UCRL-11215

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BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

September through November 1963

Berkeley, California

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UCRL-11215
UC-4 Chemistry
TID-4500(24th Ed.)

UNIVERSITY OF CALIFORNIA
Lawrence Radiation Laboratory
Berkeley, California

AEC Contract No. W-7405-eng-48

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Work done under the auspices of the
U. S. Atomic Energy Commission

Printed in USA. Price 75 cents. Available from the
Office of Technical Services
U. S. Department of Commerce
Washington 25, D.C.

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January 20, 1964

1. THE PURIFICATION OF CARBOXYDISMUTASE

P. W. Trown

Introduction

Attempts to purify the enzyme carboxydismutase with modern enzyme techniques were described in a previous quarterly report.[1] Although some purification was achieved, no homogeneous preparation was obtained and the problem of continuous deactivation of the enzyme during purification was not overcome. It was shown that the deactivation problem was in part due to the conditions in which the enzyme was kept, and a search was made for conditions under which spontaneous irreversible deactivation was at a minimum. It was found that reduced glutathione and Versenol (both at 10^{-3} M) offered considerable long-term protection of the enzyme against deactivation as did relatively high concentrations (up to 0.25 M) of ammonium sulfate. Similar high concentrations of sodium chloride deactivated the enzyme irreversibly. These reversible and irreversible deactivations, as well as the protection, have now been related to the anionic content of the salts causing them. In addition, potassium phosphate has been shown to behave in a similar way to ammonium sulfate. The nature of the reversible deactivation (or inhibition) of the enzyme has also been investigated.

When conditions were determined in which carboxydismutase was reasonably stable over a period of 1 week, further attempts were made to purify the enzyme. Although analytical data are not yet complete, it now appears that these attempts were successful.

Experimental Procedures and Results

Procedures for the isolation of partially purified carboxydismutase from spinach and for determination of protein concentration and carboxydismutase activity were as described previously.[1]

1. P. W. Trown, Bio-Organic Chemistry Quarterly Report, UCRL-10934, May 1963, p. 44.

The Relative Effects of Ammonium Sulfate, Ammonium Chloride, Sodium Sulfate, and Sodium Chloride upon the Long-Term Stability of Carboxydismutase

Carboxydismutase (0.2 mg/ml) was stored at 0° in solutions containing (a) 0.25 M $(NH_4)_2SO_4$, (b) 0.25 M NH_4Cl, (c) 0.25 M Na_2SO_4, (d) 0.25 M NaCl. Each solution also contained 0.01 M tris, pH 7.6; reduced glutathione, 10^{-3} M; and Versenol, 10^{-3} M. A control solution of the enzyme, containing only tris, glutathione, and Versenol, was also stored under the same conditions. After 24, 48, and 92 hr respectively, 100-µl samples were withdrawn and assayed as described previously.[1] After 92 hr 1-ml samples were withdrawn and dialyzed overnight at 4° against 0.01 M tris, pH 7.6, containing reduced glutathione, 10^{-3} M, and Versenol, 10^{-3} M. Samples of each of the five dialyzed and five undialyzed solutions were assayed 116 hr after the beginning of the experiment. Figure 1-1 shows the variation of carboxydismutase activity with time, and following dialysis. Since ammonium chloride and sodium chloride parallel each other in their effects on the enzyme, as do ammonium sulfate and sodium sulfate, it is evident that the effects observed are due to the anions of the respective salts.

Inhibition of Carboxydismutase by Sulfate Ion

The rate of fixation of CO_2 by carboxydismutase was measured at six different concentrations of ribulose-1, 5-diphosphate (RuDP) in the absence and presence of (a) 0.02 M $(NH_4)_2SO_4$ and (b) 0.1 M $(NH_4)SO_4$. Figure 1-2 shows a Lineweaver-Burk plot[2] of the results. The three lines appear to intersect on the 1/S axis at a negative value; this indicates that sulfate ion does not compete with RuDP for an active site on the enzyme. However, to demonstrate this unambiguously, more

2. H. Lineweaver and D. Burk, J. Am. Chem. Soc. 56, 658 (1934).

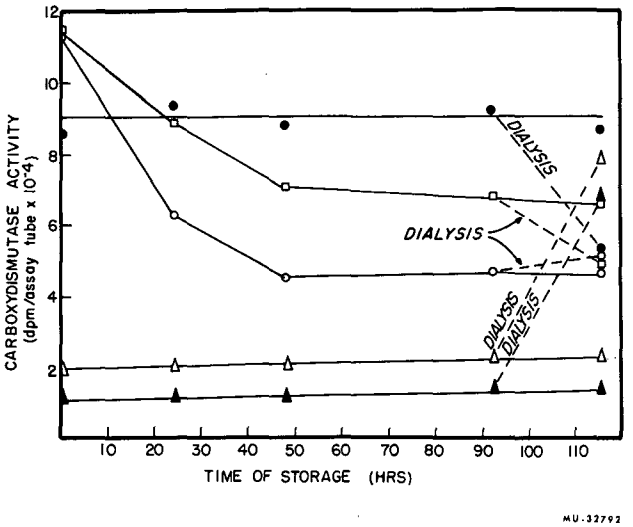

Fig. 1-1. Effects of ammonium sulfate, ammonium chloride, sodium sulfate, and sodium chloride on the stability and activity of carboxydismutase: ▲, 0.25 M $(NH_4)_2SO_4$; O, 0.25 M NH_4Cl; △, 0.25 M Na_2SO_4; □, 0.25 M NaCl; ●, control.

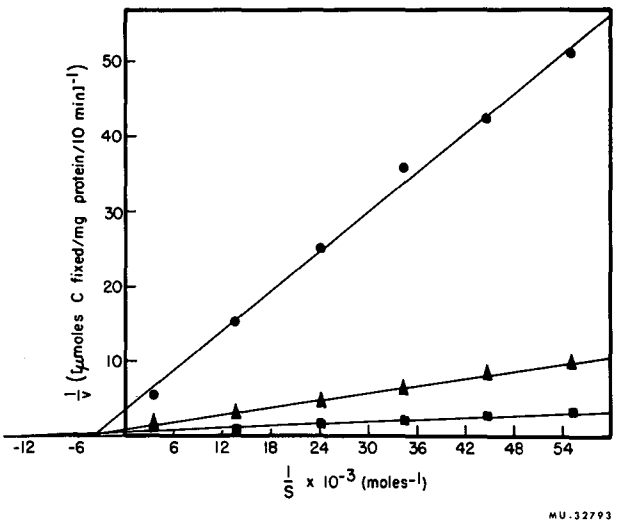

Fig. 1-2. Lineweaver-Burk plot for the inhibition of carboxydismutase by ammonium sulfate. V = reaction velocity (μmoles C fixed per mg protein per 10 min), S = concentration of RuDP (mM). ■, no ammonium sulfate; ▲, 0.02 M $(NH_4)_2SO_4$; ●, 0.1 M $(NH_4)_2SO_4$.

measurements would be required in the region where 1/S is small and S is therefore large. Unfortunately, as was shown by Weissbach, et al, and as is indicated for the lowest value of 1/S in the present experiment, high concentrations of RuDP are inhibitory for carboxydismutase.[3] Meaningful results cannot therefore be obtained in the crucial kinetic region.

The Effect of Potassium Phosphate Buffer at pH 7.6 upon the Stability and Activity of Carboxydismutase

Carboxydismutase (0.56 mg/ml) was stored at 0° in the presence of potassium phosphate buffer, pH 7.6 at (a) 0.2 M, (b) 0.05 M, and (c) 0.01 M. Each solution also contained 10^{-3} M tris, pH 7.6; reduced glutathione, 10^{-3} M; and Versenol, 10^{-3} M. A control solution containing only enzyme, tris, glutathione, and Versenol was also stored under the same conditions. At various times, samples were withdrawn and assayed for carboxydismutase activity. After 75 hr, samples (1 ml) were withdrawn and dialyzed against a solution containing 10^{-3} M tris, pH 7.6; reduced glutathione, 10^{-3} M; and Versenol, 10^{-3} M. After the experiment had been in progress for 95 hr, the four dialyzed and four undialyzed solutions were assayed. Figure 1-3 shows the variation of carboxydismutase activity with time, and following dialysis. Potassium phosphate at this pH behaved in a way similar to sulfate ion, causing reversible deactivation of the enzyme. However, in this experiment none of the dialyzed solutions that had contained potassium phosphate showed a higher activity than the control solution after dialysis. On this evidence it seems that potassium phosphate, while reversibly deactivating carboxydismutase, does not exert such a protective influence as does sulfate ion.

Inhibition of Carboxydismutase by Potassium Phosphate at pH 7.6

The rate of fixation of CO_2 by carboxydismutase was measured at six different concentrations of RuDP in the absence and presence of (a) 0.08 M and (b) 0.04 M potassium phosphate at pH 7.6. Figure 1-4 shows a Lineweaver-Burk plot of the results. As may be seen, a straight line is not obtained for 0.08 M potassium phosphate and that for 0.04 M potassium phosphate may also be slightly concave downwards. Such behavior is probably due to multiplicity of mode of attachment to, and of action of the inhibitor on, the enzyme. In such circumstances it is impossible to determine by this method whether the inhibition is competitive or noncompetitive. A possible explanation of these phenomena is that phosphate is able in some way to reverse the inhibition that occurs at high concentrations of RuDP.

3. A. Weissbach, B. L. Horecker, and J. Hurwitz, J. Biol. Chem. 218, 795 (1956).

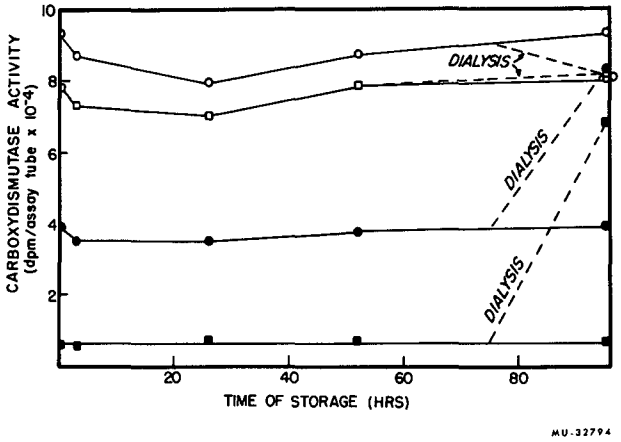

Fig. 1-3. Effect of potassium phosphate buffer at pH 7.6 on the stability and activity of carboxydismutase: O, control; □, 0.01 M phosphate; ●, 0.05 M phosphate; ■, 0.2 M phosphate.

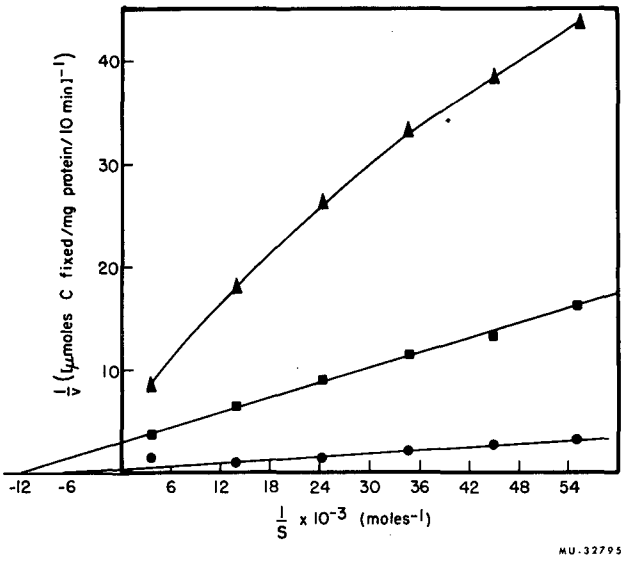

Fig. 1-4. Lineweaver-Burk plot for the inhibition of carboxydismutase by potassium phosphate buffer, pH 7.6. V = reaction velocity (μmoles C fixed per mg protein per 10 min), S = concentration of RuDP (mM). ●, no phosphate; ■, 0.04 M phosphate; ▲, 0.08 M phosphate.

The Purification of Carboxydismutase by Gel Filtration on Sephadex G-200

In a previous report, the partial purification of a crude preparation of carboxydismutase obtained by lysing spinach chloroplasts was described.[1] A careful fractional precipitation with ammonium sulfate produced material that had high specific activity but was inhomogeneous as shown by ultracentrifugation. It was not possible under the conditions used to purify this material further, either by gel filtration, zone precipitation, or ion-exchange chromatography, and still retain full activity. The stability of carboxydismutase in relatively high concentrations of $(NH_4)_2SO_4$, and the improved separation often attained in gel filtration by use of high-ionic-strength eluents, suggested that chromatography on Sephadex G-200 be tried again under conditions somewhat different from those previously described.[1]

Sephadex G-200 was equilibrated at room temperature for 48 hr with a solution containing 0.1 M tris, pH 7.6; 0.2 M $(NH_4)_2SO_4$; reduced glutathione 10^{-3} M; and Versenol 10^{-3} M. After removal of fine particles by sedimentation, the Sephadex was poured into a glass column and allowed to settle and form a gel bed 44×4.7 cm diam. The column ("G-200-1") was then transferred to the cold room (4°), where all subsequent operations were carried out. A solution of carboxydismutase derived from the 35 to 45% saturated $(NH_4)_2SO_4$ cut of a fractional $(NH_4)_2SO_4$ precipitation (see Ref. 1) was used in this experiment and was applied to the top of the gel bed. The solution (7.5 ml) contained approximately 500 mg of protein plus 0.1 M tris, pH 7.6; 0.2 M $(NH_4)_2SO_4$; reduced glutathione, 10^{-3} M; and Versenol, 10^{-3} M. Even application of the sample with a minimum of disturbance of the gel bed was achieved by passing the solution of protein through a glass capillary inserted through the rubber stopper at the top of the column and extending below the liquid level to a point just above the gel bed. The more dense protein solution formed a thin layer that rapidly passed into the column with very little mixing with the eluent standing above it. The column was eluted with a solution containing the same concentrations of tris, $(NH_4)_2SO_4$, glutathione, and Versenol as used in equilibration of the Sephadex. Four-ml fractions were collected and aliquots diluted for measurements of optical density and carboxydismutase activity. The results of these measurements are shown in Fig. 1-5.

