UCRL 10479

University of California

Ernest O. Lawrence Radiation Laboratory

BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

June through August 1962

Berkeley, California

DISCLAIMER

Research and Development

UCRL-10479
UC-4 Chemistry
TID-4500 (17th Ed.)

UNIVERSITY OF CALIFORNIA
Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

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Printed in USA. Price $1.25. Available from the
Office of Technical Services
U. S. Department of Commerce
Washington 25, D.C.

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Contents

*Preceding Quarterly Reports: UCRL-10350, UCRL-10156

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June through August, 1962

M. Calvin, Director

Edited by R. B. Park and Kenneth Sauer

Lawrence Radiation Laboratory and Department of Chemistry
University of California, Berkeley, California

September 26, 1962

1. SYNTHESIS OF COMPOUNDS FROM $^{14}CO_2$ BY CHLORELLA IN THE DARK FOLLOWING PREILLUMINATION

J. A. Bassham and Martha Kirk

In the preceding quarterly report we described light-dark transient changes in concentration of sugar phosphates in Chlorella,[1] as determined by studies with $^{14}CO_2$ in our steady-state apparatus.[2]

Since experiments of this type show only the changes in total concentration but not the kinetics of the newly incorporated carbon, it would be advantageous to do parallel experiments in which the $^{14}CO_2$ is added only upon turning off the light, particularly if some quantitative comparison could be made between the rate of carbon uptake in the light and the rate of appearance of labeled carbon in the dark immediately following illumination. Development of quantitative techniques for doing studies of $^{14}CO_2$ fixation during photosynthesis[2,3] made such a study more feasible. We report here the results of a study of the appearance of ^{14}C in specific compounds immediately following illumination, and mention another result from our previous light-dark transient study.

Tamiya, Miyachi, and Hirokawa measured the capacity of the plant to fix carbon-14 dioxide in the dark following preillumination, which they call R.[4] They found that cyanide inhibits the reaction between R and CO_2 and also accelerates the nonphotochemical decay of R. Since it has been found that cyanide reacts irreversibly with ribulose diphosphate in the plant, thereby destroying its ability to react with carbon dioxide, we are inclined to think that the major part of the CO_2-fixing capacity of preilluminated algae (R) is represented by ribulose diphosphate, together with those cofactors that bring

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1. J. A. Bassham and Martha Kirk, in Bio-Organic Chemistry Quarterly Report, UCRL-10350, May 1962, p. 42.
2. J. A. Bassham and Martha Kirk, Biochim. Biophys. Acta 43, 447 (1960).
3. D. C. Smith, J. A. Bassham, and Martha Kirk, Biochim. Biophys. Acta 48, 299 (1961).
4. H. Tamiya, S. Miyachi, and T. Hirokawa, in Research in Photosynthesis, ed. by H. Gaffron et al. (Interscience Publishers, Inc., New York, 1957), p. 213.

about the formation of ribulose diphosphate in the dark following illumination. For example, ATP would cause a phosphorylation of some initial ribulose monophosphate to give ribulose diphosphate, thereby contributing to R. Another portion of R would be the carbon fixation by phosphoenolpyruvate and TPNH to form malic acid. Miyachi et al. not only showed that R decays rapidly in the dark and is quite independent of the levels of TPNH and DPNH, but also produced a very interesting fact that the levels of these reduced co-factors actually increase in the absence of CO_2 when the light is turned off.[5] Miyachi studied the effect of sulfhydryl reagents on R.[6] Although p-chloro-mercuribenzoic acid, like iodoacetic acid and arsenite, lowered the level of R, it did not affect the absolute rate constant for the reaction between R and CO_2. Since purified carboxydismutase,[7] the enzyme responsible for carboxylation of ribulose diphosphate to give two molecules of PGA, is highly sensitive to this reagent, Miyachi concluded that R could not be identical with ribulose diphosphate. Isolated carboxydismutase, however, appears to have much less activity than that required for carboxylation in vivo, and there are many other lines of evidence that lead us to believe that the in vivo operation of the carbon-reduction cycle must have certain characteristics different from those of the isolated enzymes.[8]

In the studies of R, the Chlorella have usually been preilluminated in the absence of CO_2, whereas in the work reported here, the Chlorella are allowed to photosynthesize under steady-state conditions prior to the administration of $^{14}CO_2$. Consequently, a direct comparison between the results of the two types of experiments is not possible. We believe, however, that the details of the kinetics of ^{14}C fixation into specific compounds which we report here will be of use in the general problem of interpreting the results of preillumination experiments.

Materials and Methods

Chlorella pyrenoidosa were grown in continous-culture tubes as described previously.[9] After being centrifuged and washed once with the medium given in Table 1-I, they were centrifuged again and resuspended in this medium to a concentration of 25 ml packed volume per liter, and placed in the illumination cell of a steady-state apparatus similar to that described previously.[2] This apparatus has been modified, however, to include both automatic pH control and automatic density control. The algae suspended in the nutrient given in Table 1-I have a pH of 4.75. The pH was controlled at 6, and 0.1 N ammonium hydroxide flowed in automatically until the pH reached the set value, after which it was added automatically from time to time as the algae photosynthesized, took up ammonium ion, and tended to become acidic. All other nutrients were added automatically in response to a signal from a photoresistor which measured the light coming through the algae.

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5. S. Miyachi, T. Oh-Hama, and H. Tamiya, Plant Cell Physiol. (Tokyo) 1, 151 (1960).
6. S. Miyachi, Plant Cell Physiol. (Tokyo) 1, 117 (1960).
7. J. Mayaudon, A. A. Benson, and M. Calvin, Biochim. Biophys. Acta 23, 342 (1957).
8. J. A. Bassham, Photosynthesis: Energetics and Related Topics, Advances in Enzymol., in press.
9. J. A. Bassham and M. Calvin, in The Path of Carbon in Photosynthesis (Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 1957).

The addition medium was the same as that in which the algae were initially suspended (see Table 1-I). Also, algae suspension was automatically withdrawn from the cell when the volume exceeded a certain amount.

Table 1-I. Nutrient solution for Chlorella.

KH_2PO_4	2.0 mM
$MgSO_4$	2.0 mM
$Ca(NO_3)_2$	0.02 mM
$FeSO_4$-Versen-ol	0.1 mM in Fe
Modified Arnon's A-4 trace-elements solution (see below)	2 ml/l
NH_4VO_3 (0.023 g/l)	1 ml/l

Modified Arnon's A-4 trace-elements solution	
$CoCl_2 \cdot 6H_2O$	0.168 mM
H_3BO_3	46.0 mM
$MnCl_2 \cdot 4H_2O$	50.0 mM
$ZnSO_4 \cdot 7H_2O$	0.771 mM
$CuSO_4 \cdot 5H_2O$	0.316 mM
MoO_3(9-.5%)	0.100 mM

Carbon-14 was added both as $^{14}CO_2$ and as $H^{14}CO_3^-$, in such a proportion as to give immediately the final specific activity of radiocarbon in both solution and gas phase.[2] The algae suspension was illuminated from one side by an incandescent tungsten lamp and from the other by a bank of eight 6-watt fluorescent lamps (four blue and four white). The incident and transmitted intensities from the tungsten lamp were 140,400 and 1,620 lux. Light intensities from the blue and white fluorescent tubes were 7,830 lux incident and 86 lux transmitted. The temperature was controlled at 20°C. The algae were placed in a steady-state apparatus and allowed to photosynthesize in the presence of 1.9% CO_2 in air for 3-1/2 hours before the start of the experiment. Just prior to the start of the experiment this gas system was closed and the concentration of CO_2 allowed to decline owing to photosynthesis to 0.6%. The light was turned off and the apparatus, already in a darkened room, was shrouded with a black cloth. At the same instant, a loop containing 4.4 cc of $^{14}CO_2$ was added to the system (total gas and liquid volume was 440 cc) and at the same time 1.0 ml of $H^{14}CO_3^-$ solution, 0.06 mM and 1.98 mC per ml, was injected through a hypodermic needle into the algae suspension. Because of the rapid bubbling of the cycling gas through the algae suspension, mixing is considered to be almost instantaneous--from experiments with dyes we estimate about 1 sec. Samples were taken every 5 sec.

All samples were analyzed by two-dimensional paper chromatography and radioautography as described previously.[10] In order that we might see all possible stable compounds, no extractions were performed, and the concentrated killed algae suspension was placed directly on the paper chromatogram. After two-dimensional paper chromatography and radioautography with x-ray film, the individual spots as well as the origin were counted by a double G-M tube, which counts on both sides of the paper simultaneously.

During the course of both experiments the rates of photosynthesis were determined just prior to administering the ^{14}C by using the small gas volume of the steady-state apparatus[2] and measuring the slopes of CO_2 and oxygen change.[2, 3] After addition of ^{14}C, the levels of ^{14}C and CO_2 were monitored, giving continuous values for the specific radioactivity.

Results

Additional results of the experiment (light-dark transient) described in the preceding report[1] are shown in Fig. 1-1. (The PGA curve was not presented before.) In all results given in this paper, the term "micromoles of ^{14}C" means the number of micromoles of carbon total corresponding to the measured amount of ^{14}C when the specific activity is that of the $^{14}CO_2$-$^{12}CO_2$ administered to the algae. In other words, it is the measured amount of ^{14}C divided by the ratio of ^{14}C to (^{14}C + ^{12}C). This method of expression is convenient for comparing the flow of labeled carbon with the total flow of carbon.

As in earlier detailed light-dark transient studies,[11] the level of PGA rises rapidly and then falls, whereas the level of ribulose diphosphate falls off to 0. However, in the earlier studies, because of the different physiological conditions, the ribulose diphosphate was the only significant pool of diphosphates. The fructose diphosphate and sedoheptulose diphosphate were almost negligible in concentration. In this study, however, the amounts of these other diphosphates are significant, and it is interesting to see that their concentration also falls to 0 when the light is turned off. (See also the discussion in the preceding report.[1])

The results of the preillumination experiment are shown in Figs. 1-2, 1-3, and 1-4. The amount of radiocarbon, in micromoles per ml algae, found in compounds and areas not shown individually at 100 sec are as follows: Pyruvic acid, 0.014; uridine diphosphoglucose, 0.013; sucrose, 0.035; the origin, 0.042. Except for phosphoenolpyruvic acid, whose behavior paralleled that of PGA, these other substances were first detected at 15 sec and increased more or less linearly to the value just given. In Fig. 1-2 sugar monophosphates that have not been resolved in this experiment are plotted together. We could detect no traces of sugar diphosphates despite the fact that radiocarbon presumably must pass through fructose diphosphate on the way to the other sugar monophosphates and sucrose. From the light-dark transient study, however, we have seen that its concentration approaches 0 when the light is turned off.

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10. A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka, J. Am. Chem. Soc. 72, 1710 (1950).
11. J. A. Bassham, K. Shibata, K. Steenberg, J. Bourdon, and M. Calvin, J. Am. Chem. Soc. 78, 4120 (1956).

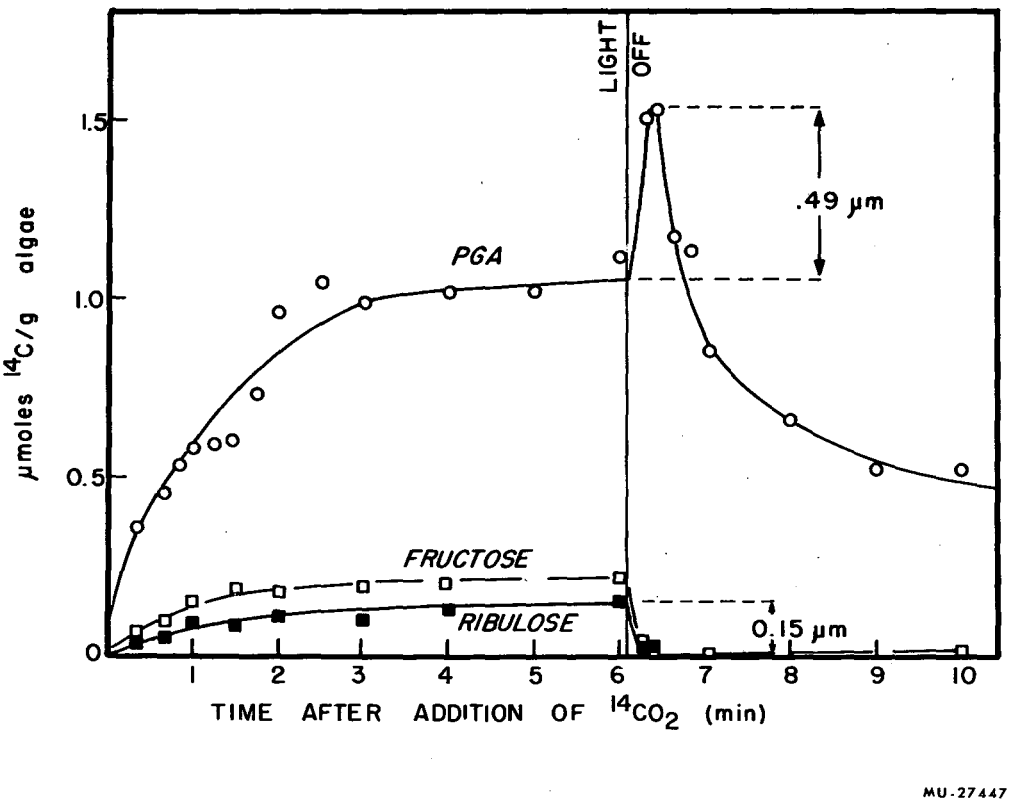

Fig. 1-1. Light-dark transients. Changes in ^{14}C labeling of compounds in Chlorella when illumination stops. PGA = 3-phosphoglyceric acid. "Fructose" and "ribulose" refer to fructose-1, 6-diphosphate and ribulose-1, 5-diphosphate, respectively.

