ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 125, 189–198 (1968)

Photoperoxidation in Isolated Chloroplasts

I. Kinetics and Stoichiometry of Fatty Acid Peroxidation

ROBERT L. HEATH[1] AND LESTER PACKER

Department of Physiology, University of California, Berkeley, California 94720

Received December 18, 1967

A photo-induced cyclic peroxidation in isolated chloroplasts is described. In an osmotic buffered medium, chloroplasts upon illumination produce malondialdehyde (MDA)—a decomposition product of tri-unsaturated fatty acid hydroperoxides—bleach endogenous chlorophyll, and consume oxygen. These processes show (*a*) no reaction in the absence of illumination; (*b*) an initial lag phase upon illumination of 10–20 minutes duration; (*c*) a linear phase in which the rate is proportional to the square root of the light intensity; (*d*) cessation of reaction occurring within 3 minutes after illumination ceases; and (*e*) a termination phase after several hours of illumination. The kinetics of the above processes fit a cyclic peroxidation equation with velocity coefficients near those for chemical peroxidation.

The stoichiometry of $MDA/O_2 = 0.02$, and $O_2/Chl_{bleached} = 6.9$ correlates well with MDA production efficiency in other biological systems and with the molar ratio of unsaturated fatty acids to chlorophyll. The energies of activation for the lag and linear phases are 17 and 0 kcal/mole, respectively, the same as that for autoxidation. During the linear phase of oxygen uptake the dependence upon temperature and O_2 concentration indicates that during the reaction, oxygen tension at the site of peroxidation is 100-fold lower than in the aqueous phase.

It is concluded that isolated chloroplasts upon illumination can undergo a cyclic peroxidation initiated by the light absorbed by chlorophyll. Photoperoxidation results in a destruction of the chlorophyll and tri-unsaturated fatty acids of the chloroplast membranes.

In 1965 we observed that isolated chloroplasts upon illumination produce substances which react with thiobarbituric acid (TBA) (1). The TBA-reactive substances frequently signify the formation of polyunsaturated fatty acid hydroperoxides (2, 3). An investigation has therefore been undertaken to determine if a cyclic peroxidative mechanism is induced by light in chloroplasts and what its relation is to electron transport and structural state.

Isolated chloroplasts should readily undergo peroxidation since the fatty acids of the lamellar system are about 75% unsaturated (4). It seems likely that peroxidative processes could be catalyzed by light and chlorophyll in the membrane (5). Other components of the electron transport system could also act as peroxidation initiators by free radical formation (6, 7). Moreover, oxygen damage (8) and solarization by high light intensity (9) known to occur within green plants could be related to photoinduced peroxidation within the chloroplast.

[1] Present address: Biology Department, Brookhaven National Laboratory, Brookhaven, New York.

METHODS

Chloroplasts were obtained as previously described (10) and incubated in 10 ml of medium containing NaCl (175 mM), Tris-Cl buffer (50 mM at pH 8) in 9 × 150-mm tubes at 25°. Illumination was with white light provided by a 500-W tungsten lamp at a flux intensity of 22 mW/cm² filtered with a Corning 1-59 IR filter. Monochromatic light at

either 590 or 680 mμ was obtained with interference filters (Baird-Atomic, 30-mμ band width); intensity was calibrated with an Eppley thermopile.

The TBA reactivity (TBAR) was determined by adding an equal aliquot of 0.5% TBA in 20% trichloroacetic acid to an aliquot of the incubation mixture containing a few grains of sand used as a boiling aid. The solution was heated at 95° for 25 minutes and then centrifuged for 1 minute in a clinical centrifuge to clarify the solution. Absorbancy was measured at 532 mμ corrected for nonspecific turbidity by subtracting the absorbancy at 600 mμ (1). The amount of malondialdehyde (MDA) was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1} (3).

The chlorophyll determination was based upon MacKinney's extinction coefficients in 80% acetone (11); four volumes of reagent-grade acetone were added to one volume of the test suspension.

A cylindrical Lucite cuvette surrounded by a water jacket and containing a Teflon-coated Clark oxygen electrode was used to measure oxygen uptake.

Fatty acid determination. Chloroplasts were sedimented by centrifugation at 12,000*g* for 10 minutes. Lipid extraction and saponification-methylation methods were carried out by established procedures (4, 12). The pellet was resuspended in a chloroform-methanol mixture (1:1, v/v), covered by oxygen-free nitrogen, and stored at −15°. After 40 hours, 0.2 volume of NaCl (0.9%) was added.

The aqueous layer was washed once with an equal volume of a chloroform-methanol-water mixture (3: 47:48, v/v), and then three times by an equal volume of chloroform. The chloroform extracts were pooled, dried with anhydrous sodium sulfate, and evaporated *in vacuo* to near dryness at 35°. The residue was dissolved in 9 ml of 5% H_2SO_4 in methanol containing 1 ml of 2,2-dimethoxypropane. The solution, covered with nitrogen, was stored for 48 hours at room temperature (22°) for methylation.