The elution pattern (as determined from optical density measurements) showed two main components, which were almost completely separated; there was also a long "tail" (not shown in Fig. 1-5) of protein-like material with an O. D. of approximately 0.2 at 280 mμ. The second peak contained all the carboxydismutase activity, and the specific enzymic activity was constant from fractions 75 to 100. These fractions were combined, then precipitated by addition of $(NH_4)_2SO_4$, and the precipitate collected by centrifugation. It was then resuspended in 0.1 M tris, pH 7.6; reduced gluta-

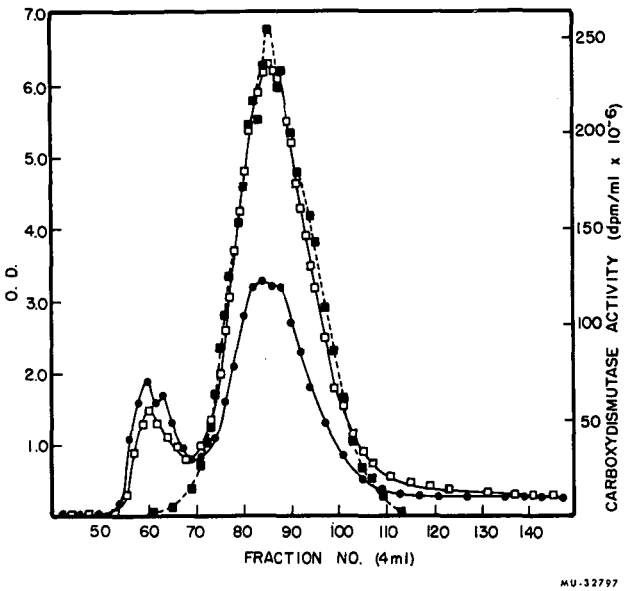

Fig. 1-5. Fractionation of carboxydismutase on Sephadex G-200: ●, O. D. at 260 mμ; □, O. D. at 280 mμ; ■, carboxydismutase activity [(dpm/ml)×10^{-6}].

thione, 10^{-3} M; and Versenol, 10^{-3} M; and dialyzed against the same solution overnight. A colorless solution of the enzyme (7.1 ml) was obtained which contained approximately 215 mg of protein, representing approximately 45% of the total protein applied to the column.

Most of the purified protein obtained from the first G-200 column (6.9 ml of the above solution) was applied a second time to the same column (now "G-200-2") and eluted again under the same conditions as those described above. Figure 1-6 shows the elution curve of this column. As may be seen, the hoped-for single peak, with optical densities and carboxydismutase activity closely parallel, was not obtained. Some uncertainties exist concerning the exact enzymic activities of all the fractions, since the several different dilutions made resulted in differing concentrations of sulfate ion, which is known to be an inhibitor (see above). Such a difficulty may be completely resolved only by dialyzing each individual fraction. Nevertheless, such uncertainties were taken into consideration, and the specific enzymic activity was reasonably constant for fractions 80-103. On the basis of the elution curve, the eluent from the column was divided as follows: fractions 47-57 combined (A); fractions 58-80 combined (B); fractions 81-103 combined (C). The protein in each portion (consisting of the combined fractions) was precipitated by addition of ammonium sulfate and stored in this form at 4° until further use.

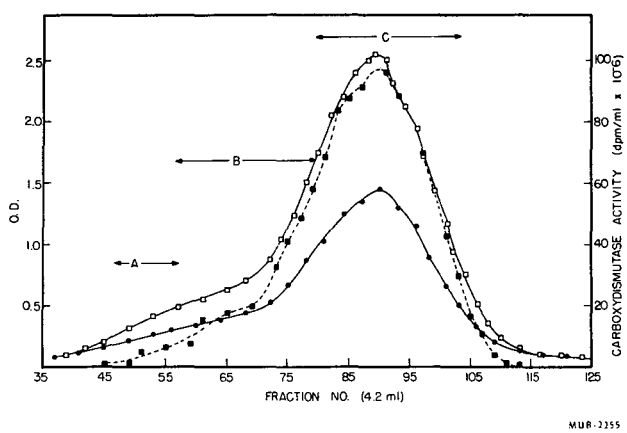

Fig. 1-6. Repeat fractionation of carboxydismutase on Sephadex G-200: ●, O. D. at 260 mμ; □, O. D. at 280 mμ; , carboxydismutase activity [(dpm/ml)×10^{-6}].

Ultracentrifugation of Material Obtained from Sephadex Column "G-200-2"

Small aliquots of each of the three portions (A, B, and C) from the second Sephadex column (G-200-2), described above, were studied in the ultracentrifuge as follows: A portion (approx 300 μl) of suspended precipitate of each portion was dialyzed separately overnight at 4° against 0.01 M tris, pH 7.6; reduced glutathione, 10^{-3} M; and Versenol, 10^{-3} M. Ammonium sulfate solution (3.0 M) was then added to bring its final concentration to 0.1 M. Each solution was studied in turn in the ultracentrifuge (Spinco Model E), by means of a double-sector cell with a filled-Epon centerpiece. All measurements were made at 4° C and 42 000 rpm, and photographs taken by means of the schlieren optics on the machine. Figure 1-7 shows the schlieren pattern obtained for portion C, 80 min after the ultracentrifuge reaches speed. With portions A and B, patterns were obtained which were similar to those of C except that small subsidiary peaks, presumably due to impurities, appeared on either side of the main peak. Sedimentation coefficients obtained from the photographs for the main peak in each of the portions were as follows: A, S_{obs} = 11.68; B, S_{obs} = 11.14; C, S_{obs} = 11.00 and 10.998 (two separate experiments).

Although a single peak in the schlieren pattern obtained upon sedimentation of a compound in the ultracentrifuge is a good indication of homogeneity, it is certainly not proof of that condition. A much more sensitive technique is that of sedimentation equilibrium, observed by the use of the Rayleigh interference optics incorporated in many Model E ultracentrifuges; this technique has recently gained popularity as a method of measuring molecular weight, because

of its potential for extremely high precision.[4] Thus an attempt was made with the dual purpose of measuring the molecular weight of carboxydismutase and testing the homogeneity of the sample obtained from the Sephadex column. Unfortunately, a series of mechanical failures in the Model E ultracentrifuge has prevented the determination of the molecular weight, but a useful test of homogeneity was possible. It appears that portion C from G-200-2 is an extremely pure sample of carboxydismutase, containing at most 5% impurities. Further information on this matter, as well as a determination of molecular weight, must await the perfect operation of the ultracentrifuge.

Summary

Carboxydismutase has been shown to be reversibly inhibited and also protected by sulfate ions, and to a lesser extent by phosphate at pH 7.6. The inhibition by sulfate is competitive with RuDP, but that by phosphate is complex and not subject to definition in competitive or noncompetitive terms.

Gel filtration on Sephadex G-200, with a high-ionic-strength eluent containing a high concentration of sulfate ion, as well as reduced glutathione and Versenol, led to a considerable purification of carboxydismutase. The resulting enzyme preparation was at least 95% pure and highly active.

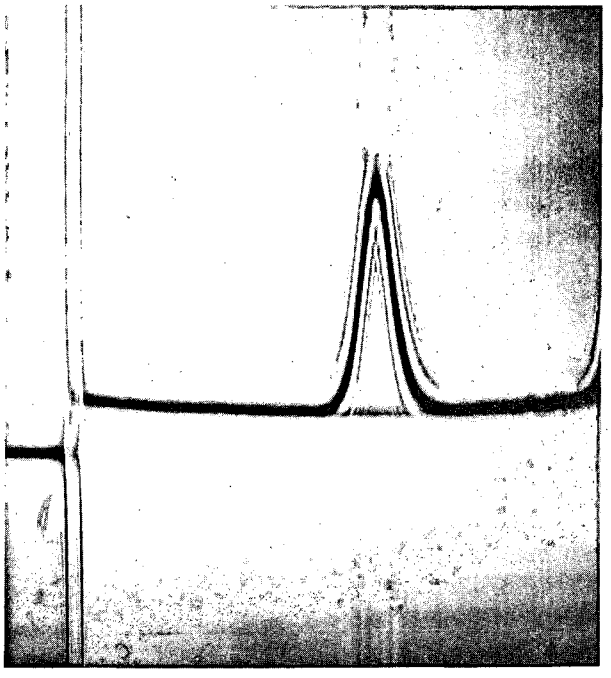

Fig. 1-7. Ultracentrifuge schlieren pattern of a preparation of carboxydismutase (portion C, Sephadex column G-200-2, see text). Rotor speed, 42 040 rpm; buffer, tris-sulfate (pH 7.6) containing $(NH_4)_2SO_4$, 0.1 M; time, 80 min after reaching speed; protein concentrations, approx 10 mg/ml. Sedimentation is from left to right.

4. H. K. Schachman, Biochemistry 2, 887 (1963).

2. A SEARCH FOR PHOTOINDUCED POLARIZABILITY CHANGES IN PHOTOSYNTHETIC AND MODEL SYSTEMS

Terry Trosper and Kenneth Sauer

Introduction

Excited electrons in a photosynthetic subunit would be expected to be more mobile than those in the ground state, and, therefore, cause an increase in the electronic polarizability of the excited unit over that in the ground state. This would be especially true if the photosynthetic unit behaved as a photovoltaic device involving charge separation and independent migration, as has been proposed by Calvin.[1,2] By experimental observation of a parameter dependent on the polarizability of the subunit, it may be possible to follow the formation of the excited state in the light.

We chose to look for changes in electronic polarizability α at optical frequencies by following light-scattering changes in a suspension of the sample. If the suspension of particles is assumed to be an ideal suspension of noninteracting macromolecules, the appropriate equation for unpolarized incident light scattered per particle or "molecule" is

$$\frac{i_\theta}{I_0} = \frac{2\pi^2 n_0^2 \left(\frac{dn}{dc}\right)^2 (1+\cos^2\theta)\, Mc}{N_A \lambda^4 r^2},$$

where I_0 is the incident intensity, i_θ is the intensity of light scattered per particle in the direction θ with respect to the incident beam, n_0 and n are the refractive indices of the solvent, and the suspension respectively, dn/dc the refractive increment of the suspension, c the concentration of the suspension, M the molecular weight of solute, N_A Avogadro's number, λ the wavelength of incident light, and r the distance from sample to detector.[3] But

$$\frac{4\pi N_A \alpha c}{M} = n^2 - n_0^2 \approx 2n_0 \frac{dn}{dc}\, c,$$

where α is the difference in polarizability between the solute and solvent.[3] Rearrangement gives

$$\frac{dn}{dc} = \frac{2\pi N_A \alpha}{n_0 M}\ ;\ \left(\frac{dn}{dc}\right)^2 = \frac{4\pi^2 N_A^2 \alpha^2}{n_0^2 M^2}.$$

Then by substitution we finally obtain

$$\frac{i_\theta}{I_0} = \frac{8\pi^4 (1+\cos^2\theta) c N_A \alpha^2}{\lambda^4 r^2 M}.$$

Any change in the polarizability of the particle will cause a change in α, and thus be reflected by changes in scattered-light intensity. Refractive index and dielectric constant are also molecular properties depending on α, but in both these cases the parameter is a bulk property of the suspension, and the contribution of the suspended particles would probably be exceedingly small. Light scattering, on the other hand, is a property primarily of the suspended particles. Contributions from the suspending medium, due principally to density fluctuations, are relatively quite small.

A suspension of bacterial chromatophore aggregates was studied. Lead sulfide and cadmium sulfide suspensions were chosen as model systems, since electrons in the excited state in these photoconductors are free to migrate throughout the crystalline structure.

Apparatus and Materials

Lyophilized bacterial chromatophore aggregates from Rhodospirillum rubrum were resuspended in deionized water and resonicated. Final concentration of the suspension was approximately (5.4×10^{-5}) gm per cm^3. The turbidity due to scattering, $\tau = -\ln (I_{tr}/I_0)$, where I_{tr}, the transmitted intensity, was 0.0198 at 630 mμ, calculated from optical density observed at 1100 mμ with a Cary Model 14 Spectrophotometer.

A lead sulfide suspension in 50% (vol/vol) aqueous glycerol was obtained by adding a few crystals of "Sulfidown" (thioacetamide, A. Daigger and Co., Chicago) to a solution of 10^{-3} M lead acetate in 50% (vol/vol) aqueous glycerol. The suspension was allowed to stand until it darkened (30 min). The supernatant taken from a clinical centrifugation did not precipitate PbS after standing several hours.

1. M. Calvin, Brookhaven Symp. Biol. 11, 160 (1959).

2. M. Calvin, J. Theoret. Biol. 1, 258 (1961).

3. C. Tanford, Physical Chemistry of Macromolecules (John Wiley and Sons, N. Y., 1961).

Solid powdered CdS (Baker and Adamson, New York), with 100% glycerol added, was ground with an agate mortar and pestle. The suspension obtained was stable for several days.

The apparatus used is illustrated in Fig. 2-1. The sample and photomultiplier (Dumont #6292) were placed in a lighttight box. The photomultiplier high voltage was obtained from a variable power supply operated at 1200 volts. The amplifier (Tektronix, type D plug-in unit) was adjusted for maximum sensitivity. The wave analyzer (Hewlett-Packard, model 302A) was tuned to the frequency at which the exciting light beam was chopped. A vacuum-tube voltmeter (Keithley model 220), with slow time constant (coax adaptor, model 2010), was used to monitor the wave analyzer output signal. In the chromatophore experiment, the signal was recorded with an x-y recorder.