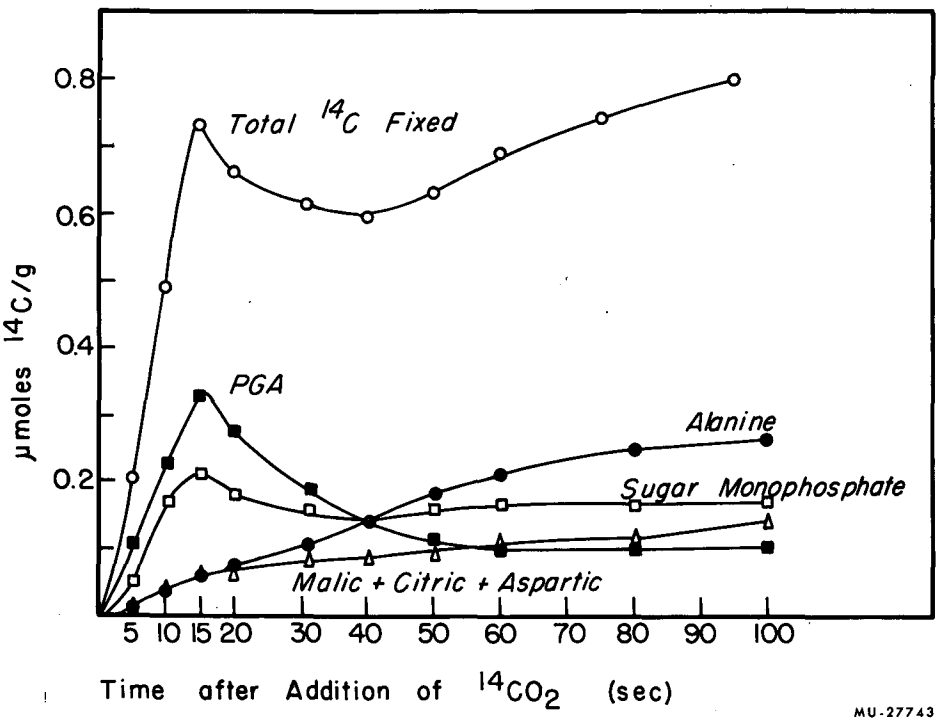

Fig. 1-2. Fixation of ^{14}C into compounds in Chlorella following pre-illumination. PGA = 3-phosphoglyceric acid; sugar monophosphates include monophosphates of fructose, glucose, sedoheptulose, ribose, and ribulose. "Total ^{14}C fixed" includes all radioactive compounds, including origin found on the chromatogram.

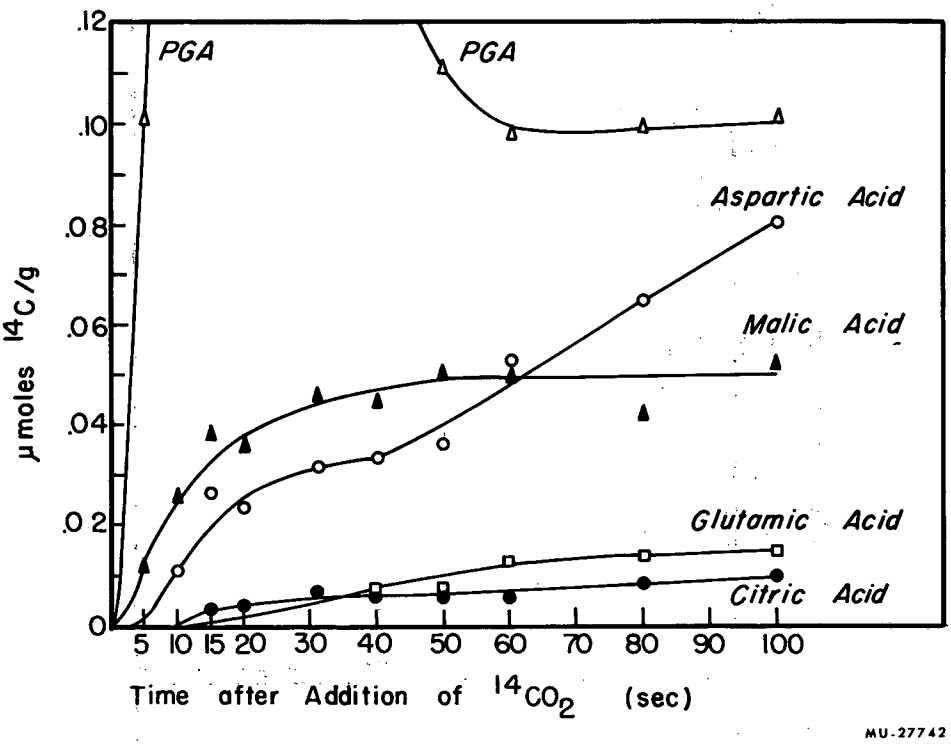

Fig. 1-3. Fixation of ^{14}C into compounds in Chlorella following preillumination.

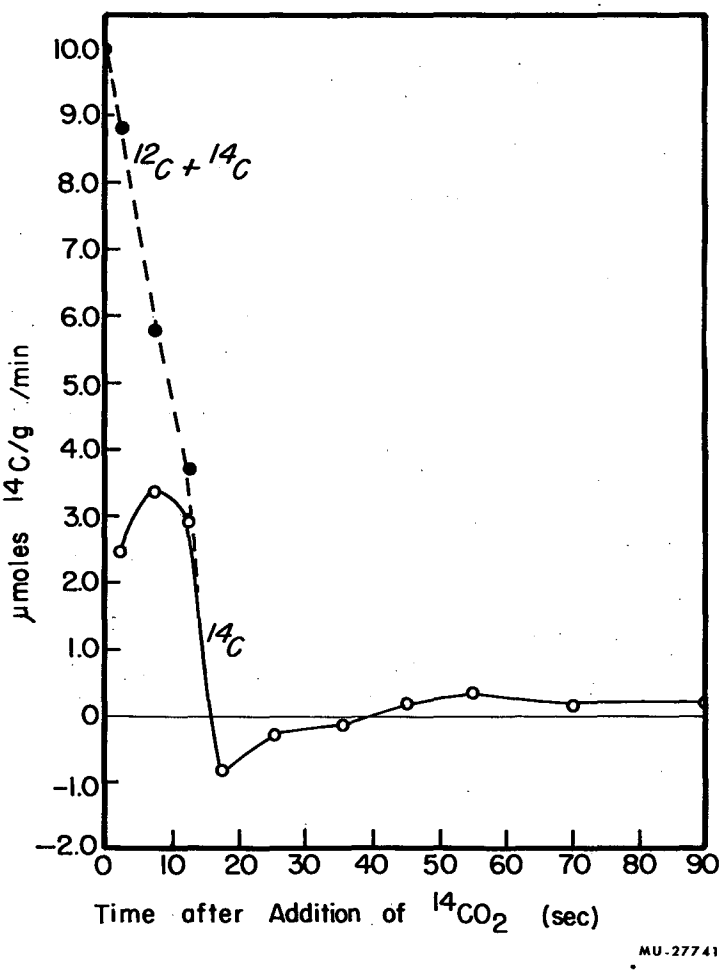

Fig. 1-4. Rate of total $^{14}CO_2$ fixation in preillumination experiment. "$^{12}C + {}^{14}C$" refers to estimated total CO_2 fixation. See text for explanation of calculation.

In looking at the total ^{14}C fixed in this preillumination experiment, it must be remembered that the radiocarbon may have to pass through unstable (or volatile) compounds before it reaches the stable compounds seen by paper chromatography. We have previously reported that the pool of such compounds is of the order of 1 to 1.5 micromoles of carbon.[2] This includes pools of bicarbonate within the cell and possible unstable substances such as CO_2-enzyme complexes. Assuming a pool of only 0.8 μM of unstable compounds, and assuming the initial rate of flow of carbon through the system was the same as existed when the light was on (10 μM of carbon per ml of algae per min), we have calculated a correction to show what the total ^{14}C fixation rate would be if there were no unstable pools preceding stable compounds. This rate is shown as a dashed line in Fig. 1-4 along with the solid line depicting the observed rate of ^{14}C uptake based on the measured 5-sec increments. It is clear from Fig. 1-4 that the rate of fixation falls very rapidly when the light is turned off in the presence of 2% CO_2.

Discussion

The results of the light-dark transient study have been discussed in the preceding report. An additional result, however, is indicated in Fig. 1-1, which shows that when the light is turned off, the increase in PGA (0.49 μM of carbon) is too great to be accounted for by carboxylation of the ribulose diphosphate present at the moment the light was turned off (0.15 μM of carbon). Carboxylation of 0.15 μM of carbon in ribulose diphosphate would give 0.18 μM of carbon in PGA, if two molecules of PGA were formed by each carboxylation. The additional PGA might be made in one of two ways:

(a) More ribulose monophosphate might be phosphorylated with ATP left over from the light or with other sources of high-energy phosphate, and the resulting ribulose diphosphate could subsequently be carboxylated. The total drop in diphosphates (ribulose, sedoheptulose, and fructose is sufficient to account for all the rise in PGA if there were some mechanism for transfer of C-1 phosphate from fructose diphosphate and sedoheptulose diphosphate to ribulose-5-phosphate. Such a mechanism is not known, however.

(b) When the light is turned off oxidation of sugar phosphates via triose phosphate dehydrogenase could take place, giving rise to PGA. The drop in dihydroxyacetone phosphate[1, 2] may indicate oxidation of triose phosphate, conversion of triose to pentose monophosphate (which might then be phosphorylated and carboxylated), or conversion to other sugars. All these possibilities should be viewed in the light of the results of the preillumination experiment.

From Fig. 1-4 we can see that the peak of radioactivity in PGA in the preillumination experiment is caused in part by the rising specific activity of carbon entering the PGA pool, as well as the accumulation of radiocarbon in this pool with time. The downward trend of both the PGA concentration in the light-dark transient experiment and the PGA labeling in the preillumination experiment is caused by the conversion of PGA to alanine as well as to malic, citric, and aspartic acids and other products. In addition it appears that some of the radioactivity in the PGA must be lost as evolved carbon dioxide. The total activity declines for a time before it reaches a slow but steady rate of increase due to carboxylation of phosphoenolpyruvic acid, giving, eventually, aspartic, malic, glutamic, and citric acids.

One of the most interesting results from the preillumination experiment is the fairly rapid incorporation of ^{14}C into sugar monophosphates (mostly hexose and sedoheptulose monophosphate) even at 10 sec exposure to $^{14}CO_2$. At 10 sec the PGA carboxyl carbon, whose pool size has been estimated at about 1μM (in an experiment at comparable light intensity[2]), has itself become only about 20% labeled. This result strongly suggests the possibility that one of the products of carboxylation is a form of bound phosphoglyceric acid, which is reduced to the level of sugar phosphate without ever coming free and equilibrating with the phosphoglyceric acid pool. There has been previous indications of this possibility, including the finding[12] that during a kinetic study of the products of carbon-14 fixation in Scenedesmus the percentage radiocarbon appearing in PGA extrapolated to only about 75% at the time of addition of the ^{14}C. It has been suggested[8] that most or all of the steps of the carbon-reduction cycle[9] of photosynthesis may take place on a multifunctional enzyme system which has the capacity for transferring carbon-compound moieties from one functional site to another.

In this proposal, ribulose-1, 5-diphosphate would react with a thiazole grouping on the enzyme similar to thiamine pyrophosphate to give a phosphoglycoaldehyde addition compound plus a phosphoglyceryl moiety which is transferred to a disulfhydryl function of the enzyme. The phosphoglycoaldehyde addition compound is then said to be carboxylated, phosphorylated (perhaps by thiamine triphosphate, reported by Yusa to be in plants[13]), and reduced. Then it is transferred to a disulfhydryl function of the enzyme, giving another 3-phosphoglyceryl moiety. Hydrolysis of these 3-phosphoglyceryl enzyme complexes gives PGA and disulfhydryl enzyme. Otherwise, they can react to give disulfide enzyme and free dihydroxyacetone phosphate, which can then react with another phosphoglyceryl sulfhydryl enzyme to give fructose-1, 6-diphosphate and a disulfide grouping on the enzyme. Other well-known reactions catalyze the transfer of glycoaldehyde moieties from fructose-6-phosphate, forming at the same time 4-phosphoerythryl sulfide-enzyme hydrosulfide.

If this proposal is correct, or even similar to the true mechanism, the cyclic carbon fixing power (R) of preilluminated algae may depend not only on levels of ribulose diphosphate, but also to some extent on some reduced functional group of the enzyme, or some reducing cofactor which may not be identical with reduced pyridine nucleotide.

The formation of malic, citric, glutamic, and aspartic acids in the dark indicates that the carboxylation of phosphoenolpyruvic acid is not dependent on cofactors from the light but is probably only light-stimulated by the presence of more phosphoenolpyruvic acid (derived from the cycle) in the light than in the dark. Formation of malic acid, which requires TPNH, does appear to stop after about one minute. Aspartic acid labeling presumably results from transamination of the C_1-C_3 carboxylation product, either by glutamic acid or by unlabeled aspartic acid.

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12. J. A. Bassham, A. A. Benson, L. D. Kay, A. Z. Harris, A. T. Wilson, and M. Calvin, J. Am. Chem. Soc. 76, 1760 (1954).
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2. THE EFFECT OF OXYGEN ON THE FORMATION OF GLYCOLIC ACID AND OTHER PRODUCTS DURING PHOTOSYNTHESIS BY CHLORELLA

J. A. Bassham and Martha Kirk

The formation of labeled glycolic acid during photosynthesis of $^{14}CO_2$ by Chlorella and by higher plants has been studied by Benson and Calvin,[1] Schou et al.,[2] Wilson,[3] Wilson and Calvin,[4] Tolbert and Zill,[5] Tolbert,[6] Warburg,[7] and Pritchard et al.[8,9] Although Tolbert and Zill reported much greater formation of glycolic acid in 1% O_2–99% N_2 than in N_2 under comparable conditions, and therefore concluded that aerobic conditions are required for glycolic acid production, there appears to have been no investigation thus far of the possible correlation between glycolic acid production as a function of oxygen pressure and the well-known effect of oxygen inhibition of the rate of photosynthesis, which is especially pronounced at low CO_2 pressure. Studies of this last effect, discovered by Warburg,[10] have been reviewed recently by Turner and Brittain.[11]

It has been suggested[4] that glycolic acid produced during photosynthesis is derived from carbon atoms 1 and 2 of the pentose phosphate intermediates of the carbon-reduction cycle.[12] More specifically, the oxidation of glycolaldehyde thiamine pyrophosphate addition compound, formed during the transketolase reaction,[13,14] has been proposed.[15,16] Since this proposed oxidation involves concommitant reduction of a disulfide (such as lipoic acid) to disulfhydryl, which might directly or indirectly be reoxidized by O_2, we have looked for effects of O_2 on the formation of glycolic acid and other early products of photosynthetic CO_2 reduction during photosynthesis as well as effects on the total CO_2 incorporation.

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2. L. Schou, A. A. Benson, J. A. Bassham, and M. Calvin, Physiol. Plantarum 3,487 (1950).
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13. R. Breslow, J. Am. Chem. Soc. 80, 3719 (1958).
14. E. C. Heath, J. Hurwitz, B. L. Horecker, and A. Ginsburg, J. Biol. Chem. 231, 1009 (1958).
15. J. A. Bassham and M. Calvin, The Photosynthesis of Carbon Compounds (W. A. Benjamin, Inc., New York, 1962).
16. J. A. Bassham, J. Chem. Ed. 38, 151 (1961).