The methylation mixture, and an equal volume of $NaHCO_3$ (0.5 M) and 1.5 volumes of ethyl ether were transfered to a separatory funnel. The ether was removed and the aqueous phase was washed three times with equal volumes of ether. The total extract was washed with $NaHCO_3$, dried with anhydrous sodium sulfate, and evaporated to near dryness at 35°. The remaining ether extract was then put on an 11 × 27-mm column half filled with silica gel, which had been previously washed with ether. The initial eluent, appearing before the green band, was found to contain fatty acid methyl esters. This procedure removed most pigments from the extract. The eluent was dried under nitrogen at room temperature, taken up in a small amount of carbon disulfide, and chromatographed (Varian Aerograph HY-FI; model 600-C); a 6-foot × ⅛-inch column of 20% diethylene glycol succinate on 60/80 mesh hand-washed Chromsorb W was used. The column was run at 156° with a flow rate of about 120 ml/minute of helium, and standardized with known mixtures of fatty acid methyl esters. The relative quantity of fatty acids was estimated by the areas under the peaks.

RESULTS

Kinetics and stoichiometry. Figure 1 shows the kinetics of formation of TBAR or MDA in illuminated chloroplasts. Several phases may be identified: a rapid initial increase of MDA during the first hour; a lag period before the onset of a linear phase; a constant rate of MDA formation between 1 and 6 hours; and a variable termination phase with its onset dependent upon the age of leaves and chloroplast preparation. Little MDA is produced in the dark. Continued production of MDA is dependent upon the presence of light. Figure 1 also shows that chloroplasts placed in the light after 2.5 hours of dark incubation show a similar MDA formation. Conversely, light-incubated chloroplasts placed in the dark cease MDA production within minutes.

Maximum MDA formation has been found to coincide with complete bleaching of endogenous chlorophyll (end of termination phase) and is directly related to the initial amount of chlorophyll (Fig. 2) since 0.13 mole MDA/mole chlorophyll is formed.

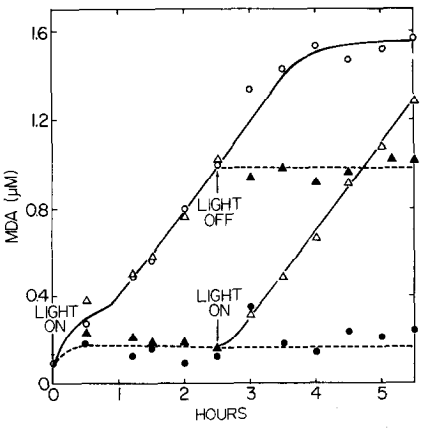

FIG. 1. Malondialdehyde formation in illuminated chloroplasts. Chloroplasts (8 μM chlorophyll) were incubated as in METHODS.

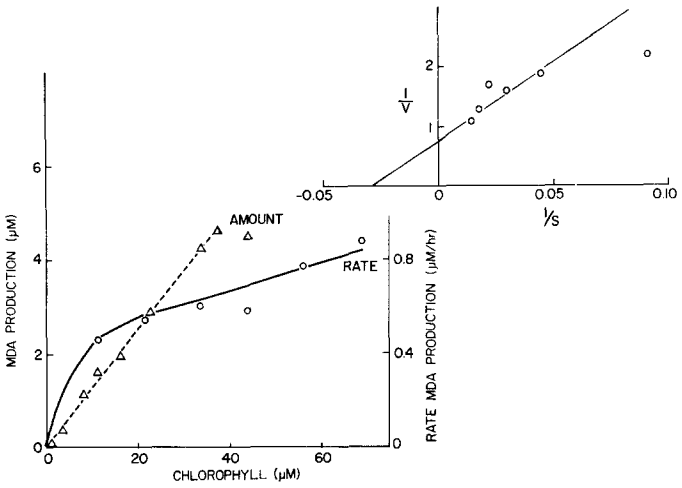

FIG. 2. Stoichiometry and kinetics of malondialdehyde in illuminated chloroplasts. Insert: Determination of kinetic coefficients of malondialdehyde production were calculated from chloroplast concentration (S) as chlorophyll (μM) and the linear rate (v) as μM/hour.

However, the linear rate of MDA production is not a linear function of chlorophyll concentration. When the inverse of the linear rate of MDA formation is plotted vs. the inverse of the chlorophyll concentration, a curve is obtained that fits the cyclic kinetic equation for peroxidation (6, 16) with the MDA production rate, at infinite chlorophyll concentration, of 1.3 μM hour^{-1} and the chlorophyll concentration at infinite MDA production rate of -30 μM (Fig. 2, insert).

Endogenous chlorophyll bleaches concurrently with the MDA production (Fig. 3). Little change in chlorophyll *a* or *b* is observed in dark-incubated chloroplasts. However, loss of chlorophyll *a* in illuminated chloroplasts is clearly observed; there is also a slower loss of chlorophyll *b*. A 1-hour lag period precedes bleaching of chlorophyll *b*, but almost no lag period exists before chlorophyll *a* bleaching commences. Bleaching occurs in a linear fashion for both chlorophylls. Although the rate of chlorophyll *a* loss (3.7 μM/hour) is greater than that of chlorophyll *b* (2.6 μM/hour), if these results are normalized by initial concentrations, the relative rate of chlorophyll *b* bleaching (0.56 /hour) is greater than that of chlorophyll *a* (0.26/hour). An average of 0.15 mole MDA is produced per mole of total chlorophyll bleached (see Table I), which correlates well with the maximum amount of MDA produced per mole of initial chlorophyll.

