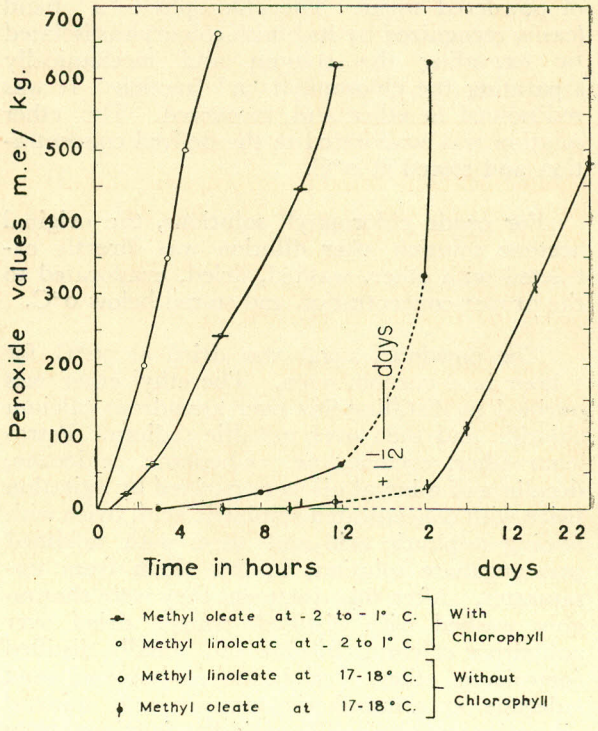

Fig. 2.—Photochlorophyll oxidation as methyl oleate and linoleate.

record with the progress of time, the peroxide values (indicating peroxide formation) of the two unsaturated materials under different conditions. Methyl linoleate has no induction period, whereas methyl oleate has definite induction period (about ¾ hr.) before initiation of oxidation in presence of light-sensitized chlorophyll. Plain

TABLE 1.—COMPARATIVE STUDIES ON PHOTO-OXIDATION IN THE PRESENCE AND ABSENCE OF CHOLOROPHYLL.

Methyl oleate				Methyl linoleate			
With chlorophyll -2 to -1 °C.		Without chlorophyll 17- 18 °C.		With chlorophyll -2 to -1 °C.		Without chlorophyll 17- 18 °C.	
Duration	P.V.	Duration	P.V.	Duration	P.V.	Duration	P.V.
1½ hrs.	20.0	6 hrs.	0.0	2¼ hrs.	199.0	3 hrs.	0.0
2½ ,,	62.0	9½ ,,	0.0	3½ ,,	350.0	8 ,,	19.0
4¾ ,,	234.0	11½ ,,	10.0	4¼ ,,	500.0	12 ,,	60.0
9 ,,	442.0	2 days	24.0	5¾ ,,	665.0	2 days	317.0
		7 ,,	107.0			2¼ ,,	618.0
11¾ ,,	614.0	15½ ,,	310.0				
		22 ,,	482.0				

photo-oxidation without chlorophyll shows for both substances induction periods during which the peroxide values increase extremely slowly. Light-sensitized chlorophyll has the advantage of greater resources of energy that make the oxidations go faster.

In another phase of the experiments, complete oxidation of methyl oleate and linoleate was attempted, and another aspect of photochlorophyll oxidation has thus been revealed by the data (Table 2). The initially slow oxidation of methyl oleate gradually increases in rate and finally keeps pace with methyl linoleate (Fig. 3). This may mean two things : (a) at the outset methyl oleate needs greater energy, not available from chlorophyll source, causing a slow rate, or (b) the energy-supply becomes sufficient in the later stages, indicating a more active form of chlorophyll. Such activated chlorophyll possesses an abundance of energy and the unsaturated fatty acid esters, methyl linoleate and oleate, could utilize only certian fractions of this energy (latter using probably greater) to rise to a maximum level of oxidation in each individual case, thereby equalizing the rates. In the same set of experiments under identical conditions, ethyl stearate did neither oxidize at 0°C., nor at 17-18°C., (Table 2 and Fig. 3). This confirms that the unsaturation and the related features of fatty acid esters are essential for the progress of photo-