Chlorella pyrenoidosa, grown in continuous culture,[17] was harvested and resuspended in 10^{-3} M $(NH_4)H_2PO_4$, 0.5 ml packed cells in 50 ml for each of the three experiments described. In each case the algae suspension was placed in a "lollypop" illuminated from each side by General Electric DXB Photospot lamps (est. 190,000 lux, incident, 5% transmitted), and allowed to photosynthesize in 1.0% CO_2 in air for 10 min. Then the algae suspension, always illuminated, was flushed with either O_2, CO_2-free air, or N_2 for 2 minutes. Then 1.0 ml of $NaH^{14}CO_3$ solution (60 micromoles and 1.28 millicuries) was added to the algae in the "lollypop," which was immediately stoppered and shaken for 30 sec. The stopper was removed and the algae flushed for 90 sec with the same gas as prior to the addition of ^{14}C so that the algae photosynthesized ^{14}C-labeled compounds for 2 min in all. The algae were then killed in methanol (final suspension 80% methanol in water). The resulting mixtures were concentrated at room temperature in vacuo and an aliquot portion of each analyzed by paper chromatography and radioautography.[17] The amount of ^{14}C in each compound was then determined with a thin-window G-M tube. Another aliquot sample was applied to a planchette in each case and the total nonvolatile radioactivity was determined. In the killed algal suspension the pH was about 7, so that sodium glycolate was not lost from the planchette. After the paper chromatograms had been prepared, the paper was somewhat acidic (second solvent: butanol-propionic acid). Consequently, a certain amount of glycolic acid was lost from the papers, and the values reported represent a lower limit. We have found previously that the loss of glycolic acid from the paper is more than proportional to the amount of glycolic acid on the paper at the start, so that the percentage loss is less in the case of the papers with small amounts of glycolic acid. The results of these experiments are given in Table 2-I. The stimulation of glycolic acid production by O_2 is very large, while smaller stimulations are seen for the formation of phosphoglycolic acid and glycine. In comparison with the total fixation of $^{14}CO_2$ under N_2, the fixation under O_2 is down 30%. The radioactivity in ribulose diphosphate is over 30% less in O_2 and that in phosphoglyceric acid is 50% less. Marked inhibition in the formation of alanine, thought to be derived rather immediately from phosphoglyceric acid,[18] is caused by O_2.

Some of the values for the CO_2-free air are intermediate between those for O_2 and N_2. In other cases the levels of compounds in air are close to either the level in N_2 or that in O_2. Since the results show only the situation at the end of 2 min of extremely varying physiological conditions, such results can be expected.

By themselves, these results could be explained as consequences of some primary effect of oxygen on the reduced cofactors formed by the light reaction. However, since ribulose diphosphate is the substrate for the principal carboxylation reaction, and since the formation of glycolic acid and phosphoglycolic acid represents metabolic paths competing with the

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

18. D. C. Smith, J. A. Bassham, and Martha Kirk, Biochim. Biophys. Acta 48, 299 (1961).

formation of ribulose diphosphate from other sugar phosphates, the data support the thesis that oxygen stimulates the oxidation of the glycolaldehyde moiety formed from carbon atoms 1 and 2 of sugar phosphates and thereby inhibits CO_2 fixation and the rate of photosynthesis.

Table 2-I. Glycolic acid and other products from photosynthesis by Chlorella (amounts in μC: ^{14}C per g algae).

	Oxygen	CO_2-free air	Nitrogen
Total ^{14}C fixed	508.0	642.7	732.9
Glycolic acid	48.18	9.18	1.98
Phosphoglycolic acid	3.35	2.05	1.65
Sugar diphosphates[a]	60.99	95.70	92.55
Phosphoglyceric acid	20.13	25.31	40.59
Alanine	35.23	50.27	81.47
Glycine	27.62	21.70	5.44
Serine	17.10	21.69	17.98
Aspartic acid	24.32	32.76	27.61
Glutamic acid	4.45	11.57	8.63
Citric acid	1.37	2.23	1.76
Malic acid	32.45	52.37	55.61

a. 95% ribulose-1, 5-diphosphate in each case.

3. PHOSPHATASE ACTION ON PHOSPHOGLYCOLIC, 3-PHOSPHOGLYCERIC, AND PHOSPHOENOLPYRUVIC ACIDS IN SPINACH CHLOROPLAST FRAGMENTS IN THE PRESENCE AND ABSENCE OF HIGH CONCENTRATIONS OF METHANOL

Johannes Ullrich

Residual phosphatase activity persists in chloroplast fragments, algae, and several other biological materials suspended in alcohol as concentrated as 80%.[1] Such a stable phosphatase could hydrolyze metabolic phosphates in experimental samples after the usual "killing" procedure of adding 4 volumes of cold alcohol. If this were so, the resulting label distribution obtained from the paper chromatographic analysis would be invalid.

For this reason, I checked a number of ^{14}C-labeled phosphates appearing during photosynthetic ^{14}C fixation which were readily prepared by eluting appropriate spots from two-dimensional chromatograms. The main interest was in the hydrolysis of three phosphorylated hydroxy-acids:

(a) Phosphoglycolic acid, which according to Richardson and Tolbert[2] is rapidly split by phosphoglycolic acid phosphatase, and which is assumed by the same authors to be the only immediate precursor of free glycolic acid in photosynthesis.

(b) 3-Phosphoglyceric acid, which can be phosphatased to free glyceric acid; however, Mortimer considered this was able to account for only part of the free glyceric acid formed in certain higher plants, e.g., soy beans.[3,4,5] Mortimer therefore deduced the existence of a different and not yet explored pathway of photosynthesis, involving glyceric acid derived from another precursor.

(c) Phosphoenolpyruvic acid, which, as a high-energy phosphate, was supposed to be easily phosphatased even in mixtures with high alcohol content, because of its higher reactivity in comparison with phosphoglycolic and phosphoglyceric acids.

Experimental Procedure

Substrates

Spots of ^{14}C-labeled compounds as listed below were cut from paper chromatograms obtained from usual photosynthesis experiments and were run in the first dimension with either phenol-water (73:27) or "semistench"[6]

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1. J. Ullrich and M. Calvin, Biochim. Biophys. Acta 57, 190 (1962).
2. K. E. Richardson and N. E. Tolbert, J. Biol. Chem. 236, 1285 (1961).
3. D. C. Mortimer, Naturwiss. 45, 116 (1958).
4. D. C. Mortimer, Can. J. Botany 38, 623 (1960).
5. D. C. Mortimer, Can. J. Botany 39, 1 (1961).
6. Semistench: 1.2 g EDTA (= $[-N(CH_2-COOH)_2]_2$) + 100 ml 27% aq. NH_3 + 950 ml H_2O + 350 ml n-PrOH + 75 ml i-PrOH + 75 ml n-BuOH + 2.5 l i-butyric acid; aged for at least 24 h before use. J. Crowley, V. Moses, and J. Ullrich, J. Chromatog. (in preparation); Bio-Organic Chemistry Quarterly Report, September through November 1962 (in preparation).

for about 25 or 40 h, respectively, and in the second dimension with butanol-propionic acid-water[7] for about 20 h.

Substrates used for the experiments

Phosphoglycolic acid	Ribose-5-phosphate
3-Phosphoglyceric acid	Glucose-6-phosphate
Phospho-enol-pyruvic acid	Fructose-6-phosphate
Dihydroxyacetone-phosphate	Sedoheptulose-7-phosphate
6-Phosphogluconic acid	Ribulose-1, 5-diphosphate
Uridine-diphosphoglucose	Fructose-1, 6-diphosphate

The spots were washed with diethyl ether to remove traces of the chromatographic solvents, and subsequently eluted with water. The activity of the substrate solutions varied within a wide range and depended mainly on the original activity of the eluted spots, the number of spots available for elution, and--to a minor extent--on the amount of water applied for the elution. When not immediately used, the substrate solutions were stored frozen.

Chloroplast fragments

Spinach chloroplasts were isolated by the aqueous procedure of Park et al.,[8] and, after resuspension in 0.025 M phosphate buffer pH 7.5, sonicated for 90 sec in a Raytheon sonic oscillator. The chlorophyll concentration of the suspension was adjusted to about 2.0 mg/ml. When not used immediately, the chloroplast fragment suspension was stored in a deep freeze. This storage resulted in a considerable loss of photosynthetic activity, but only slightly affected the phosphatase activity under investigation.

Incubation experiments

The above substrate solutions were mixed with equal volumes of 0.2 M glycylglycine-Na buffer, pH 7.5. To samples (0.1 ml) of these mixtures, contained in small screw cap vials, 0, 0.2, or 0.8 ml of methanol was added to make the alcohol concentration in the incubation mixtures 0, 50, or 80%, respectively. After adjustment to the desired temperature, 0.1 ml of chloroplast fragment suspension was added to each vial (zero time), and the samples were allowed to stand in weak daylight for the desired incubation times. The incubation was then stopped by heating the closed vials in boiling water for 3 to 4 min; methanol was added when necessary to bring the final concentration in each case to 80% for this procedure. The total samples were later spotted onto the origins of one-dimensional paper chromatograms which were run in "semistench" for about 15 h to the edge of the paper (45 to 50 cm). The spots, localized by radioautography on single-coated x-ray film, were subsequently counted for relative activity by an automatic spot-counting machine constructed

7. Equal volumes of (a) 3.7 l n-BuOH + 250 ml H_2O; and (b) 1.8 l propionic acid + 2.2 l H_2O mixed immediately before use.

8. R. B. Park, N. G. Pon, K. P. Louwrier, and M. Calvin, Biochim. Biophys. Acta 42, 27 (1960).

and described by V. Moses and K. K. Lonberg-Holm in this Laboratory.[9] The two tubes of this machine usually count about 6% of the total ^{14}C disintegrations occurring in spots in Whatman No. 4 paper, which was used for the described experiments.

Results

Sugar phosphates

Incubation of the sugar phosphates listed above with freshly prepared chloroplast fragments at room temperature without addition of methanol for 10 to 60 min resulted in the formation of a variety of other metabolic products, among them sucrose, sugar phosphates different from the starting material, amino acids, and glycolic acid, which was of interest for another investigation. In no case could the dephosphorylated starting material be identified on the one-dimensional chromatograms made from the samples. In the presence of 50% or 80% methanol there was no detectable reaction, either at 0° or at room temperature.

Phosphoglycolic acid

Incubation of phosphoglycolic acid with freshly prepared chloroplast fragments at room temperature without addition of methanol led to rapid hydrolysis of the ester bond, as is shown in Table 3-I. No other products appeared on the chromatograms, which, after drying, were sprayed with saturated $NaHCO_3$ solution in order to prevent the glycolic acid from volatilizing during the film exposure. The losses were kept very small by this procedure.

Incubation of phosphoglycolic acid with previously frozen chloroplast fragments in a mixture containing 50% methanol at 0° gave approximately the same rate of cleavage. This is demonstrated in Table 3-II. Performance of the same experiment in a methanol concentration of 80% yielded no free glycolic acid.

Phosphoglyceric acid

The hydrolysis of phosphoglyceric acid was found to be, under all circumstances, much slower than that of phosphoglycolic acid. In the absence of methanol, fresh chloroplast fragments at room temperature formed as great a variety of compounds from phosphoglyceric acid as from sugar phosphates, so that quantitative measurements could not be made on one-dimensional chromatograms. The amount of free glyceric acid observed was small in any case.

Incubation with frozen, stored chloroplast fragments at 0° in mixtures containing 50% methanol resulted in a slow hydrolysis of phosphoglyceric acid (Table 3-III) without the formation of any other compound. In 80% methanol, under otherwise the same conditions, no hydrolysis of phosphoglyceric acid could be detected.

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9. V. Moses and K. K. Lonberg-Holm, in Bio-Organic Chemistry Quarterly Report, UCRL-10112, March, 1962.

Table 3-I. Cleavage of phosphoglycolic acid at 22° in the absence of methanol.

Time (min)	Activity (counts/min) Glycolic acid	Phosphoglycolic acid	Σ	Glycolic acid (%)
0	0	3260	3260	0
2	1620	1500	3120	52
5	2580	610	3190	81
10	3020	260	3280	92
20	3140	70	3220	98
60	3150	20	3170	100

Table 3-II. Cleavage of phosphoglycolic acid at 0° in 50% methanol.

Time (min)	Activity (counts/min) Glycolic acid	Phosphoglycolic acid	Σ	Glycolic acid (%)
0	15	1225	1240	1
0.5	180	1100	1280	14
1	470	790	1260	37
2	520	700	1220	43
3.5	595	710	1305	46
5	825	450	1275	65
7.5	1030	210	1240	83
10	1210	80	1290	94
20	1230	20	1250	98
60	1270	30	1300	98

Table 3-III. Cleavage of phosphoglyceric acid at 0° in 50% methanol.

Time	Activity (counts/min) Glyceric acid	Phosphoglyceric acid	Σ	Glyceric acid (%)
0	65	5810	5875	1
10 min	105	5350	5455	2
1 h	190	5220	5410	4
5 h	400	4160	4560	9
25 h	1270	3670	4940	26
100 h	3640	2030	5670	64

Phosphoenolpyruvic acid

In the absence of methanol, phosphoenolpyruvic acid was metabolized by the previously frozen chloroplast fragments at room temperature to several compounds (fewer than with sugar phosphates or phosphoglyceric acid as starting material; so they could be separated by one-dimensional chromatography), as shown in Table 3-IV. Among them were pyruvic acid and alanine, its transamination product. There was also an impurity always showing up in the same quantity (X).

In mixtures containing 50% methanol, the rate of hydrolyses of phosphoenolpyruvic acid at 0° was of the same order as that of phosphoglyceric acid under the same conditions. Table 3-V shows the results. No free pyruvic acid could be detected in samples treated in the same way, but containing 80% methanol.

Discussion

Of the phosphates examined, only phosphoglycolic acid is phosphatased at a very high rate, at least in mixtures containing from 0 to 50% methanol. It is quite likely that the crucial phosphatase, if not destroyed immediately by heating, often remains active for a while even in 80% methanol, especially when fresh chloroplasts or algae are used for the experiments instead of the frozen, stored ones which have already been damaged to a certain extent by the freezing and thawing procedures. These data suggest that a considerable fraction of the glycolic acid found in photosynthesis experiments is due to cleavage of phosphoglycolic acid after the still rather common "killing" procedure. This procedure consists of simply dumping the samples, withdrawn from a lollipop or another incubation apparatus, into 4 volumes of cold methanol, storage for some time (a few minutes up to several hours), and subsequent heating which definitely kills all the enzymes.

The results of this investigation furthermore strongly support Tolbert's theory[2] that when the samples are properly killed all the glycolic acid is derived from phosphoglycolic acid, formed first and then phosphatased during the experimental incubation period itself by the above phosphatase. The latter has a very high activity and seems to be specific for organic phosphates of low molecular weight, like simple alkyl phosphates[1] and phosphoglycolic acid. It might even be identical with Tolbert's recently isolated phosphoglycolic acid phosphatase,[2] which has not yet been tested in the presence of high alcohol concentrations.

Phosphoglyceric acid, on the other hand, is phosphatased so slowly, even in the absence of methanol, that the results of this work cannot be used, as far as isolated spinach chloroplasts are concerned, either for or against Mortimer's proposal[3, 4, 5] of another pathway in photosynthesis to explain the appearance of appreciable quantities of free glyceric acid. But the results show that there is not much danger of getting additional free glyceric acid by the action of the surviving phosphatase after "killing" samples with cold methanol and heating them several hours later.