The rate and time lag of chlorophyll bleaching also depend upon the chloroplast concentration. While the linear rates of bleaching increase for both chlorophyll *a* and *b* with increasing chlorophyll, the absolute rates decrease. In addition, the kinetics of chlorophyll *a* bleaching agree with a cyclic kinetic equation for peroxidation (6, 16) with a rate of chlorophyll *a* loss at an infinite chlorophyll concentration of 8 μM/hour^{-1}, and the chlorophyll concentration at infinite chlorophyll *a* bleaching rate is -30 μM. The kinetics cannot be easily determined for chlorophyll *b* bleaching. The lag time for chlorophyll *a* bleaching is rapid, and it appears as if there is no change with increasing chlorophyll concentration. However, the chlorophyll *b* bleaching lag time undergoes a gradual lengthening.

The existence of a concurrent MDA production and chlorophyll bleaching provides evidence for lipid peroxidation. In addition, oxygen uptake is also observed in illuminated chloroplasts with similar kinetics. A typical oxygen uptake curve at intensities of 160 mW/cm^2 exhibits the first two phases, lag and linear. The termination phase has not been clearly observed due to relatively rapid exhaustion of oxygen in the

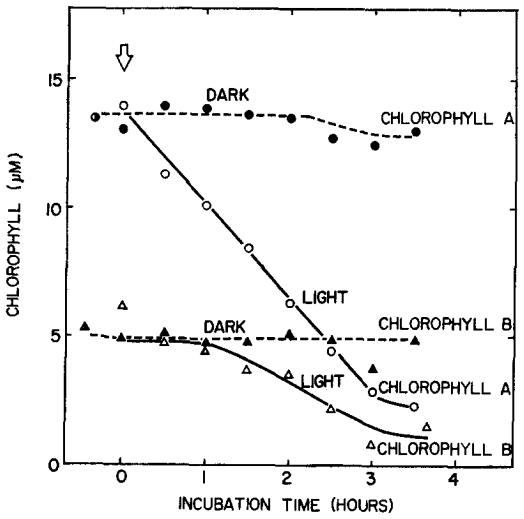

FIG. 3. Bleaching of chlorophylls *a* and *b* in illuminated chloroplasts. Chloroplasts (18 μM chlorophyll) were incubated as in METHODS.

solution at high light intensity. In dark-incubated chloroplasts an extremely low rate of oxygen consumption is observed. In contrast to the lag time observed for chlorophyll *b* bleaching, there is nearly no dependence of the lag time for oxygen consumption upon the chlorophyll concentration. The linear rate of oxygen uptake follows the cyclic kinetics equation for peroxidation with an oxygen uptake rate at infinite chlorophyll of 14 μM/minute and a chlorophyll content at an infinite oxygen uptake rate of −50 μM.

Table II summarizes the calculated stoichiometries of MDA, O_2, and chlorophyll change. The comparison shows an average ratio of MDA to chlorophyll of 0.14. The MDA to oxygen uptake ratio is 0.020. The oxygen uptake to chlorophyll loss ratio, which is indicative of the number of unsaturated fatty acids present per initial chlorophyll, is about 7. This stoichiometry varies about 10% among chloroplast preparations.

Fatty acid composition. Lipid peroxidation is an attack upon unsaturated fatty acid components of chloroplasts, and an analysis of fatty acids revealed distinct differences between light- and dark-incubated chloroplasts. The major change in chloroplasts as a result of dark incubation is an increase in relative abundance of all peaks relative to C16:0. The spectrum of fatty acids present after 4 hours light incubation, however,

TABLE II

STOICHIOMETRY OF MALONDIALDEHYDE PRODUCTION, CHLOROPHYLL LOSS, AND OXYGEN UPTAKE IN ILLUMINATED CHLOROPLASTS

Trial	MDA / Chlorophyll[a]	MDA / Oxygen	Oxygen / Chlorophyll
1	0.10	0.014	7.1
2	0.10	0.016	6.0
3	0.12	0.017	6.8
4	0.16	0.023	6.9
5[b]	0.22	0.028	7.8
Average	0.14	0.020	6.9

[a] Chloroplasts (31 μM chlorophyll) were incubated as in METHODS at 160 mW/cm² white light intensity.

[b] Calculated from the rate during the linear phase of oxygen uptake, MDA production, and chlorophyll loss.

TABLE I

STOICHIOMETRY OF MALONDIALDEHYDE PRODUCTION AND CHLOROPHYLL BLEACHING IN ILLUMINATED CHLOROPLASTS

Incubation (hours)[a]	MDA (μM)	Chlorophyll loss (μM)	MDA/ chlorophyll	Chlorophyll *a* loss (μM)	MDA/ chlorophyll *a*	Chlorophyll *b* loss (μM)	MDA / chlorophyll *b*
0.5	0.16	1.1	0.15	1.0	0.16	0.1	1.6
1.0	0.40	2.9	0.14	2.5	0.16	0.4	1.0
1.5	0.66	4.7	0.14	3.7	0.18	1.0	0.7
2.0	0.80	5.7	0.14	4.1	0.19	1.6	0.5
2.5	1.17	6.5	0.18	5.5	0.21	1.0	1.2
3.0	1.40	9.4	0.15	7.2	0.19	2.2	0.6
3.5	1.87	1.02	0.18	8.1	0.23	2.1	0.9

[a] Chloroplasts (16 μM chlorophyll) were incubated as in METHODS.