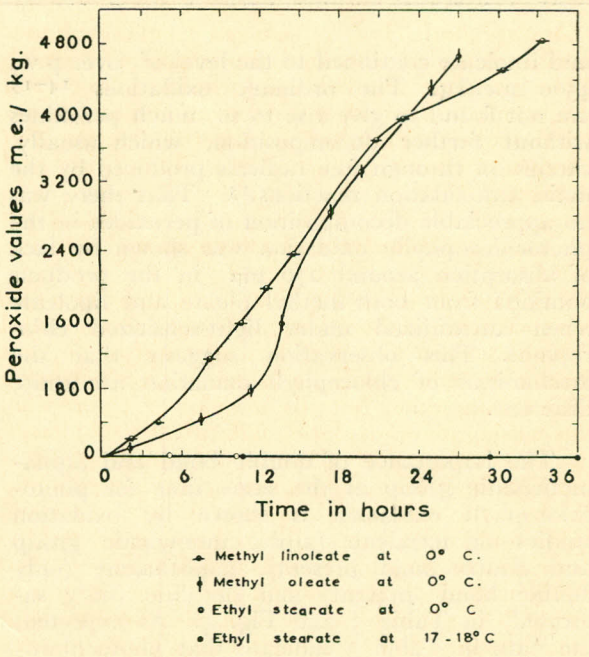

Fig. 3.—Comparative photochlorophyll oxidation of the saturated and unsaturated fatty acids esters.

oxidation. There is another significant phenomenon to be noted in these experiments. The accumulation of peroxides in both methyl oleate

TABLE 2.—PEROXIDE FORMATION DURING LIGHT-SENSITIZED CHLOROPHYLL OXIDATION.

Methyl oleate (0°C.)		Methyl linoleate (0°C.)		Ethyl stearate	
Time (in hrs.)	Peroxide number (m.e./kg.)	Time (in hrs.)	Peroxide number (m.e./kg.)	Time (in hrs.)	Peroxide number (m.e./kg.)
$2\frac{1}{2}$	54.0	$2\frac{1}{4}$	200.0	(Temperature = 0°C.)	
7	405.0	$4\frac{1}{4}$	500.0	5	0.0
$11\frac{1}{2}$	790.0	$8\frac{1}{4}$	1130.0	10	0.0
$13\frac{1}{2}$	1460.0	$10\frac{1}{2}$	1560.0	Temperature = 17-18°C.	
$17\frac{1}{4}$	2810.0	$12\frac{1}{2}$	1955.0	$11\frac{1}{2}$	0.0
$19\frac{3}{4}$	3330.0	$14\frac{1}{2}$	2375.0	$29\frac{1}{2}$	0.0
25	4280.0	$20\frac{3}{4}$	3655.0	36	0.0
27	4670.0	$22\frac{3}{4}$	3920.0		
		$30\frac{1}{2}$	4600.0		
		$33\frac{1}{4}$	4850.0		

and linoleate continued to the level of over p.v., 4500 m.e./kg. The ordinary oxidations [14-15] are not found to give rise to so much peroxides without further decomposition, which usually, carries on through free radicals produced by the main autoxidation reactions.[15] That there was no appreciable decomposition of peroxides in the photochlorophylly oxidation was shown by lack of absorption around 270 mμ in the products obtained from both methyl oleate and linoleate when autoxidized under light-sensitized chlorophyll. This observation suggests that the mechanisms of chlorophyll oxidation are quite different.

The importance of double bond and alpha-methylenic group at the same time for photochlorophyll oxidation is shown by oxidation studies on tetralene (alpha methylenic group and double bond, present), naphthalene (only double bond present) and decaline (fully saturated) in Table 3 and Fig. 4. As expected, the data in Table 3 indicate that photochlorophyll oxidation proceeded only with tetralene at 0°C. and other substances resisted such oxidation. The negligible effects of oxidation on decaline at 17-18°C. may perhaps be due to impurities. Fig. 4 shows the rather remarkable progress of oxidation in case of tertralene.