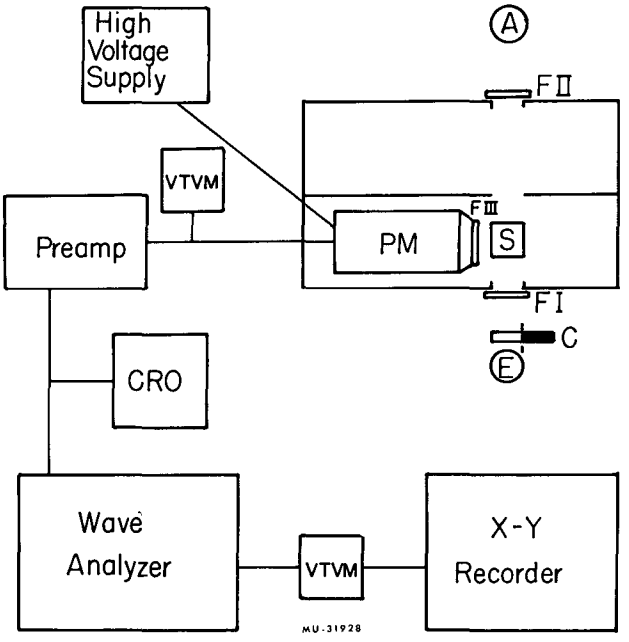

Fig. 2-1. Apparatus for measuring light-scattering changes. A, analyzing light source; E, exciting light source; C, mechanical chopper; S, sample cuvette; PM, photomultiplier; FI, II, III, filters used to separate analyzing and exciting light.

The intensity of exciting light reaching the sample cuvette was measured with a silicon photodiode (Hoffmann model 120C solar cell) and a millimicrovoltmeter (Keithley, model 149). The solar cell had been calibrated by means of a thermopile (Reedeer, model RBL-500) with a 300-W tungsten lamp (GE CZX) at 115 V used as a source. The light beam from this source was passed through a Bausch and Lomb monochromator. The thermopile was calibrated originally with standard Lamp #C-424 from the National Bureau of Standards. [4]

Method

Filter systems were selected in each case so only analyzing light would reach the photomultiplier, as shown in Table 2-I.

With the wave analyzer tuned to the chopping frequency, signals for four different situations were observed: (a) dark signal, (b) only analyzing light, (c) only exciting light, and (d) both analyzing and exciting lights. Since exciting light only was chopped, the apparatus was designed to detect only scattered light fluctuating at this frequency. Because of white noise in the analyzing light source and an imperfect filter system, cases (b) and (c), as well as case (a), gave a measurable signal on the wave analyzer. Thus the meaningful signal change was obtained by taking $\{(d) - [(b) + (c) - \sqrt{2}(a)]\}$; phase differences introduced by the circuit were disregarded.

Results

The experiment with chromatophore aggregates was carried out as a preliminary investigation only; on the recorder case (b) gave a negative signal, which indicated a polarity reversal in the circuit. The small change $[(d) - (c)]$ observed was 10^{-4} of the total light scattered. Unfortunately, the noise level of the wave analyzer was of the same magnitude as this small change. With the lack of adequate controls and repeated experiments, the observed change cannot be considered significant.

Neither photoconducting suspension gave a signal on the wave analyzer for case (d) that was significantly larger than the background signal $[(b) + (c) - \sqrt{2}(a)]$. The dc oscilloscope signal, representing the total scattered-light intensity, was 70 and 3400 mV for PbS and CdS respectively. The ac wave-analyzer signal for case (d), representing modulation of the total scattering at the tuned frequency, was 0.22 ± 0.16 and 0.7 ± 0.2 mV (rms) respectively for the two samples, whereas the background values were 0.15 ± 0.30 and 0.9 ± 0.6 mV (rms). Rapid large fluctuation of the needle made more accurate reading of the wave-analyzer scale impossible, so the ac noise level was of the same order of magnitude as the ac signal. Comparison of the ac and dc signals indicates that the modulation was less than 1 part in 370 for PbS and 1 part in 1500 for CdS. Therefore, any change in light scattering due to exciting light was no larger than these values.

4. Kenneth Sauer, in Bio-Organic Chemistry Quarterly Report, UCRL-10634, Jan. 1963, p. 23-41.

Table 2-I. Filter systems.

Chromatophore aggregates	A	500-W tungsten projection lamp (GE type CZX) run off regulated dc power supply; Bausch and Lomb monochromator set for 650 mμ.
	E	500-W tungsten bulb.
	FI	Interference filter, 430 mμ; Corning bandpass filters #4303 and #9782, no transmission between 600 and 1250 mμ.
	FII	Interference filter, 660 mμ, tilted to pass 650 mμ band.
	FIII	Interference filter, 649 mμ; Corning cutoff filter #2418, transmits wavelengths above 610 mμ.
	C	Motor-driven sector wheel, 150 cps chopping frequency.
PbS	A	Hg-arc lamp, pek 107, and regulated supply, Huggins Laboratories, model 951.
	E	500-watt tungsten bulb.
	FI	Interference filter, 631 mμ; Corning cutoff filter #2412, transmits wavelengths above 600 mμ.
	FII	Corning bandpass filter #5433, maximum transmission 430 mμ, cutoff filter #3389, transmits wavelengths above 400 mμ.
	FIII	Interference filter, 439 mμ; Corning bandpass filter #5433; infrared cutoff filter #5900.
	C	Motor-driven sector wheel, 25 and 50 cps chopping frequencies.
CdS	A	As for chromatophores, but monochromator selecting 639 mμ.
	E	Hg-arc lamp, pek 107, and regulated power supply, Huggins Laboratories, model 951.
	FI	Corning bandpass filters #5860 and #5840, maximum transmission at 360 mμ
	FII	Corning cutoff filter #2418.
	FIII	Interference filter 639 mμ; infrared cutoff filter #1-60; Corning cutoff filters #2418 and #2412.
	C	Motor-driven sector wheel, variable frequency, 0 to 250 cps.

Discussion

By the assumption of uniform particle size and an ideal suspension, the average molecular weight of the chromatophore aggregates was calculated from light scattering and spectrophotometric data[3] by substitution into the equations

$$\frac{Hc}{\tau} = \frac{1}{M}, \text{ and } H = \frac{16\pi}{3} \cdot \frac{2\pi n_0^2 \left|\frac{dn}{dc}\right|^2}{N_A \lambda^4} .$$

By rearrangement, the molecular weight is found to be

$$\frac{3N_A\lambda^4 \quad \tau}{32\pi^2 n_0^2 \left|\frac{dn}{dc}\right|^2 C} .$$

Now τ was found to be 0.0198 at λ = 630 mμ and a concentration of (5.4×10^{-5}) gm per cm^3, n_0 was 1.33, and N_A Avogadro's number. When the refractive increment was assumed to be the same for chromatophores as for quantasomes, dn/dc = 0.18 cm^3/gm was used.[4] The computation gave an average molecular weight of $M = 570\times10^6$ for the chromatophore aggregates. This value represents the weight of a lipoprotein membrane, roughly 10^{-9} cm^2 in area and 100 Å thick, which dimensions are reasonable for membranous bacterial fragments. The polarizability of a spherical dielectric chromatophore in water was calculated from the equation $4\pi N\alpha = 2n_0$ (dn/dc) c, where N is the number of solute particles per unit volume, $N = N_A c/M$.[3] Then $\alpha = n_0$ (dn/dc)$M/2\pi N_A$ and by substitution, $\alpha_{chr} = 3.6\times10^{-17}$ cm^3 is obtained.

The photoconducting particles were at the limit of resolution of the light microscope--i.e., their diameter was roughly less than or equal to 1000 Å. The polarizability of a dielectric sphere of radius r and dielectric constant ϵ_2 in a dielectric medium of dielectric constant ϵ_1 is given[5] by $\alpha = [(\epsilon_2 - \epsilon_1)/(2\epsilon_1 + \epsilon_2)]\ r^3$. At optical frequencies not coinciding with an absorption band, the dielectric constant may be replaced by the square of the refractive index, so $\alpha = [(n_2^2 - n_1^2)/(2n_1^2 + n_2^2)]\ r^3$. By substitution of $n_{PbS} = 3.91$, $n_{CdS} = 2.53$, $n_{gly} = 1.47$, and $n_{50\%\ gly} = 1.408$, values of $\alpha_{PbS} = (8.7\times10^{-17})cm^3$ and $\alpha_{CdS} = (4.9\times10^{-17})$ cm^3 were obtained.

In the light, the model particles become conducting spheres; the assumption was made that chromatophores with excited state, more mobile electrons were also conductors. The polarizability of a conducting sphere in a polar medium is $\alpha = r^3$ (Ref. 5). Substitution of r = 500 Å to obtain the polarizabilities of the three conducting particles gave $\alpha_{chr} = \alpha_{PbS} = \alpha_{CdS} = (1.25\times10^{-16})cm^3$.

In all three cases, these rough approximations imply that the ac signal observed with the wave analyzer should have been of the same order of magnitude as the dc signal observed on the oscilloscope. The absence of such a signal could have been due to several factors. For example, the steady-state concentration of excited particles might have been a very small fraction of the total number of scatterers in the sample, either because of nonsaturating light intensities or a very rapid decay time for excited states. (The lifetime of the lowest excited singlet state of chlorophyll in vivo is only (1.5×10^{-8}) seconds, determined by fluorescence decay.[6]) For the chromatophores, the intensity of exciting light reaching the sample cuvette was (5.1×10^{-5})watts/cm^2, at 430 mμ; and for the PbS and CdS suspensions it was (3.12×10^{-2})watts/cm^2 at 631 mμ and (4.6×10^{-4})watts/cm^2 at 365 mμ, respectively. The optical density of the chromatophores was calculated from spectrophotometric data to be 0.0158 at the exciting wavelength, so the intensity of absorbed light was (1.8×10^{-6})watts/cm^3. Assuming that the chromatophores were photoconducting particles in the light, the density of free carriers--in this case, the density of excited particles--was calculated[7] from the equation $n = f\tau$, where f is the number of excitations per second per unit volume and τ is the lifetime of the excited state. If the quantum efficiency of the process were unity, $f_{chr} = (1.8\times10^{-6})$watts/$cm^3$ = (3.9×10^{12})quanta/sec/cm^3. If Brody's data[6] were used for the excited-state chlorophyll, the lifetime, τ is approximately 1.5×10^{-8} sec, and there would have been (3.9×10^4) excited particles per cm^3 in the steady state. But the sample suspension had (5.7×10^{-13})particles per cm^3, based on the calculated molecular weight and measured density. Under these assumptions only 7×10^{-10} of the particles were excited at any time, and these would not have caused a large enough change in the polarizability of the suspension to be observed with the apparatus used. To the extent that the lifetime of free electrons or holes (or both) in excited chromatophores is longer than the fluorescence lifetime, the fraction excited under steady illumination will be correspondingly increased. Similar

5. C. J. F. Böttcher, Theory of Electric Polarization (Elsevier Publishing Company, New York, 1952).

6. S. S. Brody and E. Rabinowitch, Science 125, 555 (1957).

7. A. Rose, in Photoconductivity Conference, Atlantic City, N. J., 1954, Ed. by R. G. Breckenridge, (John Wiley and Sons, Inc., N. Y., 1956), p 1.

calculations for the photoconducting suspensions ($n \geq 10^{15}$ is required for conductivity, and $\tau_{max} = 10^{-5}$ sec,[8] so $f \geq 10^{20}$ would be necessary) indicated that only 1/1000 in PbS and 1/10 000 in CdS of the necessary exciting intensity was used. Again, it must be concluded that the fraction of excited particles in the suspension was too small to cause a noticeable effect.

8. R. P. Chasmar, ibid., p. 463.

The above calculations were based on rough estimates of size, and on assumptions of conductivity. The values quoted may easily be in error, so that actual differences in ground-state and excited-state particle polarizabilities would be much smaller.

The authors would like to thank Mr. I. D. Kuntz, Jr., for extensive help in using his apparatus for the light-scattering experiments.

3. THE RADIATION-INDUCED REACTION OF THYMINE WITH ETHANOL IN DEAERATED AQUEOUS SOLUTIONS

Joan Friedman

Benzophenone readily sensitizes carbonyl compounds to ultraviolet irradiation. In an effort to find similar sensitization to ionizing radiation, we tried irradiating thymine in the presence of benzophenone with γ rays. Since benzophenone is insoluble in water and thymine is only very slightly soluble in alcohol, the reaction was carried out in a mixed solvent of water and ethanol. The solutions studied were degassed to avoid the well-known formation of hydroperoxides, obtained when pyrimidines are irradiated in aerated aqueous solution. Under our experimental conditions, approximately 70% of the thymine initially present yielded two products, neither of which was the starting material, and the formation of these products was not dependent on the presence of benzophenone, but of ethanol. Further experiments, in which ^{14}C-labeled ethanol and unlabeled thymine were used, showed that both products contained the ethanol moiety as well as the thymine moiety. These Products were further characterized by their uv absorption spectra; but, as yet, no assignment of structure can be made.

Experimental Procedures

A. Irradiation of Thymine-^{14}C with Ethanol in the Presence of Benzophenone

Five λ of a thymine-2-^{14}C solution (New England Nuclear, 0.10 mC and 0.475 mg in 1 ml of sterile water) was added to 500 λ of a "cold" thymine solution (Nutritional Biochemicals Corp., 1.020 mg/ml) and 5 λ of benzophenone in ethanol (1.81 mg/ml) in a small test tube with a constricted neck. The solution was degassed on a vacuum line by repeated freeze-pump-thaw cycles. The tube was then sealed under vacuum and irradiated in the Co^{60} source for 107 min (6.65×10^6 rads/hr, total dose 11.9×10^6 rads). After irradiation, an aliquot, usually 100 λ, was chromatographed on paper, with propanol-ammonia-water as the first solvent and butanol-propionic-acid-water as the second. [1] x-Ray films were put in contact with the chromatograms for 2 days and then developed. Two other experiments were run in a similar fashion, except that the thymine was labeled with ^{14}C in the methyl group. Also, in one case, 35 λ of benzophenone in ethanol was used instead.