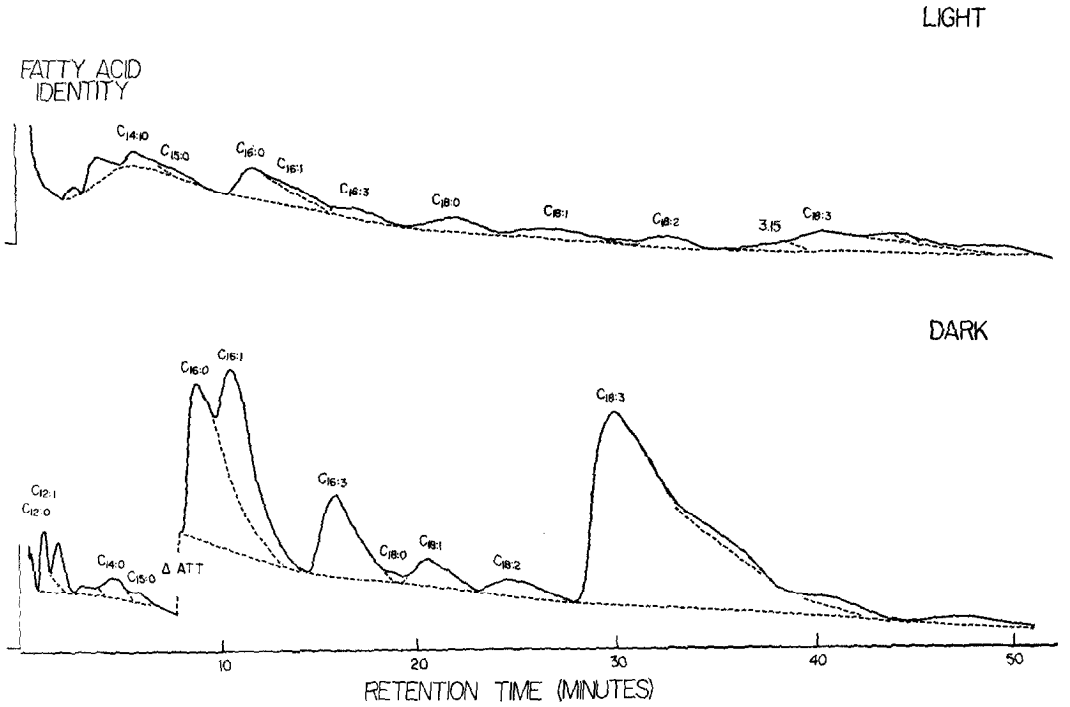

FIG. 4. Fatty acid composition of control and illuminated chloroplasts. Chloroplasts (40 μM chlorophyll) were incubated in NaCl (175 mM), Na-borate (50 mM at pH 8.9) at either 0 or 22 mW/cm² and 25° for 4.5 hours.

changes drastically, and many new peaks are apparent. Figure 4 shows that the C14:0, C15:0, C16:2, and C18:0 peaks have become prominent. Table III shows the time course for changes in fatty acid composition during incubation. A control for zero incubation time is given for comparison. A substantial decrease in C16:1, C16:3, and C18:3 and an increase in compounds with peaks above C18:3 is observed.

The relative efficiency of MDA production can be calculated from the relative amounts of tri-unsaturated fatty acids (C16:3 and C18:3) which disappear, divided by the total amount of dark fatty acid component. Fatty acid loss per chlorophyll can be estimated from an average fatty acid content (14). The efficiency of MDA production of tri-unsaturate loss was calculated to be 0.024, which is in agreement with the observed stoichiometry. Tri-unsaturated and C16:1 fatty acids are mainly involved in peroxidative loss.

Induced fatty acid peroxidation. Malondialdehyde production by illuminated chloroplasts in the presence of exogenous lipid was examined to afford a method of determining relative permeability and effectiveness of these lipids at the site of the MDA production. Additions of methyl linolenate to a standard chloroplast mixture produced an "induced" amount of MDA (MDA produced by linolenate + chloroplasts −MDA produced by linolenate) much higher than MDA formed from endogenous lipids. The induced formation of MDA is shown in Fig. 5a. The excess amount of MDA produced increases more slowly at low concentrations of added methyl linolenate than after an addition of 1 mM or greater methyl linolenate. The dependence of the induced reaction upon the chloroplast concentration is shown in Fig. 5b, where MDA produced after 1.5 hours is plotted against the amount of added chloroplasts. The MDA production curve for chloroplasts only exhibits the normal curve, but the curve with linolenate (0.8 mM) rapidly rises, peaks, and then falls. The amount of induced MDA formation shows a stimulation at low chloroplast concentration or high lipid concentration, but as the chloroplast

TABLE III

FATTY ACID COMPOSITION OF CHLOROPLASTS

Fatty acid identified	Relative abundance (to C16:0)						
	Time of incubation						
	0 hour	1 hour			2.5 hours		
		Light	Dark	Dark-light	Light	Dark	Dark-light
C10:1	0.03	0.09	0.02	−0.07	0.05	0.11	0.06
C12:0	0.02	0.07	0.02	−0.05	0.03	0.04	0.01
C14:0	0.01	0.03	0.05	0.02	0.05	0.09	0.04
D15:0	0.11	0.12	0.13	0.01	0.20	0.50	0.30
C16:0	1.00	1.00	1.00	0.00	1.00	1.00	0.00
C16:1	0.99	0.78	1.01	0.23	0.68	0.90	0.22
C16:2	0.04	0.02	0.02	0.00	0.01	0.03	−0.02
C16:3	0.80	0.69	0.78	0.09	0.61	1.09	0.48
C18:0	0.03	0.03	0.03	0.00	0.05	0.02	−0.03
C18:1	0.14	0.16	0.19	0.03	0.19	0.15	−0.04
C18:2	0.18	0.19	0.20	0.01	0.27	0.31	0.04
C18:3	3.85	3.38	4.32	0.94	3.11	5.92	2.81
C18:3*	0.15	0.52	0.19	−0.33	0.29	0.14	−0.15
C18:3+	0.10	0.28	0.05	−0.23	0.10	0.00	−0.10