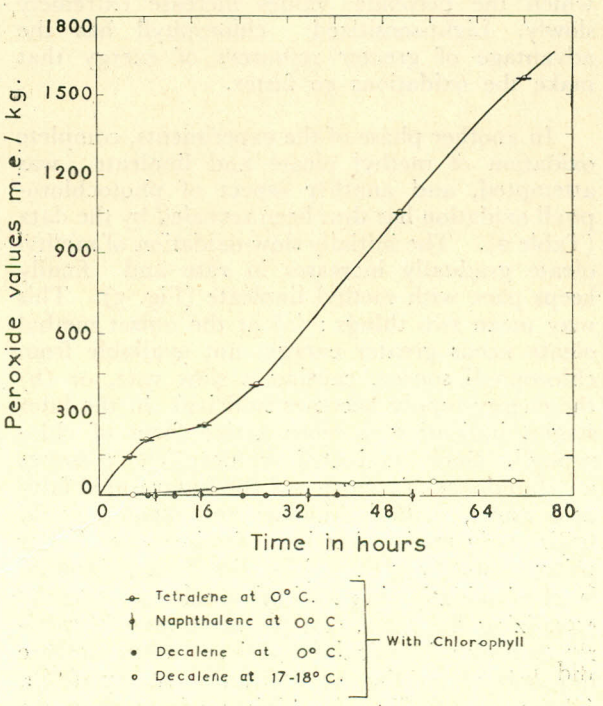

Fig. 4.—Photochlorophyll oxidation of certain specific chemicals : tetralene, naphthalene and decalene.

TABLE 3.—THE ROLE OF ALPHA METHYLENIC GROUP IN PHOTO-CHLOROPHYLL OXIDATION.

Tetralene (0°C.)		Naphthalene (0°C.)		Decalene	
Time (in hrs.)	Peroxide number (m.e./kg.)	Time (in hrs.)	Peroxide number (m.e./kg.)	Time (in hrs.)	Peroxide number (m.e./kg.)
6	150.0	10	0.0	(Temperature = 0°C.)	
8¼	170.0	17½	0.0	8, 12, 24	0.0
18½	240.0	35½	0.0	28, 40	
26¼	390.0	52½	0.0	17 — 18°C.	
50¼	1010.0			6	0.0
72½	1570.0			19	12.0
				31½	28.0
				42	25.0
				56½	25.0
				70½	26.0

The foregoing results indicate that the alpha methylenic group in conjunction with its adjacent double bond forms complex with chlorophyll under sensitization by visible light. Such chlorophyll complex (reducing) would then interact with similar chlorophyll-oxygen complex (oxidizing) to form hydroperoxides and original chlorophyll, as described in the introduction. Moreover, this property of complex formation is not appreciably affected by the presence of other pigments in the leaves (xantophylls, carotenes, etc.).

Further work directed towards an exposition of the mechanisms of chlorophyll oxidation in the presence of visible light is in hand.

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References

1. K. Meyer, Cold Spring Harbor Symposia Quant. Biol. **3**, 341 (1935).
2. E. I. Robinowitch, *Photosynthesis and Related Processes* (Interscience Publishers, Inc., New York, 1945), vol. 1, chapters 16-19.
3. E. C. Wassink, *Advances in Enzymology and Related Subjects of Biochemistry*, Vol. 1, edited by F. F. Nord (Interscience publishers, Inc., New York, 1951), p. 91.
4. J. Weiss, Trans. Faraday Soc., **42**, 116, 133 (1946).
5. J. C. Ghosh and S. E. Sengupta, J. Indian Chem. Soc., **11**, 65 (1934).
6. R. Livingstone, D. Sickle and A. Uchiyama, J. Phys. & Colloid Chem., **51**, 775 (1947).
7. J. Franck, Naturwissenschaften, **21**, 252 (1933).
8. L. Michaelis, J. Biol. Chem. **92**, 211 (1931)
9. N. Uri, J. Am. Chem. Soc., **74**, 5808 (1952).

10. A. Weissberger and E. Proskaner, *Organic Solvents* (Oxford Press, 1935), p. 111; E. C. Baby and W. B. Tuck, J. Chem. Soc., 107, 1058 (1902).
11. P. Morton, *Laboratory Techniques in Organic Chemistry* (McGraw Hill Book Co., New York, 1938), p. 64.
12. R. Adams, V. Vorrhees and R. L. Shriner, *Organic Syntheses* (John Wiley and Sons, Inc., New York, 1948), coll. vol. I, second edition, p. 463.
13. N. A. Khan, Biochim et Biophys. Acta, **16**, 159 (1955).
14. A. Robertson and W. A. Waters, Trans. Faraday Soc., **42**, 202 (1946).
15. E. H. Farmer, ibid., **42**, 228 (1946).; J. L. Bolland and G. Gec, ibid., **42**, 236, 244 (1946).
16. W. O. Lundberg and J. R. Chipault, J. Am. Chem. Soc., **69**, 833 (1947).