Table 3-IV. Conversion of phosphoenolpyruvic acid at 22° in the absence of methanol.

Time (min)	Activity (counts/min) Alanine	Y[a]	Pyruvic acid	P-enol-pyruvic acid	X	Σ	Alanine	Pyruvic acid (%)	Alanine + pyruvic acid (%)
0	0	0	155	1620	170	1945	0	8.0	8.0
2	45	40	215	1500	270	2070	2.2	10.4	12.6
5	90	50	335	1330	275	2080	4.3	16.1	20.4
10	180	80	470	1210	345	2285	7.9	20.7	28.6
25	435	155	475	450	350	1865	23.3	25.5	48.8
60	300	290	300	130	245	1675	42.4	17.9	60.3

a. Unidentified spot

Table 3-V. Cleavage of phosphoenolpyruvic acid at 0° in 50% methanol.

Time	Activity (counts/min) Pyruvic acid	Phosphoenol-pyruvic acid	X	Σ	Pyruvic acid (%)
0	180	1720	225	2125	8.5
10 min	165	1660	200	2025	8.2
60 min	260	1510	210	1980	13.1
5 h	335	1380	230	1945	17.3
25 h	875	880	250	2005	43.7
50 h	1380	420	245	2045	67.5

Although phosphoenolpyruvic acid, in the absence of methanol, is metabolized at a moderate rate to pyruvic acid and further to alanine, the hydrolysis of this substance in alcoholic mixtures, like that of phosphoglyceric acid, was found to be too slow to interfere seriously with the results obtained by the usual "killing" procedure and subsequent paper chromatography. This is even more true for all the sugar phosphates tested.

Summary

Sonically broken spinach chloroplasts, before and after storage in the frozen state, were checked for their ability to phosphatase ^{14}C-labeled phosphoglycolic, phosphoglyceric, and phosphoenolpyruvic acids, and a variety of sugar phosphates, in aqueous mixtures at room temperature, and in 50% and 80% aqueous methanol at 0°.

Only phosphoglycolic acid was found to be hydrolyzed very rapidly under the conditions examined, except in 80% methanol. The other organic phosphates tested were metabolized in the absence of methanol to a number of other compounds usually built up during photosynthesis; in 50% methanol they were hydrolyzed either very slowly (phosphoglyceric and phosphoenolpyruvic acids) or not at all (sugar phosphates), in 80% methanol they remained unchanged.

4. ABSORPTION SPECTRA OF SCATTERING SAMPLES. I. AN EVALUATION OF THREE DIFFERENT SPECTROPHOTOMETRIC TECHNIQUES USING CHLORELLA

Kenneth Sauer

The determination of the true in vivo absorption spectra of photosynthetic organisms has been the subject of much experimental investigation. Extensive surveys of the problems involved have been prepared by Rabinowitch[1, 2] and by French.[3] Whole leaves and suspensions of chloroplasts, algae, photosynthetic bacteria, etc. give rise to appreciable and often overwhelming amounts of light scattering resulting from refractive index gradients that exist in these systems. This study is aimed at the evaluation of a new experimental approach to the problem of obtaining absorption spectra undistorted by scattering.

The green alga Chlorella pyrenoidosa has been the subject of the majority of the most recent studies of the effects of light scattering on the measurement of absorption spectra of photosynthetic systems. For this reason Chlorella is a useful organism to use as a test of the present technique. The early transmission and absorption spectra using conventional spectrometers showed considerable variation in the positions and relative magnitudes of the various absorption bands. The wavelength of the main red chlorophyll absorption maximum was variously reported[1] between 668 and 680 mμ, for example.

Since 1954 a number of different experimental approaches have been studied in the effort to overcome the effects due to light scattering. One of the simplest and most widely used of these resulted from the use of a diffusing screen (opal glass, filter paper impregnated with paraffin oil, etc.) as described by Shibata, Benson, and Calvin.[4] The opal glass technique applied to Chlorella resulted in an absorption spectrum greatly improved with respect to the direct transmission measurements and approaching the quality obtained with pigment extracts in organic solvents. Amesz, Duysens, and Brandt have proposed a modification of the opal glass technique using a fluorescent scattering screen,[5] which is particularly useful at wavelengths below 315 mμ, where opal glass becomes opaque. A different approach was used by Barer, in which he adjusted the refractive index of the suspending medium by addition of a soluble protein until it matched the refractive index of the suspended particle.[6] The resulting improvement in the discrimination

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1. E. I. Rabinowitch, in Photosynthesis (Interscience Publishers, Inc., New York, 1951), vol. II, part 1 pp. 672-730.
2. E. I. Rabinowitch, ibid, part 2, pp. 1841-1867.
3. C. S. French, in Encyclopedia of Plant Physiology A. Pirson, Ed. (Springer Verlag, Berlin, 1960), vol. V/1, pp. 252-97.
4. K. Shibata, A. A. Benson, and M. Calvin, Biochim. Biophys. Acta 15, 461 (1954).
5. J. Amesz, L. N. M. Duysens, and D. C. Brandt, J. Theoret. Biol. 1, 59 (1961).
6. R. Barer, Science 121, 709 (1955).

of algal suspensions is limited by the fact that the algal cell is not a homogeneous particle with a single refractive index, but contains a pigmented chloroplast whose refractive index varies significantly with wavelength, especially in the region of absorption bands of the chloroplast pigments. Studies of these complications by Latimer[7] and by Charney and Brackett[8] have shown that the variation of refractive index with wavelength in the vicinity of pigment absorption bands is such that scattering from Chlorella is greater on the long-wavelength side and less on the short-wavelength side of such a band, even when the refractive index of the medium is adjusted to match that of the intracellular material exclusive of the chloroplast. Consequently, transmission spectra obtained for such "adjusted" suspensions are always distorted to some extent. In some instances spectrophotometers using Ulbicht spheres[9, 10] or wide-angle microscope attachments[8] have been used to obtain absorption spectra of scattering materials. In spite of the manifold problems associated with these techniques, they have yielded some of the best absorption spectra to date.

Experimental Procedure

In this study a relatively new device commercially available as an attachment for a Cary Model 14 spectrophotometer has been evaluated. The apparatus, Model 1462 scattered-transmission accessory (Applied Physics Corp., Monrovia, Calif.), utilizes an end-window photomultiplier of 2 in. diameter in close proximity to the sample and reference cuvettes. The attachment is used in place of the normal sample compartment and photomultiplier housing of the spectrophotometer, and the optical arrangement is such that transmitted and scattered light in the forward direction are measured over a large solid angle.

For these measurements an RCA 6217 photomultiplier, exhibiting good sensitivity in the far red, was used. With this detector reliable spectra with good resolution (slit widths less than 0.2 mm) can be obtained from 300 to 900 mμ. The range can readily be extended into the ultraviolet region to at least 200 mμ if a comparable uv-sensitive photomultiplier (e. g. Dumont 7664) is used. The sample and reference cuvettes were of standard design having square horizontal cross section of 10×10 mm inside dimensions and having four transparent sides. The sample and reference cuvettes were side by side, separated by about 1 mm, and the distance from the centers of the cuvettes to the photosensitive surface of the detector was about 1/2 in. Since the effective diameter of the photosensitive surface was 1-11/16 in., the solid angle subtended was about Π radians with respect to the center of the cuvette.

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7. P. Latimer, Plant Physiol. 34, 193 (1959).
8. E. Charney and F. S. Brackett, Arch. Biochem. Biophys. 92, 1 (1961).
9. G. S. Rabideau, C. S. French, and A. S. Holt, Am. J. Botany 33, 769 (1946).
10. Govindjee, C. Cederstrand and E. Rabinowitch, Science 134, 391 (1961).

A sample of Chlorella pyrenoidosa in its culture medium was centrifuged at 600 × g for 5 min, the precipitated algae resuspended in an equal volume of distilled water and recentrifuged, and the final precipitate resuspended in 1.25 times the original volume of water for the spectrum measurements.

Results and Discussion

The spectra obtained by three different experimental techniques are summarized in Fig. 4-1. The same suspension of Chlorella pyrenoidosa was used for each spectrum. The upper curve represents the transmission spectrum obtained in the conventional manner, in which the detector photomultiplier (1P28) subtends only a small angle in the forward direction from the sample. Water in an identical cuvette was used as the reference material. The spectrum using opal glass (middle curve) was obtained by placing pieces of opal glass adjacent to the reference and sample cuvettes at the sides toward the photomultiplier. The scattered-transmission spectrum (lower curve) was obtained by using the scattered-transmission accessory described above. A general upward displacement of curves to higher absorbances is due to light scattered out of the measuring beam and not reaching the detector. This effect is particularly evident at wavelengths longer than 750 mμ, where pigment absorption is negligible. The transmission spectrum is the most seriously affected by scattering losses. Although the use of opal glass brings about a considerable reduction in the magnitude of the apparent absorbance due to scattering, it is clear from the figure that a significant further improvement is obtained by using the scattered-transmission technique.

Some further important advantages of the scattered-transmission method should be noted. Use of opal glass in both beams of the spectrometer has the consequence that the intensity of light incident upon the photomultiplier is greatly reduced in comparison with either of the other two methods. The reduction of the reference beam intensity, in particular, has the effect of causing the automatic slit-control mechanism of the spectrophotometer to open the slits considerably at all wavelengths. Not only is the spectral resolution adversely affected as a consequence, but the range of wavelengths over which measurements can be made at the same instrument settings is considerably contracted. Under the conditions used the slit opened to its maximum value of 3.0 mm at 350 mμ at the lower end and 710 mμ at the upper end of the spectrum. When the scattered-transmission attachment was used the slit widths at the same two wavelengths were less than 0.01 mm. This difference is especially significant when, as in photosynthetic systems, one is interested in absorption properties in spectral regions where existing detectors have inherently low sensitivities.

The displacement of the red absorption maximum of chlorophyll a from 679 mμ in the scattered-transmission spectrum to 683 mμ in the transmission spectrum results from the variation of scattering with wavelength in the neighborhood of an absorption band.[7,8] The increase in resolution and decrease of scattering distortions resulting from the use of the scattered-transmission accessory permits the observation of spectral features that are smoothed out or covered up in the other spectra. In the lower curve in Fig. 4-1 it is possible to discern some structure in the red absorption band, especially a fairly strong shoulder at 672 mμ on the side of the main peak at 679 mμ.

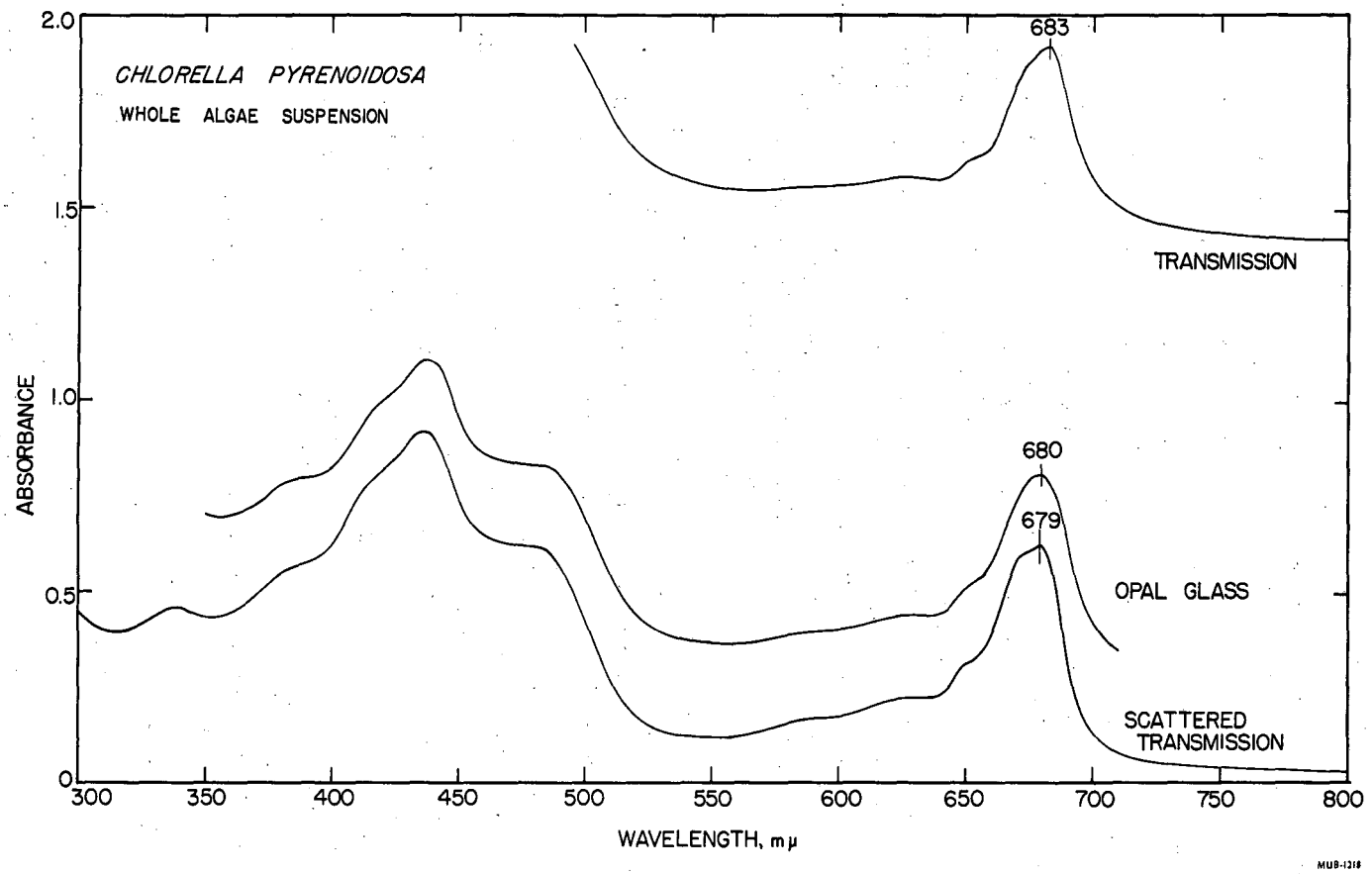

Fig. 4-1. Chlorella absorption spectra.
Upper curve: transmission spectrum using standard sample and detector compartments of Cary Model 14 spectrophotometer.
Middle curve: opal glass adjacent to sample and reference cuvettes on exit sides; same spectrophotometer arrangement.
Lower curve: scattered transmission spectrum using Cary Model 14 R spectrophotometer with Model 1462 scattered-transmission attachment.
Same suspension in each case; 1-cm cuvettes with four clear quartz sides.

Duysens has discussed the flattening of absorption bands due to the enhanced mutual shading of the pigment molecules in the suspension. [11] Subsequently, Amesz, Duysens, and Brandt found that for Chlorella the flattening amounted to only 1% if all the scattered light within 70° of the forward direction is seen by the detector. [5] With the scattered-transmission accesory as used here the corresponding angle was 60°; thus, when this technique is used the extent of flattening should be little different from that observed by Amesz, Duysens, and Brandt.