Chloroplasts (34 μM chlorophyll) were incubated and extracted as in METHODS. Identity was determined from retention time of standards (relative to C16:0), except for C18:3* (time = 4.7) and C18:3 + (time = 5.1).

concentration increases or the lipid concentration decreases, the stimulation declines.

pH Dependence. Figure 6 shows MDA production in buffered solutions at different pH values. Above pH 8.7 the amount of MDA produced is maximal and constant to pH 9.7; hence, the value of MDA produced at pH 8.8 is normalized to 1.00 for each trial. The minimum production of MDA lies in a broad pH plateau between pH 6 and 7.

Ionic strength. The salt concentration was normally held at 0.175 M for most experiments; lipid peroxidation changed only slightly with variations in ionic strength. The linear rate of peroxidation somewhat decreases with decreasing salinity (25% lower at 0 M). There is no effect of NaCl on peroxidation from 0.2 to 0.8 M.

Light intensity. The production of MDA is light-dependent (Fig. 1); hence, it would be predicted that light exerts an important role upon the observed kinetics of peroxidation. Examples are: (*a*) The rate of chlorophyll *a* bleaching has a square root dependency to light intensity (Fig. 7a) where the logarithm of the linear rate of chlorophyll *a* loss plotted against the logarithm of the white light intensity gives a straight line whose slope is 0.50 ± 0.01. (*b*) The dependence on light intensity of chlorophyll *b* bleaching is different from that of chlorophyll *a* bleaching (Fig. 7b). The linear rate of chlorophyll *b* bleaching is proportional to the light intensity above a "threshold" of 10–12 mW/cm². Chlorophyll *b* bleaching also can be saturated at light intensities about 90–100 mW/cm² and seems to exhibit different characteristics from chlorophyll *a* bleaching. (*c*) The linear rate of MDA production and oxygen uptake is also dependent upon the square root of incident white light intensity beyond a certain "threshold."

The relative effectiveness of two different wavelengths of light have been compared as follows. Chlorophyll bleaching is initiated

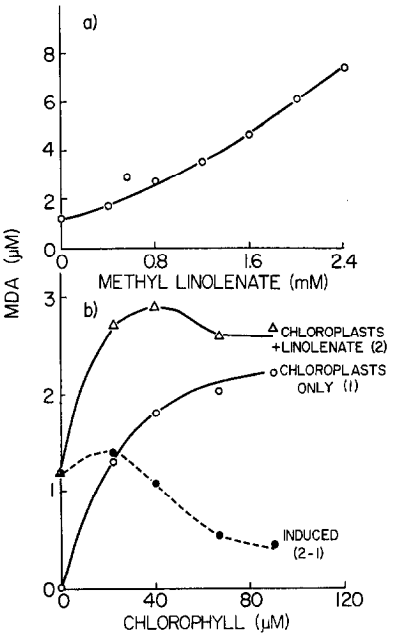

FIG. 5. Induced malondialyde production by methyl linolenate in illuminated chloroplasts. (*a*) Methyl linolenate dependence. Chloroplasts (26 μM chlorophyll) were incubated as in METHODS for 1.5 hours in the presence of methyl linolenate as indicated. (*b*) Chloroplast dependence. Chloroplasts (at indicated chlorophyll concentration) were incubated as in METHODS for 1.5 hours. Methyl linolenate concentration, where indicated, was 0.8 mM.

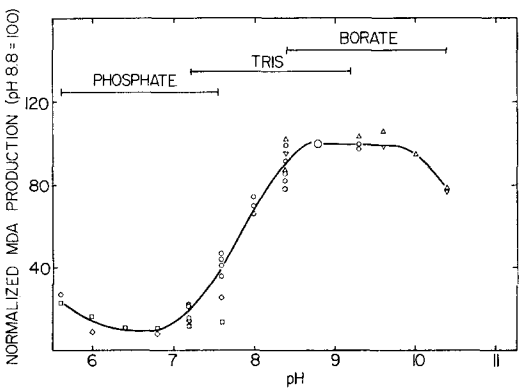

FIG. 6. The pH dependence of malondialdehyde production in illuminated chloroplasts. Chloroplasts (13 μM chlorophyll) were incubated for 2 hours as in METHODS. The pH range was analyzed with the following buffers: sodium phosphate from pH 5.7 to 7.7, Tris-HCl from pH 7.3 to 9.3, and sodium borate from pH 8.6 to 10.4.

by using white light at high intensity. After bleaching has begun, the white light is extinguished while simultaneously providing monochromatic light at 680 or 590 mμ. At 680 mμ chlorophyll *b* bleaching is maintained at the same rate as in white light, but at 590 mμ bleaching of chlorophyll *b* is inhibited. For the same photon flux of 30 nano-einsteins/cm^2/second, 680 mμ light is 40% more effective than 590 mμ light in bleaching chlorophyll *a*.

Temperature studies. The lag time of the oxygen uptake decreases with increasing temperature. The range of lag times is from about 48 minutes at 7° to less than 1.5 minutes at 42°. This relation, calculated by a least-square fit, follows an Arrhenius plot (logarithm of the inverse lag time vs. inverse absolute temperature). Activation energy for each trial is shown in Table IV (column 2) with an average of +17 kcal/mole.