B. Irradiation of Thymine-^{14}C with Ethanol in Water

As a control, four samples of thymine were irradiated in EtOH-H_2O solutions (no benzophenone present). The first sample contained 35 λ of ethanol, and thymine-2-^{14}C was used. Thymine-methyl-^{14}C and 5 λ of ethanol were used in the other three samples. The last sample received 7 min more irradiation (7.75×10^5 rads) than the others. But in all other respects, the sample preparations and irradiations were carried out as described in A.

C. Irradiation of Thymine-^{14}C in Water Alone

Three experiments were carried out as above, with ethanol omitted. In one, thymine-2-^{14}C was used; in the other two, thymine-methyl-^{14}C was used.

D. Irradiation of Thymine with Ethanol-1-^{14}C

Five hundred λ of "cold" thymine solution (described above) was added to 8.77 λ of ethanol-1-^{14}C (316 μC/ml and 0.391 μC/mg). Two 200-λ aliquots were withdrawn and irradiated as described in A.

E. Ultraviolet Spectra of Products

The two major products of the reaction of thymine with ethanol were eluted from a paper chromatogram with water. An adjacent blank area was eluted also with water and served as the reference in the recording spectrophotometer (Cary Model 11).

Results and Discussion

As can be seen in Table 3-I, the presence of benzophenone in the irradiation mixture does not influence the products, nor does the position of the carbon label. Of the activity not accounted for by compounds A and B (for the experiments in which ethanol was present), approximately 25% represents volatile products and the remainder, minor products with smaller Rf values than compounds A and B. When thymine was irradiated in the absence of ethanol, approximately 28% of the activity was absent from

1. Joan Friedman, Bio-Organic Chemistry Quarterly Report, UCRL-11046, November 1963.

Table 3-I. Formation of labeled products, A and B, after γ irradiation (11.9 Mrads) of various thymine-ethanol-benzophenone mixtures (water was present in all experiments).

Experiment	Thymine (μM)	Ethanol (μM)	Thymine left after irradiation (μM)	Original ^{14}C in A (%)	Original ^{14}C in B (%)
I. Thymine-^{14}C with benzophenone in ethanol					
1	4.06	85.6	0	30.3	36.7
2	4.06	599.2	0	32.2	42.3
3	4.06	84.0	0	25.4	36.6
II. Thymine-^{14}C with ethanol					
1	4.06	599.2	0	35.2	49.1
2	4.06	84.0	0	25.1	38.6
3	4.13	85.6	0	25.1	38.9
4[a]	4.13	85.6	0	26.6	29.3
III. Thymine-^{14}C in water alone					
1	4.06	0	2.28	0	0
2	4.13	0	0.82	0	0
3	4.13	0	0.70	0	0
IV. Thymine with ethanol-^{14}C					
1	4.05	150.0	--[c]	2.54[b] (or 3.82 μM)	1.77[b] (or 2.65 μM)
2	4.05	150.0	--[c]	2.68[b] (or 4.00 μM)	1.52[b] (or 2.28 μM)

a. This sample inadvertently received 6.5% more radiation (total 12.7 Mrads) than any of the other samples.

b. Note that this percentage is based on the ethanol activity.

c. Not applicable.

the paper chromatograms from the second and third trials, but essentially all the activity remained on the paper in the first trial. This discrepancy–between (a) the first trial and (b) the second and third–in the amount of activity volatilized and the amount of starting material remaining after irradiation cannot as yet be explained.

The Rf values of spots A, B, and thymine in the solvents used are as follows (averages from all experiments with maximum deviations):

Compound	Rf in prop-ammonia	Rf in bu-prop
A	0.90 ± 0.03	0.89 ± 0.06
B	0.82 ± 0.02	0.75 ± 0.02
Thymine	0.75 ± 0.05	0.75 ± 0.04

Because of the similarity in Rf values of thymine and compound B compound B was eluted from one of the papers and cochromatographed with approximately 100 γ of thymine. The thymine was visible as a uv-absorbing spot, and compound B was located by radioautography. Because the two spots did not coincide, compound B is not thymine. This is also shown by the presence of the radioactive ethanol moiety in spot B.

The uv spectrum of compound A shows a maximum at about 265 mμ, a minimum at about 250 mμ. The spectrum of compound B shows a maximum at about 270 mμ, a minimum at about 253 mμ.

Of importance to note in this reaction of thymine with ethanol is the high yield of compounds A and B, and the fact that no thymine remains unreacted. Reactions that produce such results are rare in radiation chemistry.

4. SOME CHEMICAL ATTEMPTS TO SUPPORT AN OXIDATIVE PHOSPHORYLATION MECHANISM

Peter M. Scott

Vilkas and Lederer have proposed a mechanism of oxidative phosphorylation (Fig. 4-1); they consider the ring opening of a chromanyl phosphate (I) to a hydroquinone phosphate (II), which can then be oxidized to a quinone (III).[1] Phosphate, according to their hypothesis, would then be reincorporated by addition to the is isomeric quinone methine (IV).

If a chromanyl phosphate were able to exist in equilibrium with a hydroquinone phosphate, it would be possible to detect this equilibrium by looking for deuterium exchange in the 3 position of the heterocyclic ring, if a suitable deuterated solvent were used. No such exchange was observed, however, in the nuclear-magnetic-resonance (NMR) spectrum of 2, 2, 5, 7, 8-pentamethylchroman-6-yl phosphate (I: R = R' = CH_3) (or 2, 2, 5, 7, 8-pentamethylchroman-6-ol) in acidic deuteromethanol (CH_3OD). Similarly there was no exchange with the di-sodium salt of the chromanyl phosphate in D_2O at pH 8.5 (approx).[2]

Another step in the hypothesis--the isomerization of the methyl quinone to the corresponding quinone methine--should also be detectable (if there is an equilibrium) by deuterium exchange at the methyl group. The recently reported[3] dimerization (in 15% yield) of vitamin $K_{1(20)}$ (V) under acidic conditions is, from consideration of the proposed structure of the dimer, taken as evidence for the formation of a quinone methine (IV) as intermediate. However, an attempt to incorporate deuterium into the nuclear methyl groups of 2, 5, 6-trimethyl-3-(3-methylbut-2-enyl)-1, 4-benzoquinone (III: R = R' = CH_3) in acidic deuteromethanol was unsuccessful (this quinone was used as a model for ease of study by NMR). There was no exchange observed with duroquinone (tetramethyl-1, 4-benzoquinone) either.

Under alkaline conditions, with deuteromethanol as solvent, deuterium exchange at the nuclear methyl groups was rapid with both quinones, indicating equilibria are established with quinone methine forms. In the isoprenoid quinone (III: R = R' = CH_3), it would appear that ring closure did not take place to any great extent during quinone methine formation under these conditions, since the NMR signal due to the =CH- proton had not markedly diminished in the time the peak due to the nuclear $-CH_3$ protons had virtually disappeared owing to exchange. Hence the quinone methine (VII), analogous to the quinone methine (VI) from duroquinone, may be expected to be in equilibrium with 2, 5, 6-trimethyl-3-(3-methylbut-2-enyl)-1, 4-benzoquinone under alkaline conditions.

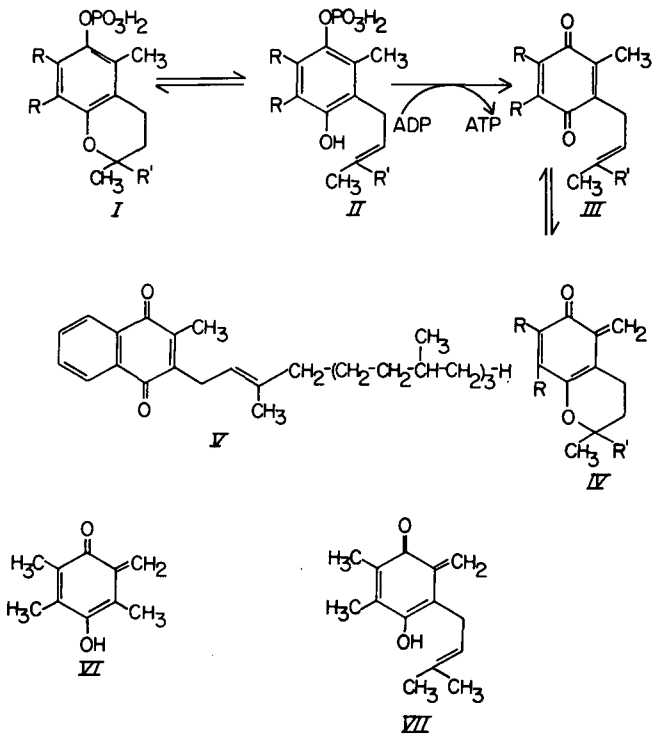

Fig. 4-1. Part of Vilkas and Lederer's scheme for oxidative phosphorylation, with chromanyl phosphate (I), hydroquinone phosphate (II), isoprenoid quinone (III), and quinone methine (IV). Formulae of vitamin $K_{1(20)}$ (V) and quinone methines (VI, VII).

1. M. Vilkas and E. Lederer, Experientia 18, 546 (1962).

2. Peter M. Scott, in Bio-Organic Chemistry Quarterly Report, UCRL-10934, July 1963, p. 57.

3. P. Mamont, R. Azerad, P. Cohen, M. Vilkas, and E. Lederer, Compt. Rend. 257, 706 (1963).

Experimental Procedures

Nuclear magnetic resonance spectra were run with a spectrometer (Varian A-60) at room temperature, with sample spinning and $Si(CH_3)_4$ as internal reference; peak positions are in τ units[4] with the number of protons involved shown in parentheses.

2, 2, 5, 7, 8-Pentamethylchroman-6-ol

The NMR spectrum (150 mg in 1.0 ml CH_3OD) had peaks at τ = 7.31, 7.42, 7.53 (2); 7.85, 7.89, 7.92 (9); 8.16, 8.27, 8.38 (2); 8.75 (6). The solution was made 0.02 N with respect to H_2SO_4, by addition of one drop of a solution containing two drops of concentrated H_2SO_4 per ml CH_3OD. No change in the peak heights or positions was observed after 3 hr.

2, 2, 5, 7, 8-Pentamethylchroman-6-yl phosphate (I: R = R' = CH_3)

The compound was prepared as described in a previous report,[2] λ_{max} (95% ethanol) 285 (ϵ = 2160), 280 (shoulder, ϵ = 1880) mμ. The NMR spectrum (120 mg in 1.0 ml CH_3OD) had peaks at τ = 7.25, 7.36, 7.48 (2); 7.80, 7.92 (9); 8.10, 8.21, 8.33 (2); 8.72 (6). The solution was made 0.03 N with respect to H_2SO_4. After 3 hr (including 1/4-hr uv irradiation in a quartz tube) the spectrum was unchanged, with no diminution in peak heights, including the group at τ = 8.21 which is due to the methylene protons in the 3 position.

2, 5, 6-Trimethyl-3-(3-methylbut-2-enyl)-1, 4-benzoquinone (III: R = R' = CH_3)

Trimethylhydroquinone (0.9 g), 2-methyl-3-buten-2-ol (1.0 g), fused zinc chloride (0.5 g), acetic acid (0.1 ml), and ether (25 ml) were refluxed for 6.5 hr, the solution washed with water and dried, and the ether removed. The mixture of products was redissolved in dry ether (120 ml) containing sodium sulfate (1 g), and oxidized by shaking with freshly prepared silver oxide (6.8 g) for 1 hr. After filtration and removal of the ether, chromatography on basic alumina (Merck) and elution of the first yellow band with benzene–petrol ether (bp 30 to 60°) (5:7, 200 ml) gave the quinone (0.60 g, 46%) as a yellow liquid (found, on a sample purified by chromatography on silica gel: C, 77.0; H, 8.2. $C_{14}H_{18}O_2$ requires C, 77.0; H, 8.3%), λ_{max} (95% ethanol): 259, 266 ($\epsilon = 1.72 \times 10^4$), 310 ($\epsilon$ = 554) mμ, IR ν_{max}: 1645 cm^{-1}. The compound ran as one spot on alumina-impregnated paper (Schleicher and Schüll 288) with Rf = 0.75 (benzene-cyclohexane, 1:1). It did not decompose on basic alumina.

The NMR spectrum (CH_3OD) had peaks at τ = 4.94, 5.07, 5.19 (1); 6.80, 6.90 (2); 8.01 (9); 8.25 (3); 8.32, 8.33 (3). The solution was made 0.02 N with respect to H_2SO_4. The spectrum, in particular the height of the $-CH_3$ peak at τ = 8.01, remained exactly the same after 30 min. Another solution (58 mg quinone in 0.7 ml CH_3OD) was made 0.014 N with respect to NaOH by addition of 0.01 ml N NaOH in D_2O. The peak at τ = 8.01 virtually disappeared after 15 min, whereas the peaks at τ = 8.25 to 8.33 remained unchanged in height at that time, though after 1 hr they were beginning to get smaller and broader. The group centered at τ = 5.07 (=CH-), though difficult to observe, did not undergo marked reduction in height in the 15 min, but had disappeared after 1 hr.

Duroquinone

A solution of the quinone (20 mg) in CH_3OD (0.5 ml) and CCl_4 (0.2 ml) was made 0.05 N with respect to H_2SO_4. The height of the $-CH_3$ peak at τ = 8.0 in the NMR spectrum was unchanged after 1 hr. On addition of 0.01 ml N NaOH in D_2O to a solution of the quinone (12 mg) in CH_3OD (0.7 ml), however, the quinone-CH_3 peak lost about 60% of its area (and about 95% of its height) in 8 min.

4. G. V. D. Tiers, J. Phys. Chem. 62, 1151 (1958).

5. CHANGES IN CONCENTRATIONS OF ^{14}C-LABELED COMPOUNDS IN PHOTOSYNTHESIZING CHLORELLA CAUSED BY ADDITION OF IODOACETIC ACID

Roger Hiller and J. A. Bassham

In previous studies it was found that 8-methyl lipoic acid and lipoic acid, when suddenly added to a suspension of Chlorella pyrenoidosa that was photosynthesizing in the presence of $^{14}CO_2$, caused sudden and large changes in the concentrations of intermediate compounds of the photosynthetic carbon-reduction cycle.[1] The concentration of 3-phosphoglyceric acid (PGA) rapidly increased, similarly to the light-dark transient,[2,3,4] but the concentration of ribulose-1, 5-diphosphate (RuDP) did not go to zero as it had in the light-dark transient. At the same time, the added disulfide compounds caused rapid positive transients in the concentrations of fructose diphosphate and sedoheptulose diphosphate--a pair of transient effects not previously observed.

