dine. After being allowed to stand overnight, the black precipitate was collected, washed well with ether, suspended in 75 cc. of ethanol and refluxed for 5 hours with 5 g. of sodium sulfite, previously dissolved in 25 cc. of water. The precipitate was removed, washed with ethanol and the filtrate was concentrated to 50 cc. Dilution with water, thorough extraction with chloroform and finally crystallization from acetone yielded 0.23 g. of the diolone XV with m.p. 222–228°. A further purified sample showed m.p. 234–236°, $[\alpha]_D$ +57°, λ_{max} 240 mμ, log ε 4.23, ν_{max} 1660 cm.$^{-1}$ and free hydroxyl band. This substance is probably identical with that of m.p. 238°, $[\alpha]^{20}D$ +51° (ethanol) described by Miescher and Klarer.[4]

Anal. Calcd. for $C_{20}H_{30}O_3$: C, 75.43; H, 9.50. Found: C, 75.80; H, 9.70.

Δ^4-Androstene-3,17-dione (XVI) from XV.—Lead tetraacetate (420 mg.; *ca.* 90% pure), previously dissolved in 8 cc. of glacial acetic acid, was added to a solution of 100 mg. of the diolone XV in 4 cc. of acetic acid and the mixture was kept at room temperature for 24 hours. Water was added, the product was extracted with ether and the extract was washed with sodium carbonate solution and water, dried and evaporated. The oily residue was chromatographed on 8 g. of neutral alumina and the fractions eluted with benzene were crystallized from a little ether. The Δ^4-androstene-3,17-dione thus produced showed m.p. 168–171° and this m.p. was undepressed on admixture with an authentic specimen (m.p. 169–171°).

17α,20-Oxido-17β-methyl-Δ^4-androsten-3-one (XVII).—17-Methylene-Δ^4-androsten-3-one (15 g.) dissolved in 90 cc. of chloroform was treated with 6.55 g. (0.9 equivalent) of perbenzoic acid in 110 cc. of chloroform and the solution was allowed to stand at room temperature for 18 hours. By this time all the peracid had been consumed. The solution was washed with aqueous sodium carbonate and water, dried and evaporated to dryness. The residue was purified most efficiently by chromatography on 500 g. of neutral alumina. The fractions eluted with benzene–hexane (8:2) and benzene on crystallization from acetone produced 9.8 g. (62%) of the oxide XVII with m.p. 179–182°. Further crystallization from acetone or methanol led to the analytical sample with m.p. 189–191°, $[\alpha]_D$ +112°, λ_{max} 240 mμ, log ε 4.24, ν_{max} 1660 cm.$^{-1}$.

Anal. Calcd. for $C_{20}H_{28}O_2$: C, 79.95; H, 9.39. Found: C, 79.87; H, 9.40.

The above conditions were the best ones found in a series of experiments carried out with different proportions of monoperphthalic and perbenzoic acid at different temperatures. The use of 0.7 equivalent of perbenzoic acid at room temperature gave some recovery of starting material, while 2.0 equivalents gave mainly a polar hydroxylic material (*cf.* ref. 24) and only little of the mono-oxide XVII.

17β-Methyl-Δ^4-androsten-17α-ol-3-one (17-Methylepitestosterone) (II).—The oxide XVII (5 g., m.p. 179–182°) dissolved in 300 cc. of dry tetrahydrofuran was reduced with 5 g. of lithium aluminum hydride in 250 cc. of tetrahydrofuran (30 minutes refluxing). The diol mixture XVIIIa and XVIIIb was isolated as described above for Xa, b and without purification was dissolved in 500 cc. of chloroform and oxidized by being shaken with 30 g. of manganese dioxide[17] for 16 hours. The dioxide was removed, washed with chloroform and the filtrate was evaporated. The crystalline residue was purified best by chromatography on 250 g. of neutral alumina. Crystallization of the fractions eluted with benzene–ether (6:4) from acetone–hexane furnished 3.21 g. (64%) of 17-methylepitestosterone with m.p. 175–177°. The analytical sample showed m.p. 181–182°, $[\alpha]_D$ +68° (ethanol), +82° (chloroform), λ_{max} 240 mμ, log ε 4.23, ν_{max} 1660 cm.$^{-1}$ and free hydroxyl band: reported[4] m.p. 182–183°, $[\alpha]^{21}D$ +66° (ethanol), +72° (chloroform).

Anal. Calcd. for $C_{20}H_{30}O_2$: C, 79.42; H, 10.00. Found: C, 79.25; H, 9.86.

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[CONTRIBUTION FROM THE DEPARTMENT OF PLANT NUTRITION, UNIVERSITY OF CALIFORNIA, BERKELEY]

Photosynthesis by Isolated Chloroplasts. III. Evidence for Complete Photosynthesis[1]

BY M. B. ALLEN, DANIEL I. ARNON,[2] J. B. CAPINDALE, F. R. WHATLEY AND LOIS J. DURHAM

RECEIVED JANUARY 21, 1955

Isolated chloroplasts, when illuminated, were found to fix CO_2 with simultaneous evolution of oxygen. This is regarded as complete extracellular photosynthesis since the observed CO_2/O_2 ratio was found to be approximately 1/1 in agreement with the well-known photosynthetic quotient of intact cells and among the products of photochemical CO_2 fixation by isolated chloroplasts were starch, phosphorylated sugars, amino acids and organic acids. Some of the factors influencing CO_2 fixation by isolated chloroplasts are described.