The linear phase of oxygen uptake shows small decreases with increasing temperature. If the linear rates are fitted to the Arrhenius equation by least-square fits (Table IV, column 3), the average energy of activation is −3.3 kcal/mole. The negative sign indicates an "antioxidant" effect of temperature whose extent seems typical for a physical process. If the linear rate is limited by the initial oxygen tension due to the temperature, this process would have a characteristic K_m. This K_m has been calculated for five trials with the average value of 320 μM indicating that the oxygen tension in solution limits the observed linear rate of oxygen uptake. This result is confirmed by the experiments showing (Fig. 8) that the inverse rate of oxygen uptake linearly depends

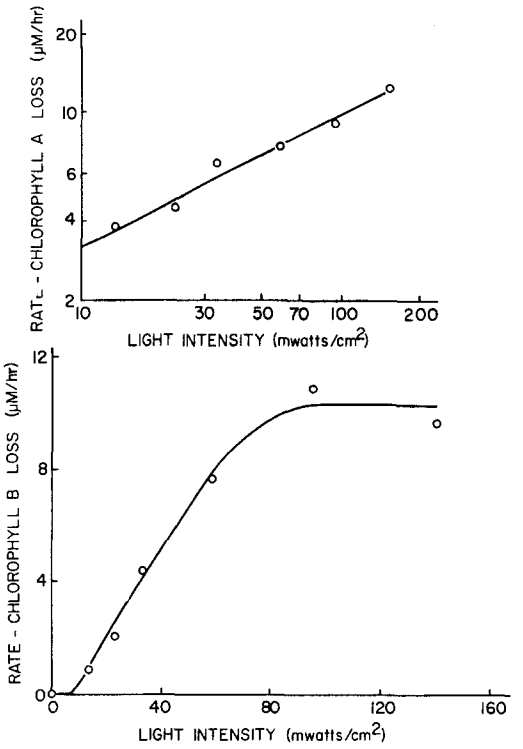

FIG. 7. Dependence of chlorophyll bleaching upon light intensity. Chloroplasts (23 μM chlorophyll) were incubated as in METHODS. *Upper:* chlorophyll *a*; *lower:* chlorophyll *b*.

TABLE IV

TEMPERATURE DEPENDENCE OF LAG AND LINEAR PHASE OF OXYGEN UPTAKE BY ILLUMINATED CHLOROPLASTS

Trial[a]	Lag phase	Linear phase	
	E_A[b] (kcal/mole)	E_A[b] (kcal/mole)	K_m[c] (mM O_2)
1	+12 (4)[d]	−6.6	<0
2	+21 (5)	−2.4	0.73
3	+23 (5)	−1.4	0.11
4	+11 (4)	−1.1	0.11
5	+26 (5)	−4.9	0.27
6	+5 (4)	−0.8	<0
7	+21 (5)	−6.0	0.36
Average	+17	−3.3	0.32
Error[e]	3	0.8	0.08

[a] Chloroplasts (70 μM chlorophyll) were incubated in NaCl (175 mM), Tris-HCl buffer (50 mM at pH 9.1) at 120 mW/cm² and temperature indicated (from 7 to 42°).

[b] E_A, energy of activation calculated by method of least squares.

[c] K_m, Michaelis-Menten kinetic constant calculated by method of least squares.

[d] Numbers in parentheses refer to the number of experimental trials at different temperatures.

[e] Error of mean.

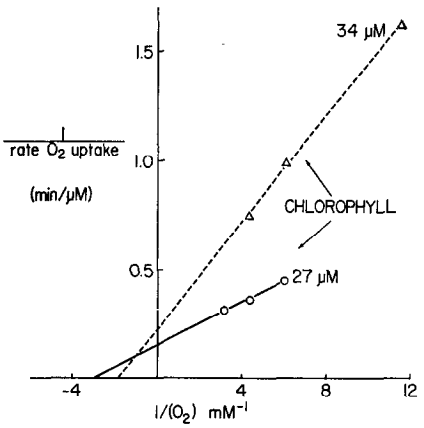

FIG. 8. Dependence of O_2 consumption by illuminated chloroplasts upon O_2 concentration. Chloroplasts (27 or 34 μM chlorophyll as indicated) were incubated as in METHODS at a light intensity of 160 mW/cm². Data were taken from linear phase of O_2 consumption.

upon the inverse of the oxygen tension. This is in accordance with a cyclic peroxidative mechanism with an average K_m of 410 μM, nearly coinciding with that found in the studies of temperature dependence.

DISCUSSION

Cyclic photoperoxidation. Photoperoxidation in chloroplasts requires light, oxygen, and endogenous fatty acids. The processes do not exactly follow the kinetics of chemical peroxidation, perhaps because the internal supply of substrate and conditions at the peroxidation sites may slightly alter the reaction (15, 16).

In biological and chemical systems the occurrence of peroxidation is usually correlated with MDA production (3, 13, 17). The efficiency of MDA production measured during mitochondrial swelling shows that the amount of MDA produced to the tri-unsaturates present is 2.8%, since the molar ratio of MDA to total fatty acids is 1:300 (18) and typical mitochondria are found to contain about 12% tri- or higher unsaturated fatty acids (19). Microsomes yield an MDA efficiency of 4.5–5% as measured by the ratio of MDA produced to oxygen uptake (20). These efficiencies of MDA formation are nearly that of the chloroplasts (see Table II). Similar ratios of MDA:O_2 and MDA:tri-unsaturates lost suggest similar mechanisms. The ratio of 1 mole oxygen taken up to 1 mole tri-unsaturate lost argues strongly for the cyclic lipid peroxidation mechanism. The interdependence of oxygen uptake, chlorophyll *a* bleaching, and MDA production fits into the peroxidation kinetics; however, the chlorophyll *b* bleaching may not be as directly connected.