In order to discover the mechanism by which these effects are produced by lipoic and methyl lipoic acids, we have undertaken studies of the effects of two other classes of inhibitors. Studies with one class of inhibitors that interfere with the photochemical production of ATP (adenosine triphosphate) and of reduced cofactors such as $NADPH_2$ are reported elsewhere in this Quarterly Report.[5] In general, the effects of such inhibitors have been very similar to the effects of turning off the light (a rise in PGA concentration, a fall in concentrations of RuDP and fructose diphosphate) and hence are quite different from the effects of the disulfide compounds. Thus it appears that the effects of these lipoic acids are not duplicated by an interruption in the photochemical production of cofactors used of the carbon-reduction cycle.

The second class of inhibitors that might reproduce the effects of the lipoic acids would comprise those that are known to act through their reaction with sulfhydryl groups of enzymes. Iodoacetic acid (IAA) was chosen as a representative of this class. Previous studies with this inhibitor on the ^{14}C fixation in photosynthesis have been reported.[6,7,8,9] It has been suggested that the primary site of inhibition in the carbon-reduction cycle is not triose-phosphate-dehydrogenase, but phosphoribulokinase,[7,8] or some undefined reaction involving the direct reduction of the carboxylation product of RuDP to the hexose level.[9]

Preliminary experiments on the inhibition of photosynthetic gaseous exchange by IAA showed that, at a concentration of 5×10^{-4} M, a marked inhibition of both CO_2 uptake and O_2 output was observable between 2 and 4 min after addition of the inhibitor.

Subsequently, the effect of IAA on the levels of ^{14}C in the carbon-reduction cycle was investigated with the steady-state apparatus.[4] Algae cells (70 ml buffer containing 2% vol/vol packed Chlorella cells) were exposed to ^{14}C for 12 min, at which time the inhibitor (final conc 4×10^{-4} M) was added. Killing, extraction, and analysis of results were as described previously.[1,4] The results are shown in Figs. 5-1 through 5-4.

Those compounds more oxidized than PGA, on or associated with the glycolytic pathway or TCA cycle, continued to increase initially in radioactivity at rates similar to those before the addition of IAA. On the other hand, compounds of the carbon-reduction cycle show a sudden decrease. PGA increases initially, then falls to a level above that of its previous steady state. RuDP maintains its steady-state level longer than any other major cycle intermediate. These results show clearly that in the present work the major site of inhibition of photosynthesis is triose-phosphate dehydrogenase.

1. J. A. Bassham, Horst Egeter, and Frances Edmonston, Biochem. Biophys. Res. Commun. 13, 144 (1963).

2. M. Calvin and P. Massini, Experientia 8, 445 (1952).

3. J. A. Bassham, K. Shibata, K. Steenberg, J. Bourdon, and M. Calvin, J. Am. Chem. Soc. 78, 4120 (1956).

4. J. A. Bassham and M. Kirk, in Microalgae and Photosynthetic Bacteria, Ed. by Japanese Soc. Plant Physiologists (University of Tokyo Press, Tokyo, Japan 1963), p. 493.

5. Edwin S. Gould, J. A. Bassham, and M. Kirk, Section 7, this Quarterly Report.

6. William Stepka, Path of Carbon in Photosynthesis (Thesis), University of California, 1950.

7. A. V. Trebst, M. Losada, and D. I. Arnon, J. Biol. Chem. 235, 840 (1960).

8. N. Calo and M. Gibbs, Z. Naturforsch. 15b, 287 (1960).

9. O. Kandler and I. Liesenkotter, Vth International Biochemistry Congress, Vol. VI, Moscow, 1961.

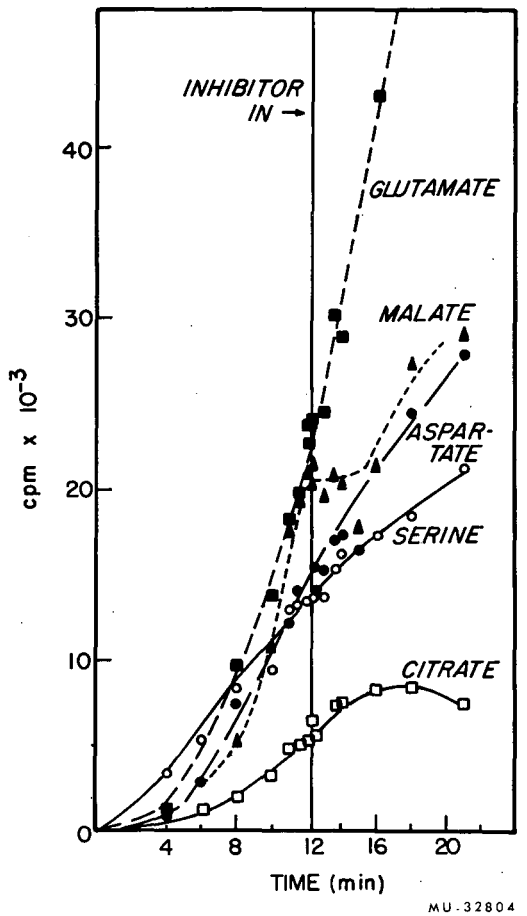

Fig. 5-1. Changes in ^{14}C level in compounds of or associated with the glycolytic pathway and tricarboxylic acid cycle on addition of 4×10^{-4} M iodoacetic acid to photosynthesizing Chlorella.

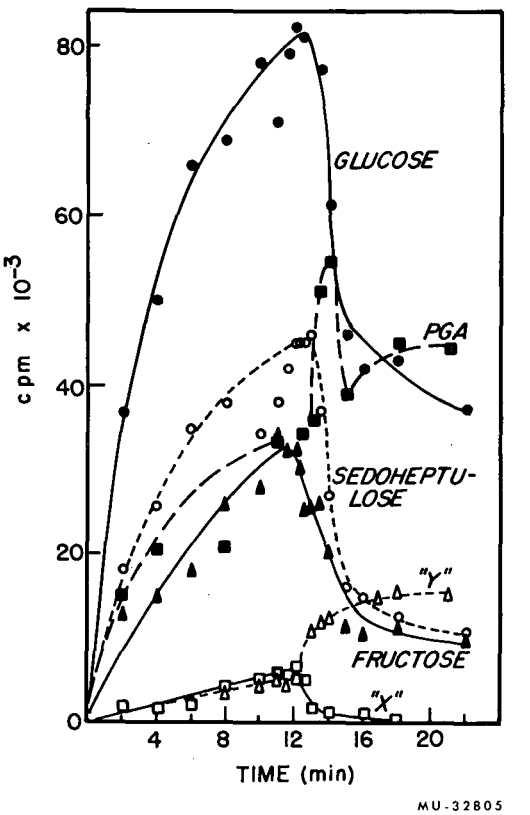

Fig. 5-2. Changes in ^{14}C level in PGA and sugar monophosphates on addition of 4×10^{-4} M iodoacetic acid to photosynthesizing Chlorella.

There is no evidence for an inhibition of either carboxydismutase or ribulokinase. Inhibition of sucrose labeling may be explained by the block in fructose phosphate production; glucose phosphate declines in activity as it is metabolized to polysaccharide.

The compounds "X" and "Y" were obtained from the combined monophosphate areas. They represent discrete spots obtained after phosphatasing with purified polydase, and in both phenol and butanol–propionic acid ran much closer to the origin than the free sugars. On addition of the inhibitor, "X" behaves kinetically, as does sugar phosphate, whereas "Y" rises similarly to PGA. Further tests are being conducted to determine the identity of "X" and "Y."

In the present work, the only observable site of carbon-cycle inhibition is triose-phosphate dehydrogenase. The continued production of RuDP, subsequent to addition of the inhibitor, points to continued photosynthetic ATP production. This may explain previously puzzling observations that the photosynthetic uptake of serine and its conversion to sucrose[10] and photosynthetic Cl^{36} uptake[11] are both stimulated by iodoacetamide. Thus, CO_2 fixation competes for ATP and reducing power with other reactions of the above type. If this hypothesis is true, it should be possible to restore to some extent photosynthetic O_2 production in the presence of IAA by addition of non-phosphorylated compounds more oxidized than PGA.

10. C. P. Whittingham, R. G. Hiller, and M. Bermingham, National Academy of Science Symposium on Photosynthetic Mechanisms in Green Plants, Airlie, Virginia, 1963 (in press).

11. P. T. Nielsen, Plant Physiol. 38, Supp. IV (1963).

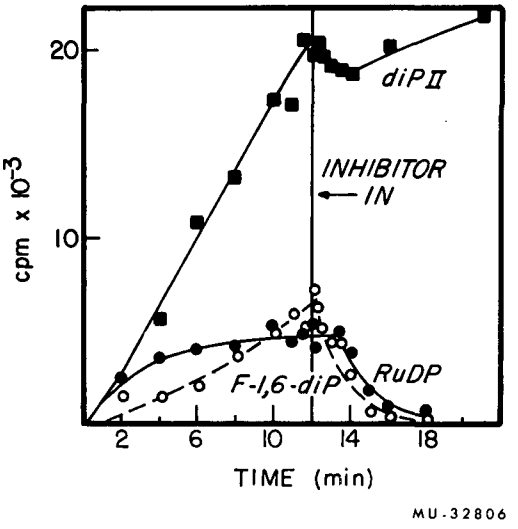

Fig. 5-3. Changes in ^{14}C level in sugar diphosphates on addition of 4×10^{-4} M iodoacetic acid to photosynthesizing Chlorella.

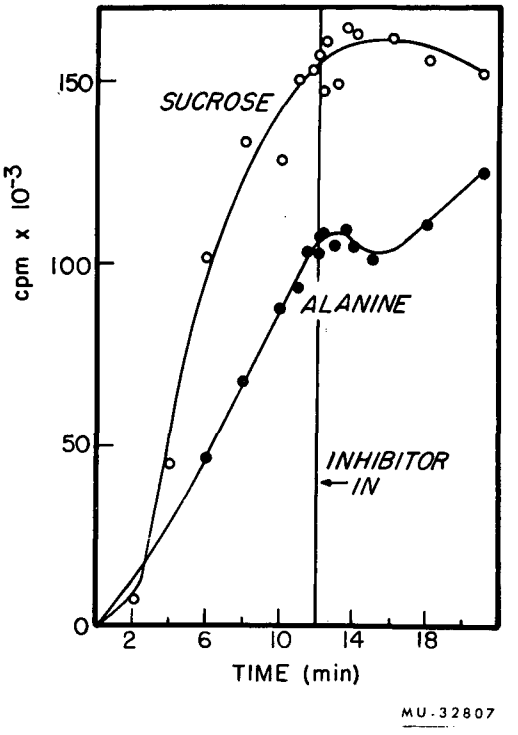

Fig. 5-4. Changes in ^{14}C level in sucrose and alanine on addition of 4×10^{-4} M iodoacetic acid to photosynthesizing Chlorella.

6. THE EFFECTS OF LIPOIC ACID ON THE REDUCTION OF CARBON DIOXIDE DURING PHOTOSYNTHESIS

J. A. Bassham, Frances Edmonston, and Martha Kirk

We have previously reported the inhibition of photosynthesis and the transient changes in the pattern of ^{14}C-labeled compounds formed during photosynthesis with $^{14}CO_2$ as a result of the addition of 8-methyl lipoic acid to photosynthesizing Chlorella pyrenoidosa. [1] It was also noted in that report that the same levels of lipoic acid caused comparable inhibition of carbon dioxide uptake and oxygen evolution during photosynthesis. We have now completed the analysis of the transient changes in the levels of ^{14}C-labeled compounds caused by the addition of lipoic acid to photosynthesizing Chlorella.

The conditions used in this current experiment and the methods of analysis are the same as those described in the previous report. [1] In particular, after 8 min of steady-state photosynthesis in the presence of $^{14}CO_2$ of constant specific radioactivity, 10 mg of lipoic acid in 200 μl of ethanol was injected into an 80-ml suspension of photosynthesizing algae. Numerous samples of the algae were taken before and after the addition of the inhibitor; they were killed in 80% methanol and subsequently analyzed by two-dimensional paper chromatography and radioautography. [1, 2] The amount of carbon-14 in each compound was determined by means of the automatic spot counter. [3]

The levels of labeled intermediates of the carbon-reduction cycle, shown both before and after addition of the inhibitor to the photosynthesizing cells, are graphed in Figs. 6-1, 6-2, and 6-3. In general, the effects are quite similar to those observed in the case of 8-methyl lipoic acid. Thus, it may be concluded that the inhibitor is acting through its disulfide function and not through competitive inhibition by a natural cofactor analog (8-methyl lipoic acid).

In the present study, however, the inhibiting effects are even more pronounced than they were with 8-methyl lipoic acid. The remarkably sudden drop in the level of 3-phosphoglyceric acid (PGA) and the initial rise in the level of ribulose diphosphate suggests more strongly than in the previous experiment that the inhibitor stops the carboxylation of ribulose diphosphate. [2] Later, the level of ribulose diphosphate falls rapidly, which indicates that the formation of this compound is also blocked in

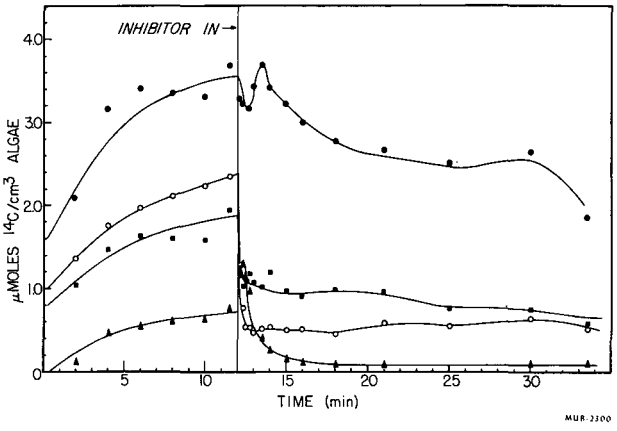

Fig. 6-1. Effects of lipoic acid on the levels of sugar monophosphates (and of ribulose diphosphate) of the carbon-reduction cycle. ●, glucose monophosphate; O, sedoheptulose monophosphate; ■, fructose monophosphate; ▲, ribulose diphosphate.