We may summarize the advantageous features of the scattered-transmission accessory as follows:

(a) by collecting scattered radiation over a wide angle it minimizes anomalous absorption band shifts or flattening;

(b) it reduces the turbidity (apparent absorbance due to light scattering) significantly below that obtained by using opal glass;

(c) it has much greater sensitivity in the sense that the photomultiplier output is approximately 10^5 times as great as with opal glass;

(d) a 300-fold reduction in the spectrophotometer slit width compared with that for opal glass results in greater resolution and in a wider usable wavelength range, and

(e) it extends the range of measurements into the ultraviolet to the limit of detector sensitivity ($\lambda < 200$ mμ).

Of particular interest in photosynthesis studies is the high detector sensitivity. This permits the study of difference spectra for scattering samples that also have high absorption.

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11. L. N. M. Duysens, Biochim. Biophys. Acta 19, 1 (1956).

5. ABSORPTION SPECTRA OF SCATTERING SAMPLES. II. SCATTERED TRANSMISSION SPECTRA OF LEAVES, CHLOROPLASTS, AND QUANTASOMES OF SPINACH

Kenneth Sauer

The scattered-transmission technique for measuring absorption spectra of scattering samples, described in the preceding paper in this report, has been used to study the absorption properties of spinach leaves and of a number of preparations derived therefrom. The principal purpose of this study is to learn the nature of the differences which exist among these spectra and to determine how close a correlation exists between the measured absorption spectra and the ability of the preparation to use light to generate chemical potential.

Absorption spectra of spinach leaves of chloroplast preparations have been reported by Rabideau, French, and Holt[1] and by Moss and Loomis.[2] In general, the quality of these spectra was not such that accurate wavelength maxima could be obtained; however, the conclusion reached by both groups was that, within experimental limitations, there seemed to be no real shifts in absorption maxima in going from whole leaves to chloroplast suspensions and even to preparations of disintegrated chloroplasts. Smith, however, had found that detergents such as bile salts and sodium desoxycholate did cause significant changes in the absorption spectra of aqueous leaf extracts.[3]

The absorption spectra shown in Fig. 5-1 were obtained by using the Cary 14 spectrophotometer with the Model 1462 scattered-transmission accessory. The upper curve represents the spectrum of a piece (10×30 mm) cut from a moderately pigmented spinach leaf about 6 in. long. The area chosen was free of veins or other obvious blemishes. The piece excised was immersed in a spectrophotometer cuvette of 1-cm^2 cross section (four clear sides) filled with distilled water. In this case a similar reference cuvette filled with distilled water contained a 10×30-mm piece of opal glass, which served to lower the recorded absorbance curve by about 0.25 unit. No correction has been made for this in the figure. The lower curve of Fig. 5-1 was obtained from a crude leaf homogenate prepared by taking 125 g of pieces of washed spinach leaves from which the stems and large veins had been removed and homogenizing for 30 sec at full speed in a Waring Blendor at 0°C in a solution containing 0.5 M sucrose, 0.1 M phosphate buffer (pH 7.4), and 0.01 M versenol. The homogenate, strained through eight layers of cheesecloth, was stored briefly at 0°C.[4] Just before the measurement a portion of the leaf homogenate was diluted 15-fold with water and the spectrum run against a reference cuvette containing water. (No opal glass was used for this or subsequent spectra.)

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1. G. S. Rabideau, C. S. French, and A. S. Holt, Am. J. Botany 33, 769 (1946).
2. R. A. Moss and W. E. Loomis, Plant Physiol. 27, 370 (1952).
3. E. L. Smith, J. Gen. Physiol. 24, 565 (1941).
4. These and subsequent preparation procedures are outlined by R. B. Park and N. G. Pon, J. Mol. Biol. 3, 1 (1961).

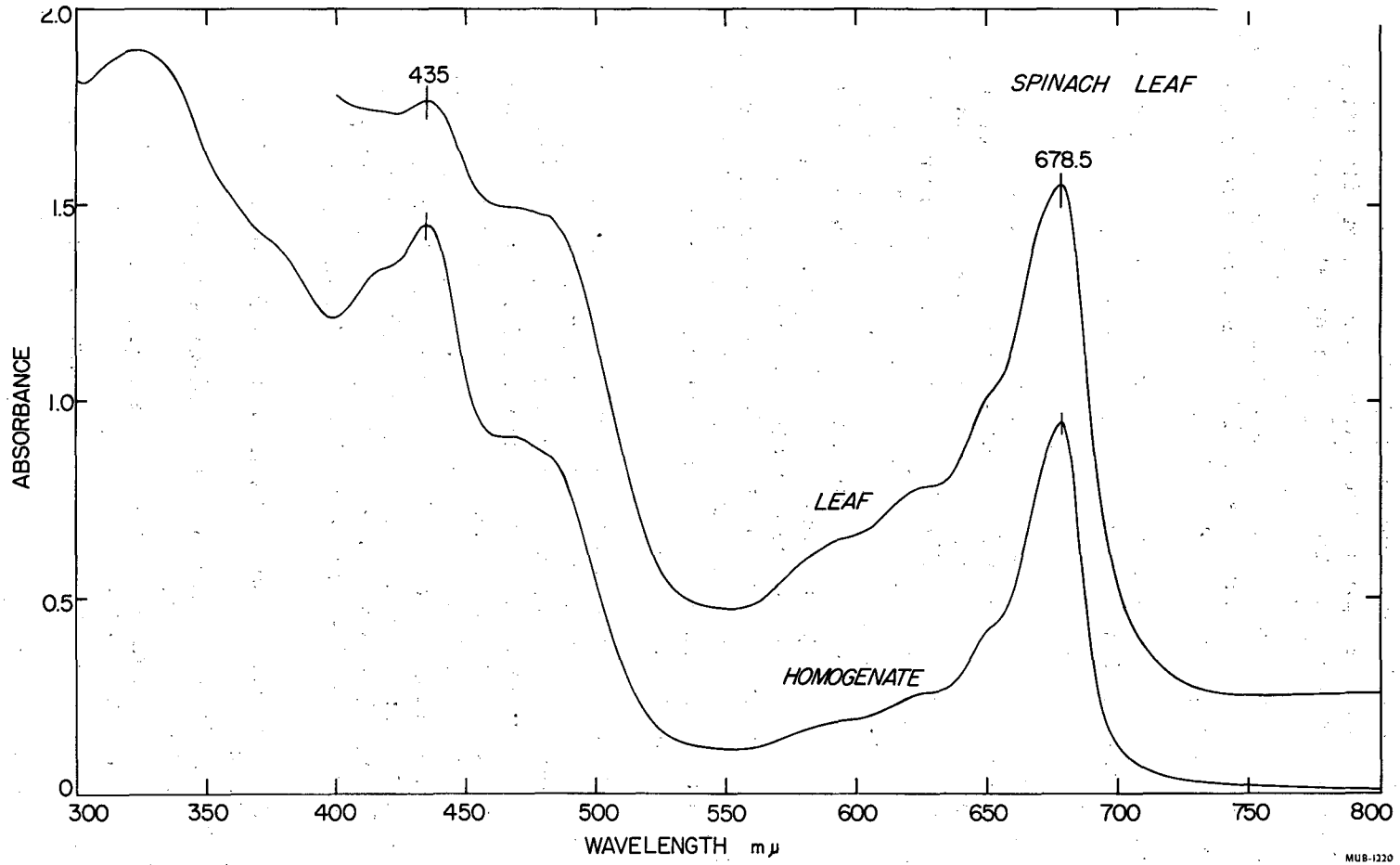

Fig. 5-1. Spinach-leaf absorption spectra from 300 to 800 mμ.
Upper curve: intact leaf immersed in water versus opal glass in water in reference beam.
Lower curve: total leaf homogenate in sucrose, phosphate buffer (pH 7.4), versenol (see text) vs water in reference beam.
Model 1462 scattered-transmission attachment used with Cary Model 14 R spectrophotometer.

The spectra shown in Fig. 5-2 were recorded for materials obtained in the following way: The crude homogenate described above was centrifuged at 200 g for 5 min at 0°. The resulting supernatant was centrifuged at 600 g for 15 min at 0°. The supernatant from this centrifugation, diluted eightfold with water, was used for the spectrum shown as a dashed curve marked "supernatant" in Fig. 5-2. The precipitate from the second centrifugation was resuspended in 40 ml 0.5 M sucrose and 10^{-3} M phosphate buffer (pH 7.4) and centrifuged again for 15 min at 600 g and 0°. The precipitate of washed chloroplasts was resuspended in 15 ml of distilled water. A 100-fold dilution with water of a portion of this suspension was used for the spectrum shown as a solid curve labeled "chloroplasts" in Fig. 5-2.

For comparison purposes, a spectrum of spinach quantasomes (free of stroma substances) is presented in Fig. 5-3. The preparation of this sample has been described previously.[5]

Of particular interest in this study are the similarities and differences among the spectra of Figs. 5-1, 5-2, and 5-3. At wavelengths throughout most of the visible and near-infrared regions ($\lambda > 425$ mμ) the similarities are more striking than the differences. The red absorption maximum of chlorophyll a occurs at a wavelength of 678.5 ± 0.5 mμ in each of the spectra shown. Thus for all materials, from the intact leaf to the preparation containing the smallest quantasome aggregates studied so far, there seems to be no measurable shift of this absorption maximum. This statement can be extended to include the monodisperse pigment protein complex of Biggins and Park prepared by sonication of extracted spinach chloroplasts in the presence of cysteine.[6] It is significant to note that activity in the form of ability to accomplish photo-induced oxygen evolution via the Hill reaction has been demonstrated for each of these preparations.

Butler, using an instrument with an end-window photomultiplier in an arrangement similar to that of the scattered-transmission accessory, has reported the absorption spectra of green bean leaves at room temperature and at -196°.[7] In the room-temperature measurements he observed the main chlorophyll a band at 678 mμ, with a shoulder at 650 mμ for chlorophyll b. The low-temperature spectra, with improved band resolution, showed additional complexity, including a long-wavelength absorption at 705 mμ. Studies using the opal-glass technique on mature leaves of spinach,[8] bean,[8, 9] and prunus[8, 9] showed the chlorophyll a maximum at 676 mμ. Studies using etiolated leaves of bean[10, 11, 12] and sugar beet[13] showed that, following

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5. Kenneth Sauer, in Bio-Organic Chemistry Quarterly Report, UCRL-10156, April 1962, pp. 54-68.
6. John Biggins and R. B. Park, in Bio-Organic Chemistry Quarterly Report, UCRL-10350, June 1962, p. 48.
7. W. L. Butler, Arch. Biochem. Biophys. 93, 413 (1961).
8. K. Shibata, J. Biochem. (Tokyo) 45, 599 (1958).
9. K. Shibata, A. A. Benson, and M. Calvin, Biochim. Biophys. Acta 15, 461 (1954).
10. K. Shibata, J. Biochem. (Tokyo) 44, 147 (1957).
11. W. L. Butler, Arch. Biochem. Biophys. 92, 287 (1961).
12. J. C. Goedheer, Biochim. Biophys. Acta 51, 494 (1961).
13. A. A. Krasnovsky and L. M. Kosobutskaya, Proc. Acad. Sci. S.S.S.R. 104, 440 (1955).

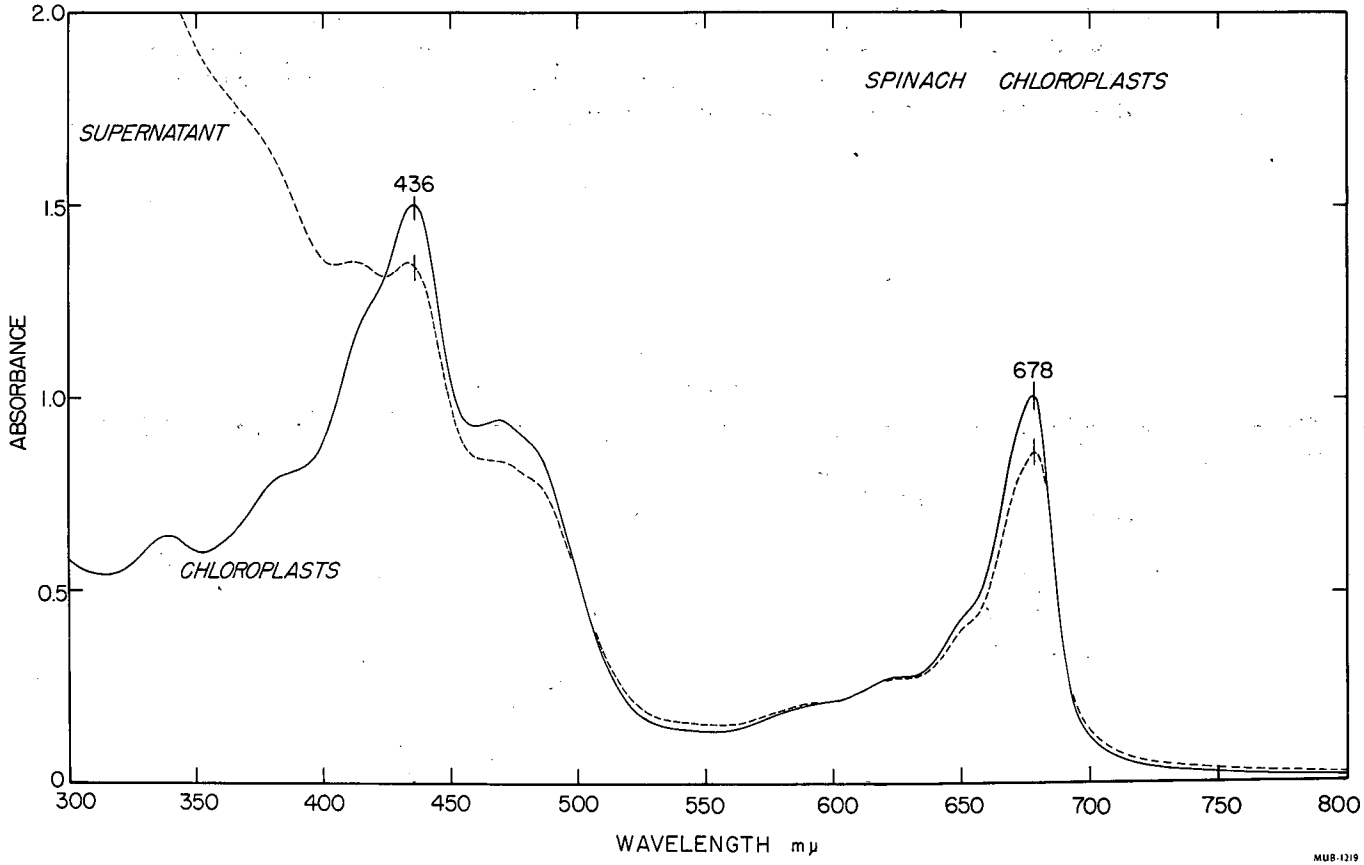

Fig. 5-2. Spinach chloroplast absorption spectra from 300 to 800 mμ.
Solid curve: washed chloroplasts.
Dashed curve: supernatant containing chloroplast fragments, mitochondria, soluble cell cofactors, etc. (see text for details).
Model 1462 scattered-transmission attachment used with Cary Model 14 R spectrophotometer.