Friend and Acton (21) have shown by the thiocyanidate method that initially there is an actual increase in hydroperoxides in the light over the dark-incubated chloroplasts; however, the dark-incubated chloroplasts have a higher concentration after longer periods of time. This is to be expected since the relative abundance of each of the compounds within the cyclic peroxidation mechanism is vastly affected by the amount of hydroperoxides present, especially as to breakdown caused by homolytic scission (6, 15, 16).

Kinetics of photoperoxidation Using Bolland's notation for the velocity coefficients of fatty acid peroxidation (16) for initiation (k_1), reaction with oxygen (k_2), the chain initiation step of hydroperoxyl radical reaction

TABLE V

VELOCITY COEFFICIENT CALCULATED FOR PEROXIDATIVE PROCESSES IN ILLUMINATED CHLOROPLASTS

Measurement[a]	Light intensity (mW/cm²)	k' [b] (aqueous phase)	$\bar{k}$ [b] (sec^{-1})
Malondialdehyde formation	20	1.1	9×10^{-5}
Chlorophyll *a* bleaching	20	1.1	7×10^{-5}
O_2 consumption	160	0.8	67×10^{-5}
O_2 consumption (as O_2 pressure)	160	2.9	43×10^{-5}

[a] See RESULTS.

[b] See DISCUSSION.

(k_3), and the termination processes (k_4, k_5, k_6), the rate of O_2 uptake or peroxide formation is given as

$$-\frac{d(O_2)}{dt} = \frac{k_3(k_1)^{1/2}}{(k_6)^{1/2}} \frac{[RH]\,[O_2]}{[O_2] + [RH](k_3/k_2)(k_3/k_6)^{1/2}},$$

where $[O_2]$ is oxygen concentration and [RH] is lipid concentration. From the kinetics of measurements at different chloroplast concentrations and the stoichiometry as in Table II, the two combinations of velocity coefficient $[(k_1)^{1/2}k_3]/(k_6)^{1/2} = \bar{k}$; $(k_3/k_2)(k_4/k_6)^{1/2} = k'$ can be determined as in Table V.

The average k' is 1.5, but this assumes the unlikely possibility that peroxidation is occurring within the aqueous phase. A more reasonable site for the peroxidation is within or near the lipophilic grana membranes. This would alter the concentration of fatty acids by a dilution factor of about 1/3000, and then the average value of k' should be 5×10^{-4}. This does not agree with the value of k' found for chemical peroxidation of ethyl linolenate of 4.4×10^{-6} (6, 16). If the two systems are equivalent, this would indicate the oxygen concentration at the site of peroxidation is only about 2 μM.

The average $\bar{k}$ can be calculated if the individual $\bar{k}$'s are reduced to the value at a constant light intensity. The average $\bar{k}$ is 14×10^{-5} second^{-1} for a white light intensity of 20 mW/cm², which is nearly 20×10^{-9} einstein/cm²/second (22). If an average absorbancy of 0.5 cm^{-1} and a quantum efficiency of 7 (from stoichiometry) are assumed, the above value agrees with the chemical value of $\bar{k}$ for a chemical system (2.3×10^{-2} M$^{-1/2}$ sec$^{-1/2}$) (6, 16). For chloroplasts at a chlorophyll concentration of 11 μM, the reaction rate is calculated as 0.4 μmole/mg chlorophyll/hour.

The velocity coefficients are near those for a chemical autoxidation of the same fatty acid (linolenic), providing the oxygen at the site during peroxidation is low (2 μM) and the quantum efficiency is high (7 moles/einstein). A quantum yield above one and a square root light dependency constitutes primary evidence for a photoperoxidation reaction (6, 16).

Chlorophyll sensitization and fatty acid loss. The dependence of chlorophyll *b* bleaching to light intensity suggests that it is not involved in primary peroxidative kinetics. The threshold of light intensity and the long lag period indicate a protective agent or action of antioxidant which must be overcome. Either a photosensitized reaction of chlorophyll directly with oxygen or a reaction with the hydroperoxides produced by the primary reaction might account for chlorophyll *b* bleaching. There are indications that chlorophyll *b* is also not in the region of electron flow in normal photosynthesis (23). Monochromatic light studies indicate that chlorophyll is sensitizing peroxidation, but it is not known whether chlorophyll *a* or *b* initiates the process.

The major fatty acids lost by peroxidation are C16:1, C16:3 and C18:3 (Table III), and the loss of tri-unsaturated compounds is reasonable by a peroxidative reaction being in good agreement with the observed efficiency of MDA production. Perhaps other unsaturated fatty acids are spared first because the tri-unsaturated compounds present a more susceptible route for peroxidation (16). Ultimately all the unsaturated compounds are attacked (see Fig. 4). This, however, does not explain the early loss of C16:1 by a peroxidative mechanism unless C16:1 is in a vulnerable position (24). On the average seven tri-unsaturated fatty acids suffer peroxidation attack for each chlorophyll destroyed, and MDA is formed with 2% efficiency.