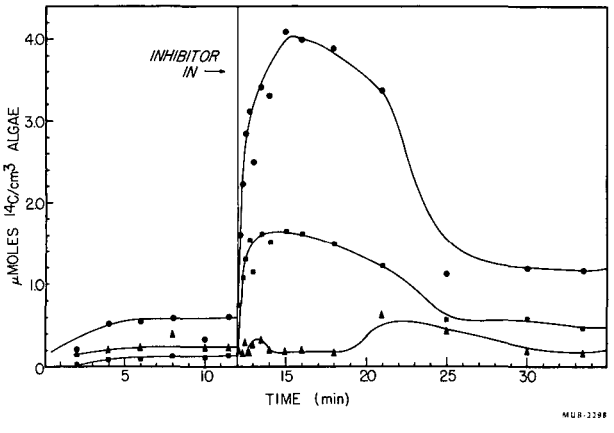

Fig. 6-2. Effects of lipoic acid on the levels of sugar diphosphates of the carbon-reduction cycle. ●, fructose diphosphate; ■, sedoheptulose diphosphate; ▲, glucose diphosphate.

1. J. A. Bassham, Horst Egeter, Frances Edmonston, and Martha Kirk, Biochem. Biophys. Res. Commun. 13, 144 (1963).

2. J. A. Bassham and M. Calvin, The Path of Carbon in Photosynthesis, (Prentice-Hall, Englewood Cliffs, N. J., 1957).

3. V. Moses and K. K. Lonberg-Holm, Analyt. Biochem. 5, 11 (1963).

some way by the inhibitor. The previously observed increases in sedoheptulose and fructose diphosphates and corresponding decreases in the monophosphates of those sugars are in this experiment more pronounced.

As yet, no unique explanation of these several effects can be given. Since the preceding report we have made several studies of the effects of other inhibitors on the levels of intermediates of the carbon cycle. These are reported in Sec. 5 of this Quarterly. One of these inhibitors, iodoacetic acid,[4] produced a rapid increase in phosphoglyceric acid and a rapid decrease in the levels of other sugar phosphates; these actions would be characteristic of an inhibition of the reduction of PGA to sugar phosphates by triose phosphate dehydrogenase. This enzyme is known to be inhibited by substances such as iodoacetic acid, which react with sulfhydryl groups. The iodoacetic acid effects are quite different from the effects of the lipoic acid, and suggest therefore that the lipoic acid effect must be specific to disulfhydryl reagents and must produce a different type of inhibition.

4. Roger Hiller and J. A. Bassham, this Quarterly Report.

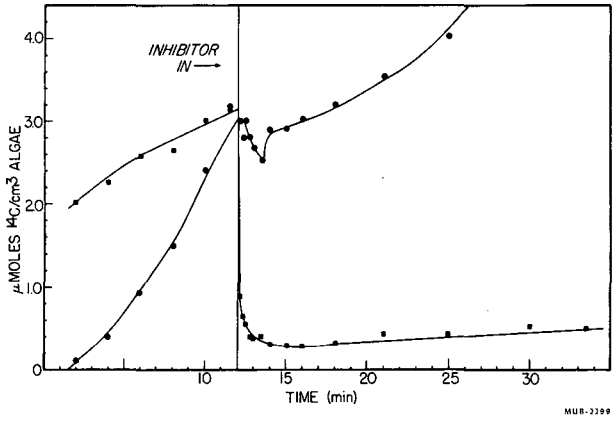

Fig. 6-3. Effects of lipoic acid on the levels of PGA and sucrose of the carbon-reduction cycle. ●, PGA; ■, sucrose.

Another class of inhibitors studied[5] comprises those which interfere with the primary photochemical production of ATP and reduced cofactors. Again the effects were very different from those of methyl lipoic and lipoic acids and resembled the effects of turning off the light, which produces an increase in PGA and a decrease in the levels of sugar phosphates. Unpublished results by Trown[6] showed no effect of methyl lipoic acid on the activity of the isolated carboxydismutase reaction--namely, the carboxylation of ribulose diphosphate to make two molecules of PGA.

Thus, the effects of methyl lipoic and lipoic acids cannot as yet be related to effects of other known inhibitors. These disulfide acids appear to produce one or more unique and specific inhibitions of reactions of the carbon-reduction cycle.

5. Edwin S. Gould, J. A. Bassham, and Martha Kirk, this Quarterly Report.

6. P. W. Trown (Lawrence Radiation Laboratory) (private communication).

7. INHIBITOR STUDIES ON THE CARBON-REDUCTION CYCLE IN CHLORELLA

Edwin S. Gould, J. A. Bassham, and Martha Kirk

Although the general outline of the photosynthetic carbon-reduction cycle had been established by the mid-1950's, many questions remain, particularly with respect to relationships between the photochemical stages of photosynthesis and the carbon-reduction pathway. Disturbance of a photosynthesizing system by darkening or by withdrawal of CO_2 leads to pronounced changes in the concentrations of the various carbon-cycle intermediates, and the study of such changes helped greatly in formulation of the cycle. More recently, analogous studies have been made on systems in the presence of chemical inhibitors capable of interfering in the precarboxylative stages of photosynthesis.

When 8-methyl lipoic acid[1] or lipoic acid[2] was added to photosynthesizing Chlorella, sudden and striking changes were observed in the levels of phosphoglyceric acid (PGA), fructose-1,6-diphosphate, and sedoheptulose-1,7-diphosphate. The drop in PGA suggested inhibition of the carboxylation of ribulose diphosphate to PGA, whereas the rise in the fructose and sedoheptulose diphosphates suggested inhibition of the phosphatasing action by which these are converted to their respective monophosphates. Since, however, there are a number of possible ways in which disulfide groups and sulfhydryl groups (derived from disulfides in biological systems) may affect enzyme action, it is difficult to pinpoint the model(s) of action of such inhibitors.

The two inhibitors employed in the present study appear to be more specific in their action. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) strongly inhibits photophosphorylation,[3] but is without effect on TPN (triphosphopyridine nucleotide) reduction in spinach chloroplasts.[4] The second inhibitor, 2,4-dichlorophenyl dimethylurea (DCMU), by analogy with the known action of its monochloro analog,[5] blocks the transfer of electrons from water (giving O_2), and stops the reduction of $NADP^+$ to NADPH in the absence of added electron donors. At the same time it blocks photophosphorylation in the absence of other added substances.[4]

Methods and Results

Preliminary experiments showed that DCMU in concentrations greater than 10^{-5} M completely inhibited CO_2 uptake and oxygen evolution by illuminated Chlorella pyrenoidosa within 1 minute after addition, but did not block respiration. With a DCMU concentration of 10^{-6} M, photosynthesis was about 65% inhibited within 6 min and about 80% inhibited in 18 min. At 10^{-7} M inhibitor concentration, there was no significant effect.

The action of the second inhibitor, CCCP, was less drastic. At 10^{-5} M, the hydrazone brought about complete inhibition of photosynthesis within about 1.6 min, but there was evi-

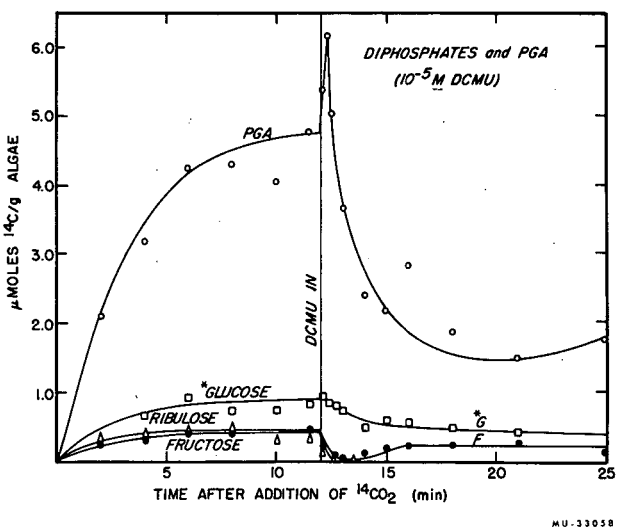

Fig. 7-1. Levels of ^{14}C-labeled sugar diphosphates and PGA in DCMU experiment. The "*glucose" curve, representing all labeled diphosphates hydrolyzed to glucose, is thought to be mainly uridine diphosphoglucose.

1. J. A. Bassham, H. Egeter, F. Edmonston, and M. Kirk, Biochem. Biophys. Res. Commun. 13, 144 (1963).

2. J. A. Bassham, Frances Edmonston, and Martha Kirk, unpublished data.

3. P. G. Heytler and W. W. Prichard, Biochem. Biophys. Res. Commun. 7, 272 (1962).

4. E. S. Bamberger, C. C. Black, C. A. Fewson, and M. Gibbs, Plant Physiol. 38, 483 (1963).

5. A. T. Jagendorf and M. M. Margulies, Arch. Biochem. Biophys. 90, 184 (1960).

dence of slight recovery (about 10%) after 10 min. No effect was observed at the 10^{-6} M level.

The procedure for kinetic studies of the changes in levels of the various ^{14}C-labeled intermediates brought about by addition of the inhibitors has been described.[1] Inhibitor concentration for these experiments was 10^{-5} M, and the pH was set at 5.0. The algae were allowed to photosynthesize for about 45 min before addition of $^{14}CO_2$, and 12 min more before addition of the inhibitor.

Figures 7-1 and 7-2 show the levels of the various ^{14}C-labeled compounds obtained from samples taken during the DCMU experiment; Figs. 7-3 and 7-4 indicate the corresponding data for the CCCP experiment. The changes in ^{14}C labeling patterns brought about by addition of the two inhibitors are strikingly similar, and both follow closely the changes observed when the light is turned off during the light-dark transient study previously described.[6]

As when the light is turned off, the sudden rise in the PGA level is significantly greater than the drop in level of ribulose diphosphate.

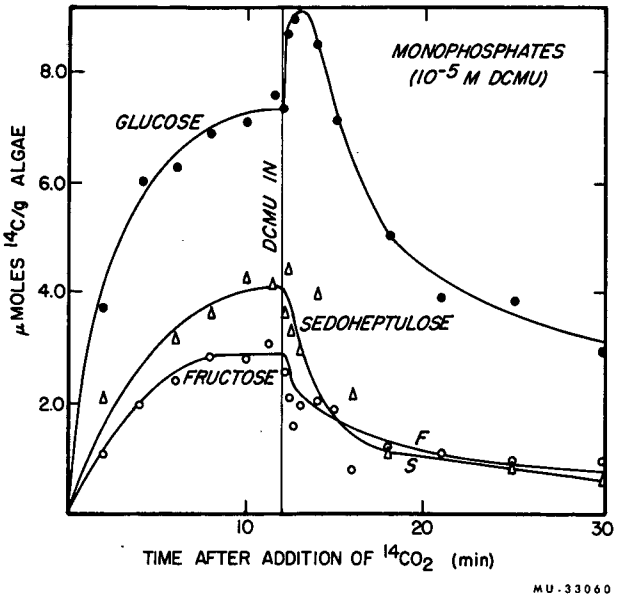

Fig. 7-2. Levels of ^{14}C-labeled sugar monophosphates in DCMU experiment.

It is reasonable to assume that the PGA increment is derived not only from ribulose diphosphate, but also from the thermodynamically favored oxidation of sugar diphosphates via triose phosphate, i.e., from reversal of the steps in reductive cycle and from further conversion of ribulose monophosphate to ribulose diphosphate followed by carboxylation. Oxidative formation of PGA is in accord also with the rapid drop in fructose diphosphate. The conversion of fructose monophosphate to PGA via triose phosphate or ribulose diphosphate requires ATP and may continue at a slow rate after the supplies of photosynthetically produced ribulose diphosphate and fructose diphosphate have been virtually exhausted. Such dark formation of PGA cannot compete with the drain of PGA into the tricarboxylic acid cycle (via phosphoenolpyruvate), and thence to glutamate and other products. Hence, after the initial spurts, the PGA levels with both inhibitors suffer the same type of decline as observed in the light-dark transient study.

In the period following additions of the inhibitors, the ^{14}C-labeling patterns for the monophosphates (Figs. 7-2 and 7-4) suggest not only that glucose phosphate increases at the expense of sedoheptulose and fructose monophosphates, but also that there has been interference with at least one process that consumes glucose phosphate during ordinary operation of the cycle. It seems reasonable that the observed increase in the glucose phosphate level, 1 to 2 min after additions of the inhibitors, arises from interference with conversion of glucose phosphate to sucrose via uridine

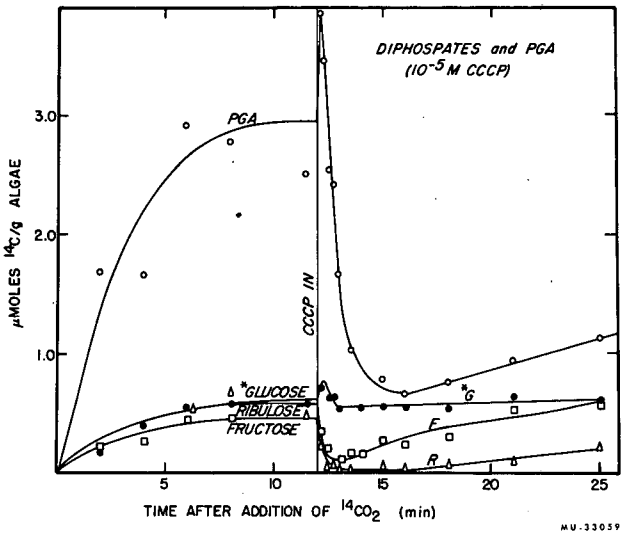

Fig. 7-3. Levels of ^{14}C-labeled sugar diphosphates and PGA in CCCP experiment. The "*glucose" curve, which represents all labeled diphosphates hydrolyzed to glucose, is thought to be mainly uridine diphosphoglucose.