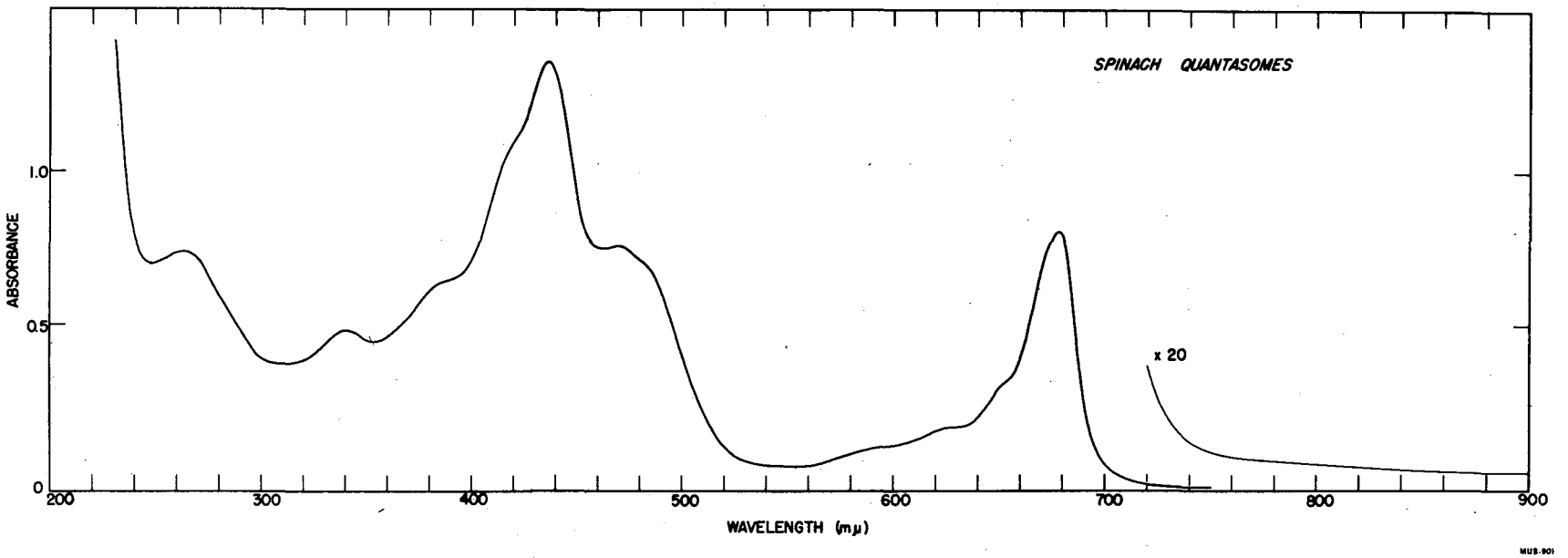

Fig. 5-3. Spinach quantasome absorption spectrum from 200 to 900 mμ. Sample prepared from sonicated spinach chloroplasts; precipitate between 45,000 g and 145,000 g resuspended and resonicated; supernatant from subsequent 20,000-g centrifugation. Curve at right represents 20-fold greater concentration (see ref. 6 for details).

absorption shifts occurring during the greening process, the main chlorophyll a band has its maximum absorption at 677 mμ. Considerable complexity in this long-wavelength absorption band for mature leaves has been reported from studies using the technique of derivative spectrophotometry, [14, 15] and, recently, direct absorption spectrophotometry. [16, 17] Except for the shoulder at about 673 mμ, which appears in all the spectra from spinach in Figs. 5-1, 5-2, and 5-3, none of these subbands has been observed for spinach when the scattered-transmission accessory was used. Recently, in two cases, it has been reported that by prolonged centrifugation of leaf homogenates it was possible to achieve partial fractionation of forms of chlorophyll that differ in their absorption maxima. [18, 19] As noted previously, [5] no such separation or absorption shift has been observed in these studies. This latter observation has been confirmed by similar experiments on sonicated chloroplasts by Butler. [20]

In the blue and the near-ultraviolet regions of the spectrum ($\lambda < 425$ mμ) there are substantial differences among the various spectra shown in Figs. 5-1, 5-2, and 5-3. These differences can be accounted for qualitatively in terms of three major fractions of the leaf cells: the chloroplast lamellar structures containing the photosynthetic pigments, the leaf mitochondria, and the matrix fluid of the cells and chloroplasts. The leaf homogenate (Fig. 5-1) contains all three components approximately in the proportions in which they occur in the intact leaf. By low-speed centrifugation they can be separated into two fractions (Fig. 5-2) in which the chloroplasts are largely precipitated and the mitochondria remain in the supernatant. The colorless intracellular substances are chiefly present in the supernatant fraction and account for its strong absorption in the ultraviolet. Similar or identical substances with strong ultraviolet absorption can be extracted by osmotic rupture of washed chloroplasts, using water. The spectrum of this extract (Ref. 5, Fig. 11-5) is similar in the near ultraviolet to that of the supernatant fraction shown in Fig. 5-2. The spectrum of quantasomes from which stroma materials have been removed (Fig. 5-3) exhibits much lower absorption in the ultraviolet. Those bands which do occur can be accounted for in terms of known absorption bands of the photosynthetic pigments and, at the shortest wavelengths, of colorless lipoprotein. [5] The ultraviolet spectra of washed chloroplasts (Fig. 5-2) and of quantasomes (Fig. 5-3) are not markedly different from one another down to 300 mμ; however, they probably show greater divergence at still shorter wavelengths (e.g. see Ref. 5, Fig. 11-2).

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14. C. S. French and R. F. Elliott, Carnegie Institution of Washington Year Book 57, 278 (1958).
15. Y. F. Frei, Biochim. Biophys. Acta 57, 82 (1962).
16. Govindjee, C. Cederstrand, and E. Rabinowitch, Science 134, 391 (1961).
17. J. B. Thomas, Biochim. Biophys. Acta 59, 202 (1962).
18. A. A. Krasnovsky, M. I. Bystrova, and A. D. Sorokina, Proc. Natl. Acad. Sci. S.S.S.R. 136, 1227 (1961).
19. J. A. Gross (Illinois Institute of Technology) private communication.
20. Warren L. Butler (USDA Research Station, Beltsville, Md.) private communication.

6. THE EFFECT OF SONICATION OF SPINACH CHLOROPLASTS ON PHOTOSYNTHETIC PHOSPHORYLATION

Charles Prevost

In their paper on the correlation of structure with function in Spinacia oleracea chloroplasts, Park and Pon described a subunit of chloroplast lamellae that had the same dimensions as Fraction I protein but contained the photosynthetic pigments and carried out the light reactions of photosynthesis.[1] These green lamellar fragments consisted of as few as eight subunits and were capable of supporting CO_2 fixation in the light when the colorless supernatant was added back.

Recent studies by Park and Pon on the chemical composition of purified chlorophyll-containing lamellae[2] yield a minimum molecular weight of 960,000 for a lamellar subunit (a quantasome) on the basis of the manganese content. Electron microscope pictures of lamellae extracted with lipid solvents suggest that the lamellae consist of proteins embedded in a lipid matrix.

Biggins has succeeded in breaking up the quantasome aggregates into still smaller units by the use of cysteine.[3] He has characterized these units in the ultracentrifuge as protein-chlorophyll complexes that have a density of 1.174 g/ml, a sedimentation coefficient of 86 Svedberg units, and a probable molecular weight between one and two million. The visible spectra were the same for the aggregates, the monomers, the chloroplasts, and the leaves. The monomers as well as the aggregates exhibited Hill activity when ferricyanide was used as oxidant.

Since these particles could affect the light reactions involving electron transport from water to an oxidant, could they not as well perform photosynthetic phosphorylation and be called true photosynthetic units? The fragments of lamellae from the washed sonicated chloroplasts were assayed for photosynthetic phosphorylation, but with no success. The procedure for obtaining the small fragments was then investigated to find out where the loss of activity occurred and how it could be prevented.

Materials and Methods

Crystalline yeast hexokinase was purchased from the California Corporation for Biochemical Research. One ml of the solution containing 10 mg protein per ml, suspended in $(NH_4)_2SO_4$, 3.0 M, at pH 7.0, was dialyzed with stirring for 6 h at 5°C against 10 liters of TRIS (trishydroxyaminomethane), 0.01 M, pH 8.0. After dilution with the buffer, the concentration was approximately 19 μg protein per 10 μl. The enzyme was then kept frozen in the deep freeze. The activity of this preparation under the conditions of the assay was roughly 900 mμM glucose phosphorylated per 19 μg protein per min; this rate is almost 100 times the maximum rate at which ATP would be produced in the photosynthetic system.

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1. R. B. Park and N. G. Pon, J. Mol. Biol. 3, 1 (1961).
2. R. B. Park and N. G. Pon, Chemical Composition and the Substructure of Lamellae Isolated from Spinacia oleracea Chloroplasts, J. Mol. Biol., in press.
3. John Biggins (Lawrence Radiation Laboratory), private communication.

Uniformly labeled glucose-^{14}C was purchased from the California Corporation for Biochemical Research. It was purified chromatographically on washed Whatman No. 4 paper as follows: 4 h in the first dimension with ethyl acetate:pyridine:water (10:4:3) and 12 h in the second dimension with butanol:propionic acid:water (7:1:2.55). The specific activity of the purified glucose was 30 mCi/mM; 4 mμM was generally used, but diluted with 120 mμM of cold glucose.

Phenazine methosulfate (PMS) was purchased from Sigma Chemical Company.

The chloroplasts, in this experiment, were prepared as follows: 200 g fresh spinach leaves was ground up in a Waring Blendor for 30 sec with 250 ml of a buffer solution containing sucrose 0.5 M, TRIS 0.01 M, ascorbate 0.01 M, EDTA 0.001 M, and $MgCl_2$ 0.02 M at pH 7.8. The juice was filtered through 16 layers of cheesecloth, and large debris was spun down for 5 min at 200 g. The supernatant was then centrifuged for 15 min at 600 g, and the precipitate so obtained consisted of whole chloroplasts. These were resuspended in a buffer solution identical to the one used for grinding except that it contained no EDTA; the chlorophyll concentration, determined by the method of Arnon,[4] was 0.3 mg/ml. Aliquots of 15 ml were sonicated for various periods of time with a Raytheon sonic oscillator at 9 kc. These were then diluted fivefold with the second buffer solution, making the chlorophyll concentration 0.63 μg per 10 μl. All these operations were carried out at 0°C.

Adenosine triphosphate was assayed by trapping the ATP produced from ADP and inorganic phosphate (Pi) with the hexokinase reaction. The enzyme hexokinase catalyzes the conversion of glucose to glucose-6-P at the expense of ATP, thus regenerating ADP in the process. The composition of the assay medium is indicated in Table 6-I. The assay was carried out in the following way: The reactants were added to the reaction vessel in a nitrogen-filled box. Ten μl of yeast hexokinase and 10 μl of a solution containing buffer, substrates, and cofactors were pipetted to the bottom of 1/2-dram vials, which had previously been flushed with nitrogen gas for 10 min. Ten μl of the chloroplast preparation was added next. The screw-cap vial was immediately closed and placed in a dark box at 0°C for exactly 10 min. The vials were then shaken with a Vortex test tube mixer and were incubated at 16°C in a water bath. The samples were illuminated from the bottom with white light of approximately 7,000 foot candles intensity as measured at the level of the reaction mixture with a Weston light meter. After exactly 5 min of incubation, 150 μl of methanol was added to the mixture and the mixture was boiled for 2 min. The mixture and two water rinses of 100 μl each were spotted on washed Whatman paper No. 4. The chromatograms were developed overnight with butanol:propionic acid:water (18:37:24.5). Glucose usually moved at least twice as far as glucose-6-P. Glucose concentration was estimated by counting the ^{14}C activity associated with the glucose and glucose-6-P spots. The ratio of glucose-6-P to glucose plus glucose-6-P, multiplied by the initial glucose concentration equals the amount of glucose-6-P present. This concentration of glucose-6-P is equal to the amount of ATP produced. Dark controls accounted for adenylate kinase activity as well as for possible phosphatases that are not light-dependent.

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4. D. I. Arnon, Plant Physiol. 24, 1 (1949).

Table 6-I. Composition of the assay medium.

Ingredient	Amount (mμM)
TRIS (trishydroxyaminomethylmethane)	600
$MgCl_2$	200
ADP	100
Pi	1000
Ascorbate	100
PMS (phenazine methosulfate)	1
Glucose, cold	120
Glucose, hot	4
Chlorophyll	0.64 μg

Total volume 30 μl; pH 8.0

Results and Discussion

The grinding medium used by Park and Pon[1, 2] contained 0.01 M versenol to remove heavy elements that could inactivate the enzymes of the isolated chloroplasts. Jagendorf and Smith, however, found that a similar chemical, EDTA (versene), causes uncoupling of photosynthetic phosphorylation.[5] This effect could be prevented by the presence of cations. They also found that soluble protein could be extracted from the chloroplasts by suspension in 3×10^{-4} M $MgCl_2$ without affecting their phosphorylation activity; moreover, the activity was partially lost when the addition of 1×10^{-4} M EDTA caused some further protein extraction. This loss was irreversible. This uncoupling was also noticed when the chloroplasts were simply washed in water. Since this was one step in the preparation of the chloroplasts from which the purified chloroplast lamellae were obtained, very little phosphorylation activity could be expected from such a washed preparation.

The next step in the preparation of lamellae fragments was the sonication of the washed chloroplasts. In order to find out whether sonication itself could further impair the already decreased photosynthetic phosphorylation, chloroplasts prepared with EDTA, but protected with magnesium and unwashed, were sonicated for various periods of time up to 6 min. The plastids and large portions of plastids were counted under a light microscope with a Levy counting chamber. The curves showing the decrease in photophosphorylation and percentage of remaining plastids are shown in Fig. 6-1. The values vary from experiment to experiment, but always show a similar loss of activity. This probably depends upon the concentration of the plastids and the efficiency of the sonicator.