Possible site of peroxidation. The activation energy for initiation of chemical autoxidation of fatty acids (6) and for the lag period of chloroplast peroxidation is about 17 kcal/mole (Table IV). Moreover, the energies of activation for system II as measured by oxygen uptake and absorbancy changes (23), and for temperature inactivation of the Hill reaction (25), are within 1 kcal/mole of autoxidation. Therefore peroxidation within chloroplasts may be linked to photosystem II inactivation.

The dependence upon temperature of the linear phase has been examined at low oxygen tension. Autoxidation of tri-unsaturated fatty acids has a K_m of only 5 μM oxygen (6, 16). The results of Franck and French (26) on photooxidation in *Hydrangea*, plotted as in Fig. 8, reveal a K_m for oxygen of 690 μM compared with 420 μM obtained in this investigation. From comparisons of peroxidation in the aqueous phase and chloroplast system, the oxygen tension at the site of peroxidation appears to be at least 100 times lower than for the aqueous phase. If the fatty acids and chlorophyll *a* were located in a nonaqueous environment, as indicated by the stability of the excited chlorophyll state (27), inhibition of photosystem II by lipases (28), and by electron microscopy fixation (29), then the low oxygen concentration might be maintained by a permeability barrier.

ACKNOWLEDGMENT

This work was supported by the National Science Foundation GB 6147 and a predoctoral fellowship (5-F1-GM-30,175-02) from the United States Public Health Service.

REFERENCES

1. HEATH, R. L., AND PACKERS, L., *Biochem. Biophys. Res. Commun.* **19,** 716 (1965).
2. SIDWELL, G. G., SALWIN, H., BENCA, M., AND MITCHELL, J. H., JR., *J. Am. Oil Chem. Soc.* **31,** 603 (1954).
3. KWON, T., MENZEL, D. B., AND OLCOTT, H. S., *J. Food Sci.* **30,** 808 (1965).
4. ALLEN, C. F., GOOD, P., DAVIS, H. F., CHISUM, P., AND FOWLER, D. D., *J. Am. Oil Chem. Soc.* **43,** 223 (1966).
5. KHAN, N. A., TOLBERG, W. E., WHEELER, D. H., AND LUNDBERG, W. O., *J. Am. Oil Chem. Soc.* **31,** 460 (1954).
6. SCOTT, G., "Atmospheric Oxidation and Antioxidants," Chapters 2-4. Elsevier, Amsterdam (1965).
7. SOGO, P., PON, N., AND CALVIN, M., *Proc. Natl. Acad. Sci. U.S.* **43,** 387 (1951).
8. KESSLER, E., *Handbuck Planzenphysiol.* **5,** 941 (1962).
9. NIELSEN, E. S., *Physiol. Plantarum* **5,** 334 (1952).
10. PACKER, L., SIEGENTHALER, P. A., AND NOBEL, P., *J. Cell. Biol.* **26,** 593 (1965).
11. MACKINNEY, G., *J. Biol. Chem.* **132,** 91 (1940).
12. LIPSKY, S. R., AND LAUNTOUN, R. A., *Methods Enzymol.* **6,** 513 (1963).
13. YU, T. C., AND SINNHABER, R. O., *J. Am. Oil Chem. Soc.* **41,** 540 (1964).
14. BENSON, A. A., *Ann. Rev. Plant Physiol.* **15,** 1 (1964).
15. BATEMAN, L., *Quart. Rev. London* **8,** 147 (1954).
16. BOLLAND, J. L., *Quart. Rev. London* **3,** 1 (1949).
17. JACOBSON, G. A., KIRKPATRICK, J. A., AND GOFF, H. E., JR., *J. Am. Oil Chem. Soc.* **41,** 124 (1964).
18. HUNTER, F. E., JR., SCOTT, A., HOFFSTEN, P. E., GUERRA, F., WEINSTEIN, J., SCHNEIDER, A., SCHULTZ, B., FINK, J., FORD, L., AND SMITH, E., *J. Biol. Chem.* **239,** 604 (1964).
19. UTSUMI, K., YAMAMOTO, G., AND INABA, K., *Biochem. Biophys. Acta* **105,** 368 (1965).
20. HOCHSTEIN, P., AND ERNSTER, L., *Biochem. Biophys. Res. Commun.* **12,** 388 (1963).
21. FRIEND, J., AND ACTON, G. J., *Brookhaven Symp.* **19,** 485 (1967).
22. SELIGER, H. H., AND MCELROY, W. D., JR., "Light: Physical and Biological Action," Chapter 1. Academic Press, New York (1965).
23. WITT, H. T., RUMBERG, B., SCHMIDT-MENDE, P., SIGGEL, U., AND SKERRA, B., *Angew. Chem.* **4,** 799 (1965).
24. ALLEN, C. F., HIRAYAMA, O., AND GOOCH, P., "Biochemistry of Chloroplasts" (Goodwin, T. W., ed.), Vol. I, p. 193. Academic Press, New York (1966).
25. BISHOP, N. I., LUMRY, R., AND SPIKES, J. D., *Arch. Biochem. Biophys.* **58,** 1 (1955).
26. FRANCK, J., AND FRENCH, C. S., *J. Gen. Physiol.* **25,** 309 (1941).
27. KAMEN, M. D., "Primary Process in Photosynthesis," Chapter 2. Academic Press, New York (1963).
28. OKAYAMA, S., *Plant Cell Physiol.* **5,** 145 (1964).
29. WEIER, T. E., STOCKING, C. R., AND SHUMWAY, L. K., *Brookhaven Symp.* **19,** 353 (1967).