6. J. A. Bassham and Martha Kirk, Microalgae and Photosynthetic Bacteria (Special issue, Plant and Cell Physiol., Japan), 1963, p. 493. In this communication, as in the present report, the term "micromoles of ^{14}C" refers to the total quantity of carbon (as measured by the ^{14}C level) incorporated into the various species after introduction of $^{14}CO_2$.

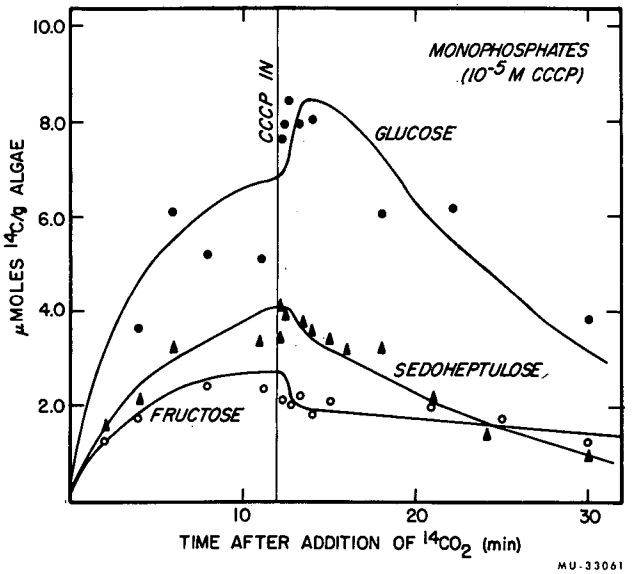

Fig. 7-4. Levels of ^{14}C-labeled sugar monophosphates in CCCP experiment.

diphosphate glucose (UDPG).[7] The nature of this interference is not clear, but the nearly constant level of UDPG observed suggests that both the formation of UDPG (which requires ATP or UTP) and its reaction with fructose monophosphate (which is depleted) are retarded upon addition of inhibitor. About 2 min after onset of inhibition, the glucose phosphate level begins to drop as the levels of all sugar phosphates decline. A very similar pattern for glucose monophosphate has been observed in the light-dark transient study and may be rationalized similarly. The view that the net increase in glucose phosphate level arises from interference with its conversion to sucrose is in accord with the observation that the sucrose level in photosynthesizing Chlorella remains very nearly constant when the light is turned off.[8]

The marked similarity between the pattern of ^{14}C distribution in the light-dark transient study[6] and the patterns derived from experiments with our inhibitors is not unexpected, and indicates merely that the manner in which the organism reacts when its supply of ATP is cut off does not depend upon the way such a cutoff is achieved. It does not matter whether inhibition results from darkening, from chemical interference (by CCCP) in the photophosphorylation sequence itself, or from removal of the photochemically produced reducing agent(s) needed to initiate photophosphorylation (DCMU); the end result is the same.

The very different patterns obtained in the experiments with lipoic and methyl lipoic acid inhibitors confirm the view that the primary action of these disulfide inhibitors is neither on the photophosphorylation sequence nor on the electron-transfer acts preceding it, but is rather on enzymes controlling one or more of the chemical steps in the reduction cycle itself.

Finally, comparison of the rate at which ribulose diphosphate (RuDP) decreases in the DCMU experiment with that in the CCCP experiment is of interest with respect to the question of a possible alternate path involving some type of reductive carboxylation, by which CO_2 may be converted to triose phosphate without coming into equilibrium with the pool of free PGA.[6] If it is assumed that such a reductive path requires a reduced cofactor such as NADPH, then such a path should be blocked by the electron-transfer inhibitor, DCMU, but not necessarily by the phosphorylation inhibitor, CCCP. If this be the case, and if the reductive path compares in importance to the well-established carboxydismutative route by which RuDP is converted to PGA in the cycle, then the drop in RuDP should be more sudden in the presence of CCCP (where there are two paths for carboxylation) than with DCMU (where there is only one path). We observe no such difference; with either inhibitor, the RuDP level drops essentially to zero within 30 seconds after inhibitor addition.

However, the steady-state level of ribulose diphosphate in this experiment was only 0.08 μM per cm^3 of algae (0.4 μM C). At an estimated carboxylation rate of 10 μM CO_2 per min, or 0.17 μM per sec, the ribulose diphosphate could be depleted in about 1 sec. Thus these results do not support nor deny the existence of a reductive carboxylation reaction. They do demonstrate that with chemical inhibition we can reproduce, in rapidity and magnitude, transients in levels of carbon-cycle intermediate compounds previously produced only by turning off the light. The information obtainable by this type of study is potentially much more meaningful than that obtained by long incubation with inhibitors (with consequent killing of the organism) prior to $^{14}CO_2$ addition.

7. See, for example, J. G. Buchanan, V. Lynch, A. A. Benson, D. Bradley, and M. Calvin, J. Biol. Chem. 203, 935 (1953).

8. M. Calvin and P. Massini, Experientia 8, 445 (1952).

8. STUDIES ON THE QUINONE COMPOSITION OF CHLORELLA PYRENOIDOSA

Hartmut K. Lichtenthaler

In earlier reports the isolation and determination of lipophilic quinones from spinach chloroplasts and quantasome aggregates were described. A rapid paper chromatographic method (Schleicher and Schüll paper No. 288) was developed, allowing the separation of lipophilic quinones and α-tocopherol from pigments such as carotenes, xanthophylls, and chlorophylls, which usually interfere. [1] The principal quinones in spinach-chloroplast lamellae were identified as plastoquinone A and B, phylloquinone (vitamin K_1), α-tocopherylquinone and its chromanol form, tocopherol. [2] The presence of several other quinones in minor concentrations was demonstrated by the neotetrazolium chloride test, which revealed quinones as red-violet bands on the paper. [1]

The quinones mentioned above were also found in chloroplasts from various other plants; their presence suggested that they are typical constituents of the photosynthetic apparatus in higher plants. From a phylogenetic point of view, green algae are relatively close to higher plants; for example, they contain the same pigments as higher plants. [3] From this one may predict that the quinones present in green algae are identical to those of higher plants. The purpose of the present investigation on the quinone composition of Chlorella pyrenoidosa is to test this prediction. This work is also important with reference to the early photosynthetic reactions, in which quinones seem to act as electron carriers.

In several independent studies, addition of individual quinones has restored the photochemical activity (Hill reaction) of petroleum-ether– or acetone-extracted freeze-dried chloroplasts. [4, 5, 6, 7] In plastoquinone A (nine isoprene units in the side chain) the position in the photosynthetic electron-transport chain, which comprises the two light reactions, seems to be established. [8] Whether the other quinones participate in the proposed scheme, either as electron carriers or possible cofactors of photosynthetic phosphorylation, is still uncertain, however.

Four plastoquinones (PQA, PQB, PQC, PQD) have been isolated from spinach chloroplasts. The structure is established only for PQA. We know that PQB has an absorption spectrum similar to that of PQA, with the maximum at 255 mμ (in ethanol); PQC and PQD possess a second peak at 261 mμ (in ethanol). Crane et al. have some evidence for a possible interconversion of plastoquinones. [9] Interconversion between α-tocopherylquinone (α-TQ) and α-tocopherol--the cyclic form (chromanol) of α-TQ--is very likely. Some of the proposed reaction mechanisms for quinones even depend on such conversions. [10] Light- and dark-treated chloroplasts show significant differences in their quinone concentrations, which could partly be explained by interconversion. [9] Studies with ^{14}C-labeled quinones should reveal an interconversion of chloroplast quinones and possibly yield further information on their function in photosynthesis.

The de novo synthesis of quinones in isolated chloroplasts from $^{14}CO_2$ substrate is slow. Chlorella pyrenoidosa, which can be grown under physiological conditions with $^{14}CO_2$ as carbon source in the steady-state apparatus, [11] would be a good pool for the production of labeled quinones. The high multiplication rate of Chlorella provides homogeneous labeling of the quinones, which then could be used, after extraction and isolation, for interconversion studies.

Preliminary studies on isolation and identification of lipophilic quinones from Chlorella pyrenoidosa, with some remarks on their labeling, are described in this report.

Methods and Results

Chlorella pyrenoidosa cells were cultured in Myer's medium[3] and harvested with the

1. Hartmut K. Lichtenthaler, J. Chromatog., 1964 (in press).
2. Hartmut K. Lichtenthaler and M. Calvin, Biochim. Biophys. Acta, 1964 (in press).
3. Joan M. Anderson, Research in Photosynthesis (Ph. D. thesis), Lawrence Radiation Laboratory Report UCRL-8870, September 1959 (unpublished).
4. N. I. Bishop, Proc. Natl. Acad. Sci. U. S. 45, 1696 (1959).
5. M. D. Henninger, R. A. Dilley, and F. L. Crane, Biochem. Biophys. Res. Commun. 10, 237 (1963).
6. D. W. Krogmann and E. Olivero, J. Biol. Chem. 237, 3292 (1962).
7. F. A. Trebst, Proc. Roy. Soc. (London) 157B, 355 (1963).
8. D. I. Arnon and A. A. Horton, Acta Chem. Scand. 17, 135 (1963).
9. F. L. Crane, (Dept. of Biological Science, Purdue University, Lafayette, Ind.) Private communication.
10. M. Vilkas and F. Lederer, Experientia 15, 546 (1962).
11. J. A. Bassham and Martha Kirk, Biochim. Biophys. Acta 43, 447 (1960).

Sharples centrifuge. The wet-packed algae were suspended in a small volume of distilled water, then extracted with acetone and acetone-chloroform mixtures. After four or five extractions, a colorless-cell residue was obtained. The combined extract solutions were concentrated under reduced pressure, taken up in a mixture of acetone and petroleum ether, and then transferred to a separatory funnel. By addition of half-saturated sodium chloride solution, all lipid-soluble substances were dissolved in the epiphase. The petroleum ether extract thus obtained was concentrated under reduced pressure and stored in the deep freeze at -10 °C until further use.

The absorption spectrum of a total lipid extract from Chlorella pyrenoidosa in diethyl ethers is represented in Fig. 8-1. Overnight storage of the highly concentrated petroleum ether extract in the deep freeze resulted in the formation of orange crystals, which were collected by centrifugation. The red-orange crystalline sediment was found by paper chromatography to consist of very pure lutein. Lutein is the main carotenoid of Chlorella pyrenoidosa, contributing 52% of the total carotenoids. The absorption spectrum of lutein, recrystallized from ethanol, is given in Fig. 8-2. The absorption maxima in diethyl ether are at 267, 445, and 472 mμ, with shoulders at about 335 and 423 mμ. The values are in good agreement with other data reported in literature.[3]

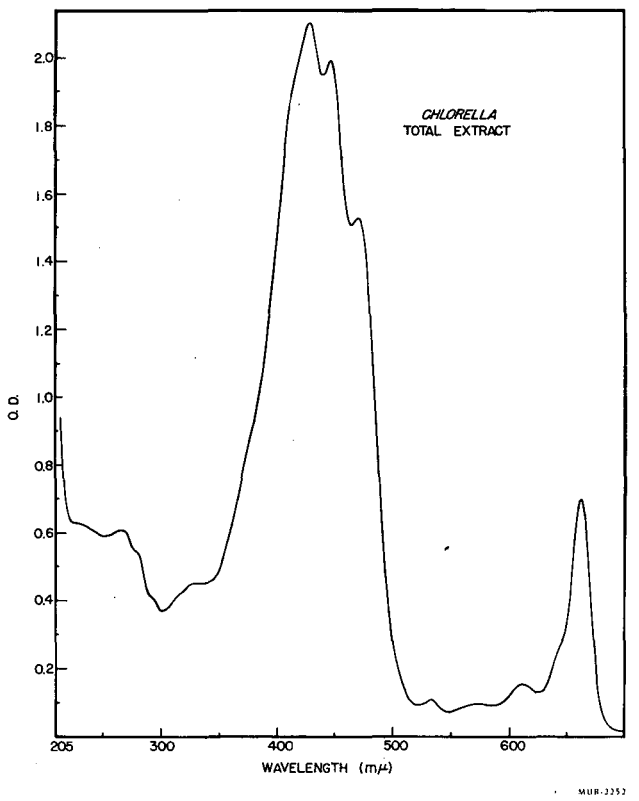

Fig. 8-1. Absorption spectrum of a total lipid extract from Chlorella pyrenoidosa, in diethyl ethers.

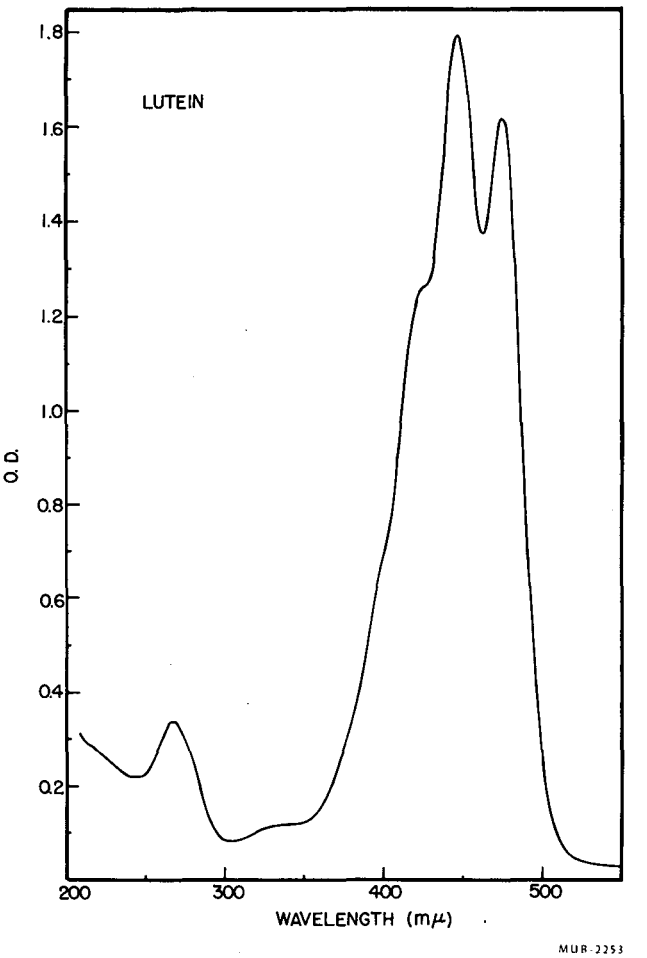

Fig. 8-2. Absorption spectrum of lutein in diethyl ethers.