5. A. T. Jagendorf and M. Smith, Plant. Physiol. 37, 135 (1962).

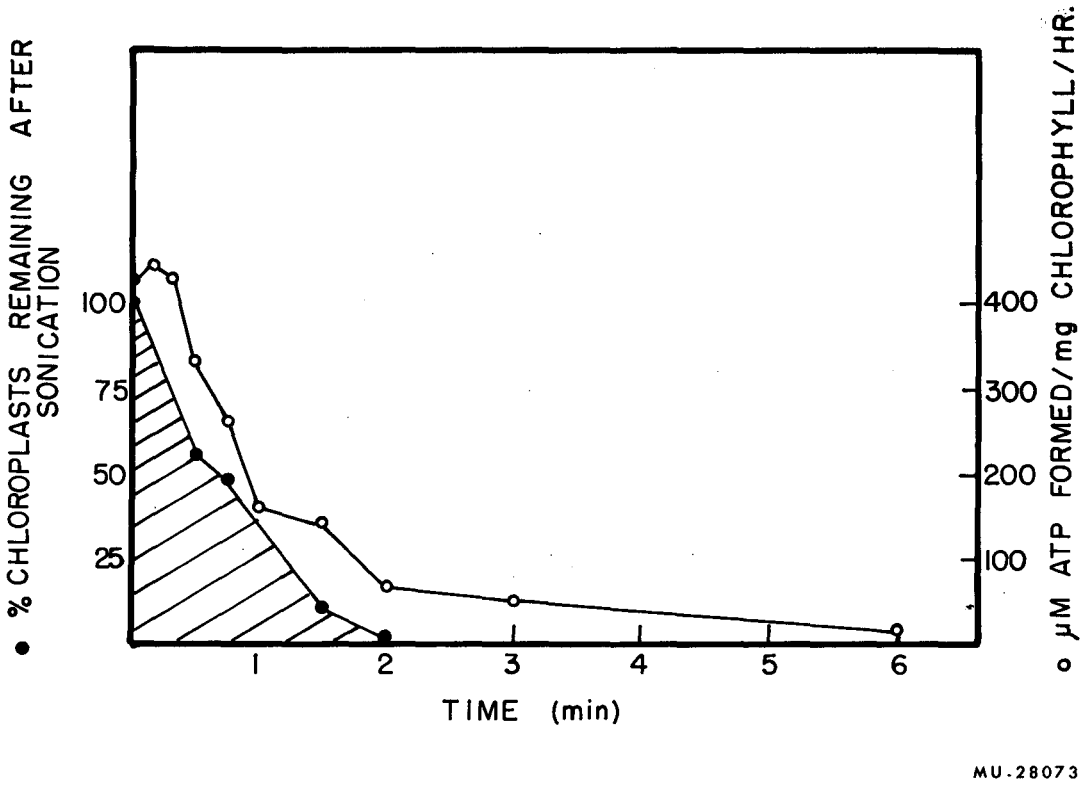

Fig. 6-1. Effect of sonication of whole, unwashed chloroplasts on photophosphorylation.

It would appear that the high rates of phosphorylation are dependent upon the intactness of the whole plastid or, at least, of visible parts of it. The fact that the loss in phosphorylating activity between 2 and 6 minutes of sonication is very much less than the loss suffered from 0 to 2 min may point to a dilution effect. This decrease in the rate could be caused by the dilution of a required enzyme or cofactor. (The contents of the chloroplast are diluted some 1,000-fold when they are broken up by sonication.) This effect could also be due entirely to the disruption of a structural entity, essential for the coupling of the electron-transport chain with the production of ATP.

7. CONCERNING THE OCCURRENCE OF α, α-TOCOPHEROL AND α-TOCOPHERYLQUINONE IN CHLOROPLASTS AND QUANTASOMES

Hartmut K. Lichtenthaler*

Many investigators have suggested that lipophilic quinones play a role in biological systems. Various mechanisms have been proposed for their participation in the electron-transport system of the photosynthetic apparatus. The quinones are also discussed as the initial acceptors of phosphate in photosynthetic and in oxidative phosphorylation. For these reactions the chromanol and chromenol forms that could transfer phosphate to ADP are required. [1] The chromanol and chromenol forms, however, have not been found in chloroplasts, nor is it really clear which quinones the chloroplasts actually contain.

From a detailed study of the lipid components of spinach chloroplasts we have now obtained more information about the quinones present that bears upon the proposed reaction mechanism. Besides vitamin K_1 and plastoquinones, α-tocopherylquinone (TQ) and its chromanol, α, α-tocopherol (T) are present in spinach chloroplasts. These compounds were also found in quantasomes, [2] the pigment-containing subunits of the grana lamellae in chloroplasts.

The compounds TQ and T were purified by aluminum oxide chromatography. T was eluted with a mixture of cyclohexane and benzene (4:1) and TQ with diethylether acetone (1:1). The eluates showed absorption maxima at 260 and 270 mμ for TQ and at 292 mμ for T, both measured in ethanol.

The two substances showed the same Rf values as the synthetic products when cochromatographed on paraffin-impregnated paper and on filter paper containing Al_2O_3. [3] They gave the known color reactions with $FeCl_3^-$ + α, α'-dipyridyl reagent and with neotetrazolium chloride. [4] After reduction with sodium borohydride the absorption at 260 to 270 mμ decreased, in the case of TQ, to give a small peak at 286 mμ. The spectrum of T was not changed. (See Figs. 7-1 and 7-2.)

TQ and T can be isolated from the original chloroplast extract solution (a mixture of acetone and cyclohexane) by means of paper chromatography. [3] This shows that both compounds are components of chloroplasts and that TQ is not formed as an artifact in the course of the purification procedure.

Neither by paper nor column chromatography could we detect coenzyme Q_{10}, which, on the other hand, was found in extracts of the whole leaves. This indicates that the isolated chloroplasts were not contaminated significantly by mitochondria.

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*NATO Fellow from University of Heidelberg, Germany.

1. V. M. Clark and A. Todd, in Ciba Symposium on Quinones in Electron Transport (J. and A. Churchill, London, 1961) p. 190.
2. R. B. Park and N. G. Pon, J. Mol. Biol. 3, 1 (1961).
3. H. K. Lichtenthler, this report, Paper 9.
4. J. Jayaraman and T. Ramasarma, J. Sci. Industr. Res. 20C, 69 (1961).

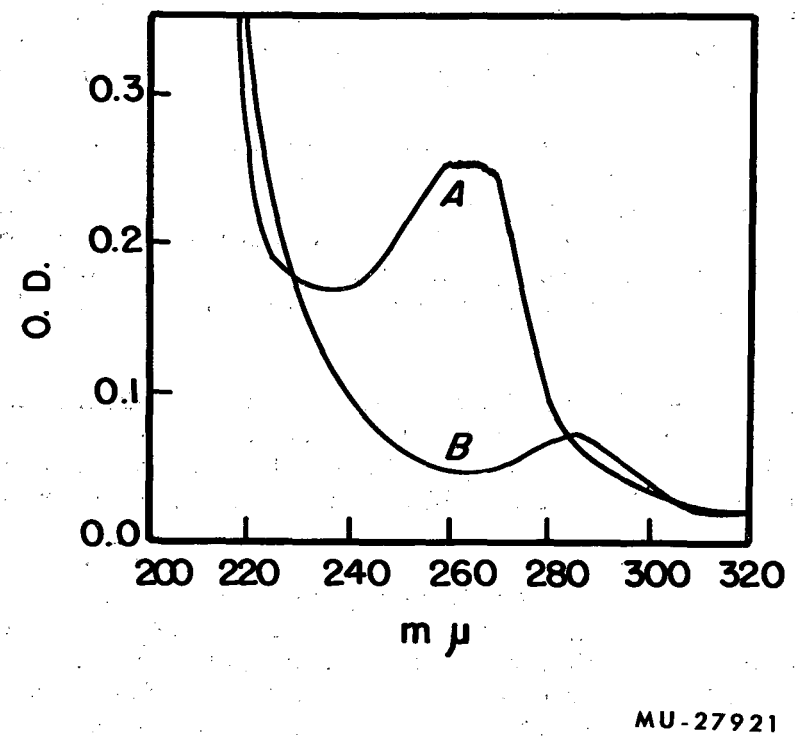
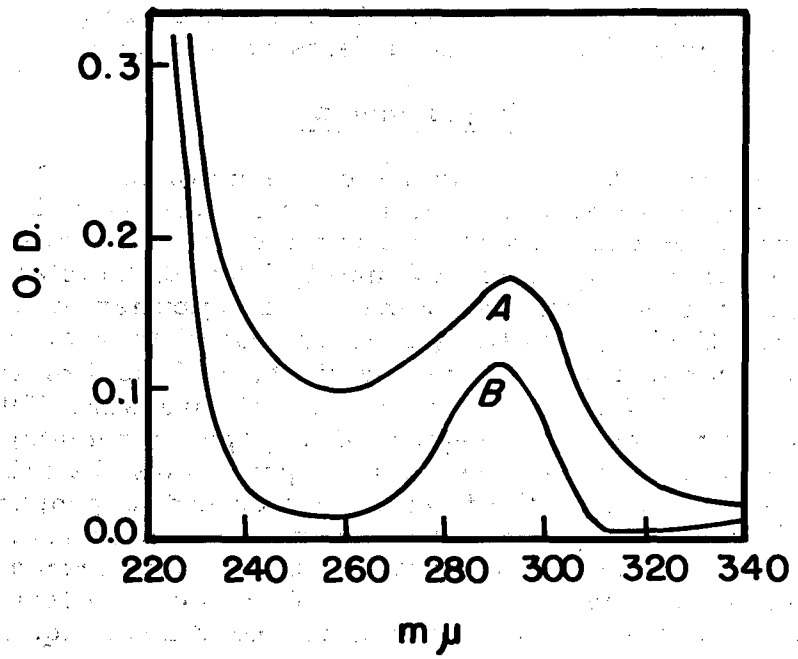


Fig. 7-1. Absorption spectrum of α-tocopherylquinone extract, isolated from spinach chloroplasts (in 95% ethanol). A, oxidized form; B, reduced with sodium borohydride.



MU-27922

Fig. 7-2. Absorption spectrum of α-tocopherol isolated from spinach chloroplasts (in 95% ethanol). A, extract solution; B, after purification.

8. EFFECTS OF ULTRAVIOLET AND GAMMA RADIATION ON THYMINE IN FROZEN AQUEOUS SOLUTION AND IN THE SOLID STATE

Joan Friedman, Richard M. Lemmon, and M. Calvin

Stimulated by the recent discovery by Beukers et al.[1] that thymine in frozen aqueous solution forms dimers when irradiated by ultraviolet light, and by the investigations in this Laboratory by C. A. Ponnamperuma on the effects of gamma radiation on nucleic acid constituents,[2] we started an investigation of the radiation chemistry of thymine in frozen aqueous solution and in the solid state. The purpose of this work is to increase our understanding of the mechanism of pyrimidine dimer formation under the influence of both uv light and ionizing radiation. Such studies are potentially important for an understanding of the biological effects of radiation.

Experimental

All our experiments were carried out using thymine-2-^{14}C (supplied by the Research Specialties Co.) with a specific activity of 1.13 mCi per mM (8.94 μCi per mg). Paper chromatography indicated the presence of a radioactive impurity, amounting to approximately 2.6% of the total activity. The ultraviolet light in most of the experiments was supplied by a 15-watt G. E. germicidal lamp (G15T8), and the γ rays by a Co^{60} source. However, the first series of experiments involving the kinetics of the dimerization reaction was performed by using the uv light from a high-pressure 500-watt Hg arc (Hanovia AH6). Products were separated by paper chromatography on Whatman No. 4 oxalic acid-washed filter paper, using the solvent systems ethanol-water (7:3 by vol) in the first dimension and isopropanol–conc. HCl–water (68:15.5:16.5 by vol) in the second. Radioautograms were made in order to locate the radioactive spots. Other paper chromatographic solvent systems that were tried, but that did not work satisfactorily, were: butanol-water, butanol–formic acid–water, and ethanol–aq. NH_4OAc.

Effects of uv light on frozen aqueous solutions

The formation of dimer as a function of time was studied as follows: Four 1-ml solutions of thymine (5×10^{-4} M) were placed in 10-ml beakers and frozen at -80°. The surfaces of the frozen solutions were irradiated for varying amounts of time by the AH6 lamp at a distance of 27 cm. The solutions were then allowed to thaw and aliquots were chromatographed, by use of the ethanol-water and isopropanol-HCl solvent systems. The spots were counted on the paper (average of both sides) with a gas proportional counter.

A study was made to compare dimer formation in fast-frozen and slow-frozen solutions. Three similar experiments were performed. In each case, a 1-ml solution of thymine (4.8×10^{-4} M) was frozen slowly by precooling for approximately 1 hour at 6° in a refrigerator, then allowing the solution to freeze slowly at -15°. The samples were frozen in this way in about 30 min.

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1. R. Beukers, J. Ijlstra, and W. Berends, Rec. trav. chim. 78, 883 (1959).
2. Cyril A. Ponnamperuma, The Radiation Chemistry of Nucleic Acids (Thesis), UCRL-10053, June 1962.

Other samples were quick-frozen by plunging 10-ml beakers each containing 1 ml of solution into liquid nitrogen. These samples were entirely frozen in about 1 min. Then, one slow-frozen and one quick-frozen sample were irradiated side by side for 30 min, with the 15-watt germicidal lamp at a distance of 5 inches. The samples were allowed to thaw and were chromatographed as above.

Effects of uv light on the crystals

The orientation of thymine molecules in frozen solutions might be similar to their configuration in the crystal lattice. If this were so, the dimer might be formed in the crystals as well as in the frozen solutions. We therefore irradiated pulverized suspensions of crystalline thymine (suspended in hexane) and looked for products. Pulverization and suspension were necessary in order to provide a large surface area on which the uv light could act. In one experiment, 0.6 mg of thymine was pulverized in a small procelain mortar, then transferred to a 10-ml beaker together with about 5 ml of hexane. The suspension was irradiated, with stirring, for 1 hour with the 15-watt lamp 5 in. from the surface of the liquid. The hexane was evaporated completely, the resulting solid dissolved in water, and an aliquot chromatographed as before. The second experiment was carried out in the same way, using 0.8 mg of pulverized thymine and an irradiation time of 5 hours.

Effects of γ radiation on frozen aqueous solutions

Two experiments were performed. In the first, two 1-ml samples of thymine solution (4.8×10^{-4} M) were frozen in 1-ml volumetric flasks. The flasks, together with dry ice, were put into the γ-ray source and irradiated for 30 min (4×10^{5} rads). One flask was removed and the other was irradiated for another 30 min (total dose, second sample, 8×10^{5} rads). The solutions were allowed to thaw and were chromatographed as before. The second experiment was carried out in the same way, with a 1-ml sample of 6×10^{-4} M thymine solution and an irradiation time of 8 hours. Irradiation was interrupted occasionally for addition of more dry ice. The sample stayed frozen throughout the irradiation. Total dose received was 4.8×10^{6} rads.

Effects of γ radiation on the crystals

Crystalline thymine (0.045 mg) was irradiated in a 1-ml volumetric flask for 2 hours. The dose received was 1.3×10^{7} rads. The solid was dissolved in water and an aliquot chromatographed as above. A second experiment was performed with 0.049 mg of thymine and an irradiation time of 20 hours. Total dose received was 1.3×10^{8} rads.