For the isolation of quinones, the lipid extract was chromatographed on Schleicher and Schüll paper No. 288, with petroleum ether or benzene as solvents. With the neotetrazolium chloride reagent the quinones were detected on the paper as red bands. Further identification was achieved by measuring the spectra of the rechromatographed, eluted bands before and after reduction with borohydride solution. Co-chromatography was also applied.

A typical paper chromatogram of a total lipid extract from Chlorella pyrenoidosa is represented as a diagram in Fig. 8-3. Quinones are indicated as dark bands. On the solvent front we find first a yellow band consisting of α- and β-carotene, which are separated sometimes in two distinct bands. The spectrum of

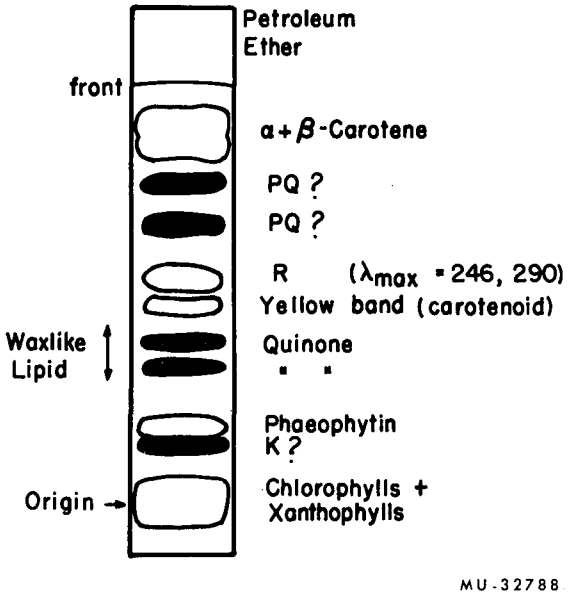

Fig. 8-3. Diagram of a paper chromatogram of a lipid extract from Chlorella pyrenoidosa. Solvent, petroleum ether; developing time, 90 min.

the first quinone band is shown in Fig. 8-4. The absorption maxima are at 292, 282, 270, and 262 mμ. Upon reduction with borohydride the absorption decreases below 277 mμ but increases at about 290 mμ. The spectrum is not typical for any known quinone; the behavior upon reduction, however, suggests a quinone of the plastoquinone type, presumably contaminated with another uv-absorbing substance.

The second quinone band has a mobility on the paper like PQA (nine isoprene units in the side chain). The spectrum (Fig. 8-5) shows the typical 255-mμ maximum. Reduction decreases this band and slightly increases the absorption at 290 mμ. It is therefore assumed that this quinone is identical with PQA.

One pink band, which showed the same mobility as α-tocopherol, appeared on the chromatogram with the Emmerie-Engel spray[1] for tocopherols. The eluted material had an uv absorption with peaks at 246 and 290 mμ (diethyl ether) [Fig. 8-6(a)]. Reduction with borohydride did not change the spectrum. These two absorption peaks could not be separated by paper chromatography in maxima of two single compounds. The ratio of the maxima, 246/290, was 4.2 and did not change upon further purifi-

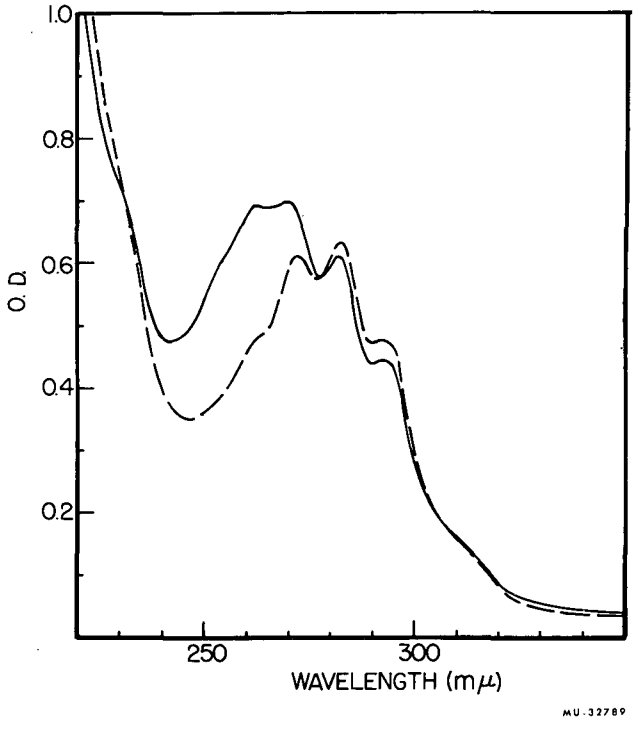

Fig. 8-4. Spectrum of the first eluted quinone band in ethanol (solid curve) and after reduction with borohydride (dashed curve).

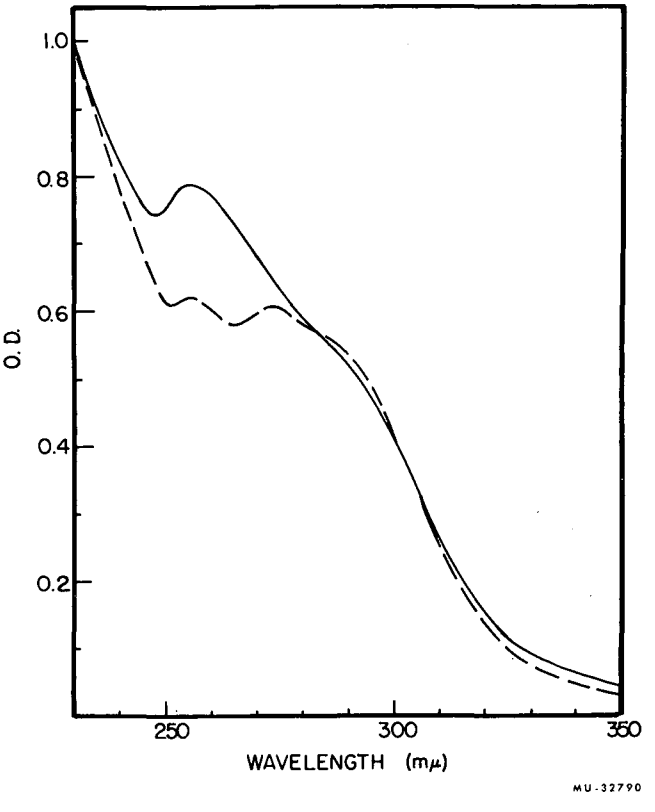

Fig. 8-5. Spectrum of second quinone band in ethanol (solid curve) and after reduction with borohydride (dashed curve).

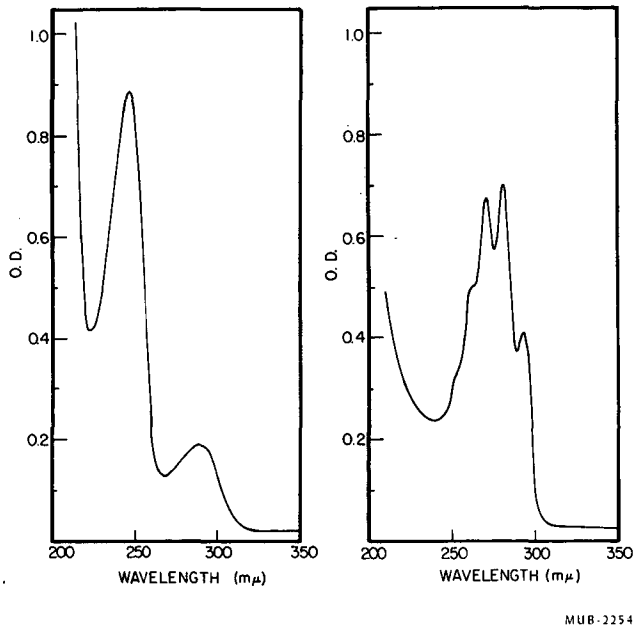

Fig. 8-6. (a) Spectrum of reducing substance $R_{(246, 290)}$ in ethanol (shown on left). (b) Spectrum of colorless waxlike lipid in ethanol (presumably ergosterol ester) (shown on right).

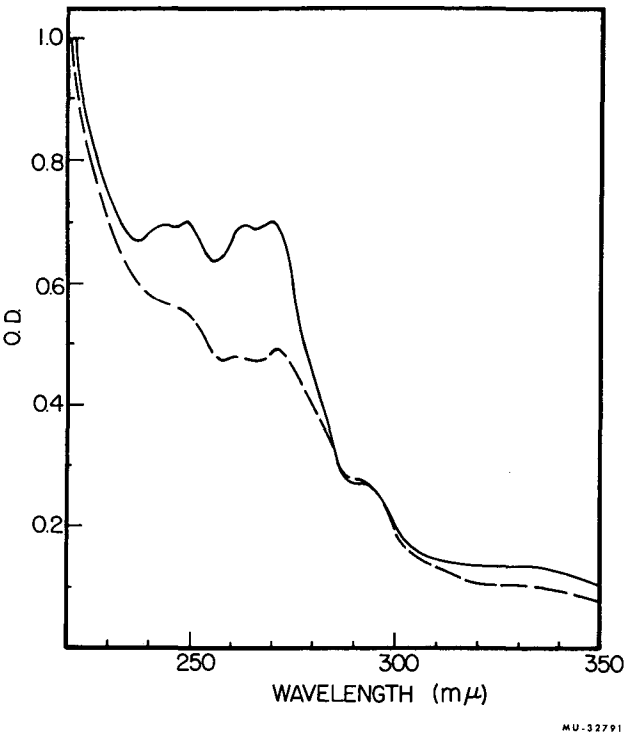

Fig. 8-7. Absorption spectrum of vitamin-K-type quinone in ethanol (solid curve) and after reduction with borohydride (dashed curve).

cation on paper. Oxidation with ceric sulfate did not result in the formation of α-tocopherylquinone as one might have expected from α-tocopherol, which also possesses a maximum at 290 mμ. Thus it seems that the peaks are both due to one as yet unknown compound that has reducing properties, since it can be detected with the Emmerie-Engel reagent. On the chromatogram it is indicated as reducing substance R (246, 290 mμ).

Below the R (246, 290) we find a waxlike lipid in a broad band, which includes also two weak quinone bands. It has not been possible so far to separate these two quinones from the waxy material present in high concentration. The colorless waxy lipid has a distinctive uv absorption with maxima at 292, 281, and 271 mμ and a broad flat shoulder at 263 mμ [Fig. 8-6(b)]. This spectrum is similar to that of the first quinone band in the reduced form. It is almost identical to the spectrum of ergosterol (λ_{max} 271, 282, 292 mμ in ethanol). Synthetic ergosterol does not move as fast on the chromatogram as the compound described here. The spectrum and mobility on the paper would suggest a derivative of ergosterol with higher lipid solubility, presumably an ergosterol ester.

The next band on the chromatogram showed grey-green and was found to be pheophytin. Just below pheophytin appeared a strong quinone band that had four absorption maxima. The spectrum is shown in Fig. 8-7. The maxima are at 244, 249, 260, and 270 mμ. They correspond to those of vitamin K_1. Reduction follows the same pattern as that of vitamin K_1. The mobility on the alumina paper, however, is completely different. Vitamin K_1, with a higher mobility than the PQ's, appears in chromatograms from spinach-chloroplast extracts shortly below β-carotene. No such band could be detected in Chlorella extracts. Spectrum and mobility of the new quinone (lower than that of pheophytin) suggest it is a vitamin-K-type quinone with fewer isoprene units in the side chain than K_1. A hydroxyl group in the side chain, similar to α-tocopherylquinone, is also possible.

Discussion

Since Chlorella pyrenoidosa has basically the same green and yellow pigments as do higher plants, one expects to find the lipophilic quinones present in spinach-chloroplast lamellae.

The results thus far indicate the presence of five principal quinones, probably two plastoquinones, two unidentified quinones associated with an ergosterol derivative, and a quinone from the vitamin-K type that is not, as in higher plants, vitamin K_1. Besides these, we find a reducing substance with maxima at 246 and 290

mμ, which can be isolated also from total spinach leaves but was not present in the chloroplasts.

Also present are two waxlike lipids, one in low concentration with high mobility, another in high concentration with lower mobility, showing the same absorption spectrum as does synthetic ergosterol. The first shows up only upon reduction of the quinone in the first quinone fraction. These compounds apparently correspond to two different esters of ergosterol.

Several quinones not yet identified show up with the neotetrazolium chloride reagent. They possess Rf values similar to some colorless lipids from which they can be separated only with difficulty or with considerable losses. These lipids, waxlike substances that are present in higher concentrations than the quinones, possess distinctive absorption bands that interfere with the quinone maxima. This makes the identification of Chlorella quinones difficult. Thus, the rate of ^{14}C incorporation in Chlorella quinones, as well as the isolation of the labeled compounds, can be achieved only with improvements in the purification procedures.

Radioactive quinone fractions were obtained from Chlorella pyrenoidosa grown under steady-state conditions and 6-hr photosynthesis with $^{14}CO_2$. However, it could not be decided whether the activity was due to quinones or labeled contaminants.

On radioautographs of paper chromatograms several dark spots appeared, which, in part, were congruent with quinone bands revealed by the neotetrazolium chloride test. This preliminary experiment indicates that 6-hr photosynthesis is sufficient to produce labeled quinones from $^{14}CO_2$.