Results and Discussion

Effects of uv light on frozen aqueous solutions

The formation of the dimer as a function of time is shown in Fig. 8-1. Apparently, after an initial sharp rise, reactions leading to the destruction of the dimer gradually become more important. In later work, we hope to

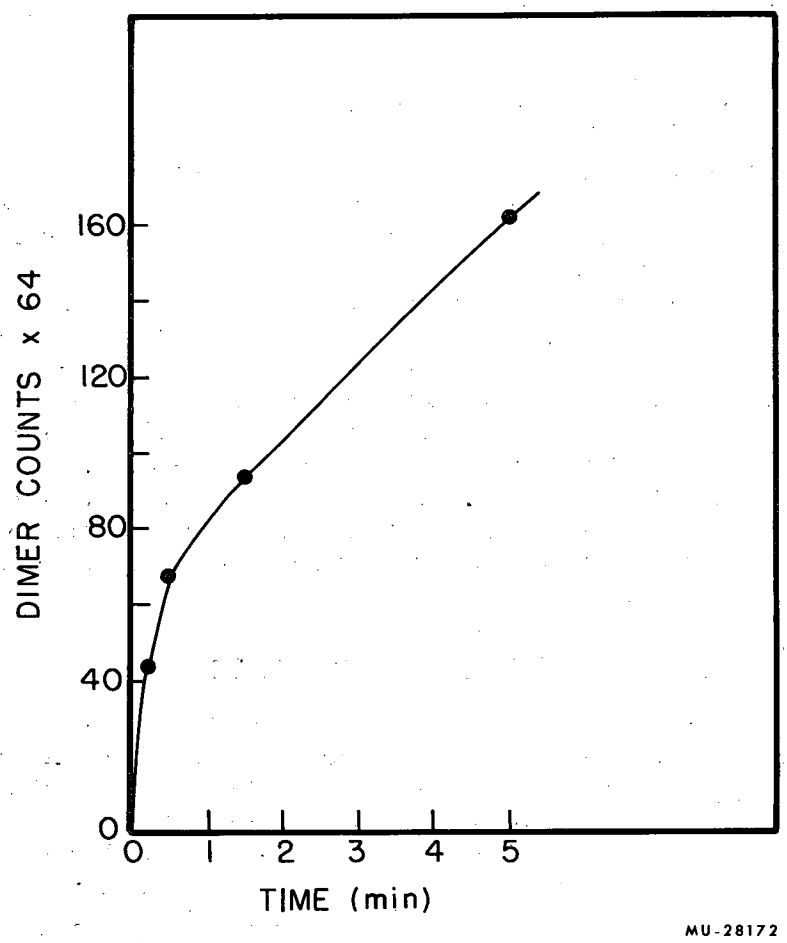

Fig. 8-1. Rate of formation of the dimer (frozen solution, 27 cm from the AH6 lamp).

study the kinetics of the reaction by varying (a) the initial concentration of thymine and (b) the concentration of the excited thymine molecule by varying the light intensity.

Table 8-I shows the results from slow-frozen and fast-frozen solutions.

Table 8-I. Comparison of dimer/thymine yield ratios for uv irradiation of thymine solutions

Experiment	Slow-frozen	Quick-frozen
A	1.823	2.453
B	1.70	2.72
C	1.02	2.20

In every case more dimer was formed in the quick-frozen solution. This is contrary to our initial expectation. We thought that the greater opportunity for thymine molecular alignment in the slowly frozen solution would lead to the higher dimer/thymine ratio. Since the reverse is true, we now presume that a larger number of smaller crystallites of thymine must form when the solution is frozen quickly, and thus a greater surface is available on which the uv light can act.

Effects of uv light on the crystals

No thymine dimer, or any other product, was detected in either experiment after chromatography and radioautography. This, however, does not exclude the possibility that unstable products may have been formed, or that the dose of uv absorbed at the surface of the crystals may have been insufficient to cause reaction.

Effects of γ radiation on frozen aqueous solutions

Under the same conditions as the previous uv irradiations, which cause dimerization, no reaction to give stable products was detected. This is probably a result of the basic difference between ionizing radiation and uv light. The former acts indiscriminately, imparting its energy to any atom it happens to strike. The latter is absorbed in molecules at certain selective sites where all the energy may be used to excite particular covalent bonds.

Effects of γ radiation on the crystals

As with uv radiation, no stable products could be detected at doses of 10^7 and 10^8 rads. It may be that our dose levels here were too high and that the dimer was largely destroyed after its formation. It is more likely, however, that little or no dimer is formed in the crystals.

In summary, we have tentatively confirmed previous suppositions that the initial formation of the uv-irradiation product of thymine in frozen aqueous solution (dimer) follows second-order rate laws. In addition, we have ascertained that γ radiation under these same conditions does not produce the dimer, at least not at doses up to 10^8 rads, and that crystalline thymine is extremely resistant to both uv and γ radiation.

9. A RAPID METHOD FOR THE IDENTIFICATION OF SMALL QUANTITIES OF LIPID-SOLUBLE VITAMINS AND QUINONES IN BIOLOGICAL MATERIAL

Hartmut K. Lichtenthaler*

Increased knowledge of the function of fat-soluble vitamins and quinones in the living organism has made their rapid separation and identification of considerable interest. Various methods for their separation by paper chromatography are reported in the literature.[1, 2, 3, 4] These methods, however, are not directly applicable to the original extracts of plant or animal tissues, since vitamins and quinones are present in very small concentrations and are masked by large amounts of accompanying lipids. These interfering substances cause poor separation of the vitamins and quinones. A preliminary purification of the extract, usually by saponification and column chromatography, is necessary before paper chromatographic methods can be applied. The preliminary treatments, however, very often lead to a partial destruction of the quinones and vitamins and thus result in considerable losses. Transformation products that were not originally detectable in the extracts may then become prominent. Furthermore, most of the fat-soluble quinones and vitamins are extremely sensitive to light and oxygen, and may be partially destroyed during chromatography (10 to 20 hours). For the study of the function and biosynthesis of these compounds it was desirable to develop a rapid paper chromatographic method for their separation and identification, applicable to biological extracts without further purification.

By testing various papers and solvents we have been able to develop a simple and rapid method for the separation of vitamins and naturally occurring quinones. In comparison with existing chromatographic methods the present technique has several advantages, resulting from the rapidity of the chromatogram development (1 to 2 hours) and the high adsorption capacity of the paper used (Schleicher and Schüll No. 967). The quantity of the whole-cell extract that can be applied to the Al_2O_3-containing paper is about 20 times what can be used with silicone- or paraffin-impregnated papers. This makes it possible to locate compounds present in extracts in such extremely low concentrations that they could not previously be detected. The substantially higher adsorption capacity is also important for the separation of the components for further analytical purposes.

Experimental Procedure

In the text and the figures the following abbreviations are used for the separated compounds:

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*NATO Fellow from University of Heidelberg, Germany

1. H. K. Lichtenthaler, Planta 57, 731 (1962).
2. J. Davidek and J. Blattna, J. Chromatog. 7, 204 (1962).
3. H. Wagner, L. Hörhammer, and B. Dengler, J. Chromatog. 7, 211 (1962).
4. J. Jayaraman and T. Ramasarma, J. Sci. Ind. Res. (India) 20c, 69 (1961).

A	= Vitamin A	Ph	= Phaeophytin
C	= β-Carotene	PQ	= Plastoquinone
Chl	= Chlorophyll a + b	Q_{10}	= Coenzyme Q_{10}
D	= Vitamin D_2	T	= D-α-Tocopherol
K_1	= Vitamin K_1	TQ	= α-Tocopherylquinone

Solvents

The choice of the solvent depended on the particular separation to be undertaken. Cyclohexane was found to be convenient for the separation of K_1 and Q_{10} from β-carotene. For the separation of the other quinones and vitamins from one another a mixture of cyclohexane + benzene (7:3) or pure benzene was used.

Application of samples to paper

In general, samples were applied to the paper from ethanol, cyclohexane, or isooctane solution. Samples in acetone were also satisfactory if care was taken to keep the spot size small.

Development of chromatogram

Ascending chromatography was used routinely. Circular chromatography worked equally well with the above solvents, and was found especially convenient when test substances were employed. All chromatograms were run at room temperature and in the dark to avoid destruction of the lipids.

Detection methods

1. Examination in daylight.
2. Examination in ultraviolet light.
3. Spraying with a saturated solution of $SbCl_3$ in chloroform (A, β-carotene, carotenoids).
4. Spraying with the $FeCl_3$-α,α'-dipyridyl reagent: a freshly prepared solution of equal volumes of α,α'-dipyridyl (0.5%) and $FeCl_3$ (0.2%) in 95% ethanol (reducing substances, tocopherol).
5. Use of the neotetrazolium spray according to Lester and Ramasarma:[5] quinones were first reduced by immersing the paper in 0.1% solution of sodium borohydride for 30 sec. Excess borohydride was destroyed by dipping the paper in 0.1 N HCl for 2 sec. The chromatogram was then immersed in a solution containing 0.25% neotetrazolium chloride and 0.25 M potassium phosphate, pH 7.0 (α-tocopherol, hydroquinones). Naphthoquinones gave an intense violet color (K_1, menadione, 1.4-naphthoquinone, and phthiocol). Benzoquinones (α-tocopherylquinone, Q_{10}) gave purple spots after heating at 100° for 60 sec.

5. R. L. Lester and T. Ramasarma, J. Biol. Chem. 234, 672 (1959).

Results and Discussion

By the procedures described above, all known fat-soluble vitamins and various naturally occurring quinones have been successfully separated. Figure 9-1 represents a typical chromatogram. The R_f values varied within narrow limits when natural extracts were applied. There was, however, no change in the order of migration.

Various other solvents such as isooctane, petroleum ether, chloroform, carbon tetrachloride, and acetone, or mixtures thereof were tested for the separation of lipid-soluble vitamins and quinones. They also provided good separation, but had no advantages over the solvents described.

Chromatography of tissue extracts

The method described here has been used successfully for examining plant extracts. It may be employed as successfully for analysis of animal tissue extracts. Figure 9-2 represents chromatograms of a freshly prepared acetone extract from spinach leaves. β-Carotene, the principal compound accompanying quinones and vitamins, runs almost with the solvent front, while the chlorophylls and xanthophylls remain practically on the starting line. Thus, these normally interfering substances do not affect the separation of the other lipid compounds.

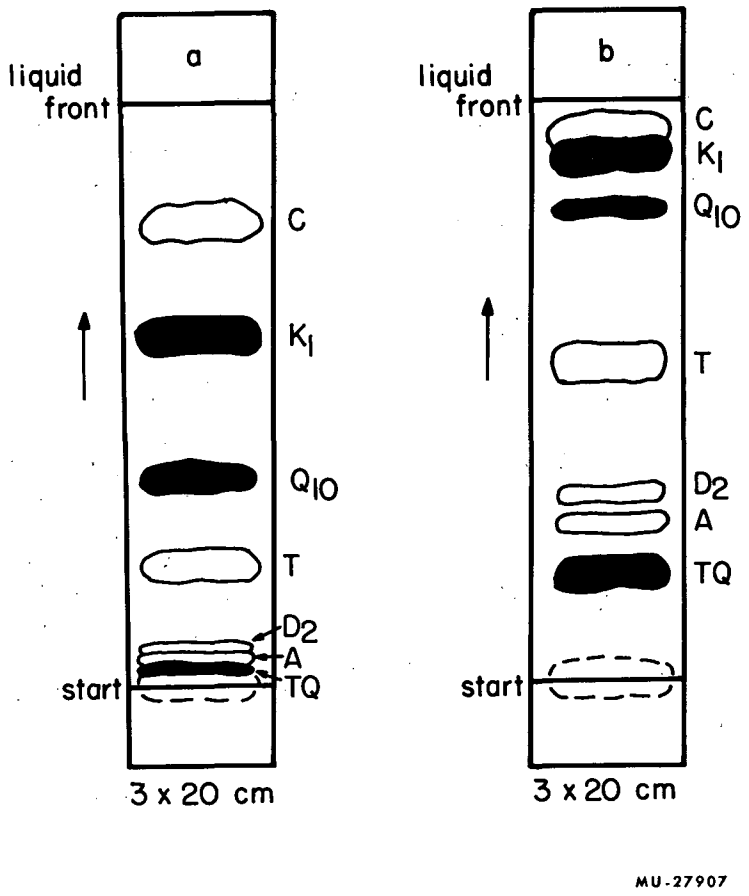

Fig. 9-1. Paper chromatogram of lipid-soluble vitamins and quinones. Solvent for a, cyclohexane; for b, benzene. Dark spots indicate quinones.

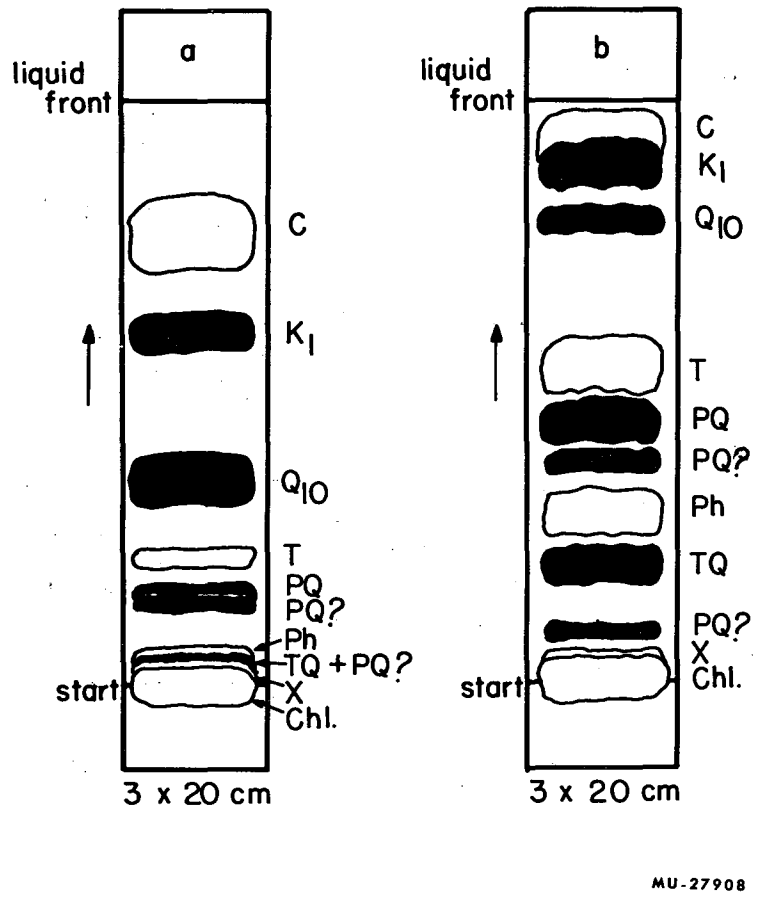

Fig. 9-2. Paper chromatogram of an acetone extract from spinach leaves. Solvent for a, cyclohexane; for b, benzene. Dark spots indicate quinones.

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