Heretofore, it has generally been accepted that "complete photosynthesis—that is, reduction of carbon dioxide to carbohydrates, and oxidation of water to oxygen, at low temperature and with no energy supply except visible light—has never been achieved outside the living cell."[3] The discovery of two new light-dependent reactions of isolated chloroplasts, CO_2 fixation[4] and photosynthetic phosphorylation[4-7] (the anaerobic esterification of inorganic phosphate, forming the pyrophosphate bonds of adenosine triphosphate), raised the question whether this extracellular photosynthetic activity of chloroplasts could be regarded as complete photosynthesis. To meet the criteria laid down by the above definition, evidence was required that CO_2 is reduced to carbohydrates by isolated chloroplasts, and that CO_2 fixation is accompanied by the evolution of oxygen. This paper presents evidence on both these points, leading to the conclusion that chloroplasts are the cytoplasmic structures in which the complete photosynthetic process is carried out, both inside and, under suitable conditions, outside the living cell. The relation of CO_2 fixation to other light-dependent reactions of isolated chloroplasts and a discussion of a general concept of photosynthesis supported by these findings will be given in another paper.[8]

(1) The substance of this paper was presented before the American Association for the Advancement of Science at its December, 1954, meeting at Berkeley, California.

(2) Aided by grants from the National Institutes of Health and the Office of Naval Research.

(3) E. I. Rabinowitch, "Photosynthesis," Vol. 1, Interscience Publishers, New York, N. Y., 1945, pp. 61, 53.

(4) D. I. Arnon, M. B. Allen and F. R. Whatley, *Nature*, **174**, 394 (1954).

(5) D. I. Arnon, F. R. Whatley and M. B. Allen, THIS JOURNAL, **76**, 6324 (1954).

(6) F. R. Whatley, M. B. Allen and D. I. Arnon, *Biochim. Biophys. Acta*, **16**, 605 (1955).

(7) D. I. Arnon, F. R. Whatley and M. B. Allen, *ibid.*, **16**, 607 (1955).

(8) D. I. Arnon, M. B. Allen and F. R. Whatley (manuscript in preparation).

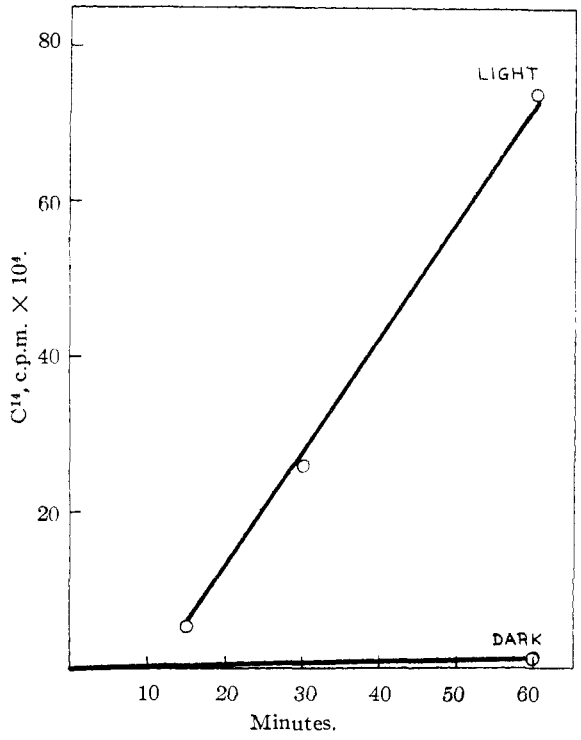

Fig. 1.—Time course of CO_2 fixation by isolated spinach chloroplasts in light and dark. Reaction mixture is described in text. The lag in CO_2 fixation during the first few minutes of illumination is attributed to a time lag in equilibration after the release of $C^{14}O_2$ gas from the sidearm (see Methods).

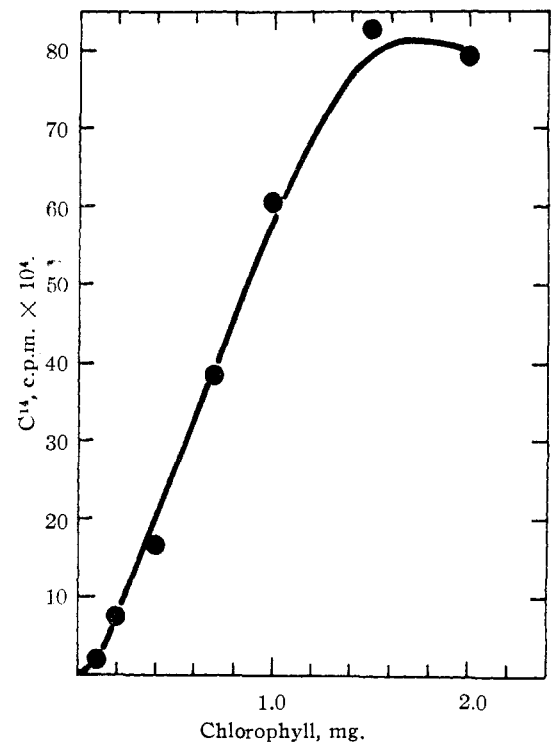

Fig. 2.—Dependence of CO_2 fixation upon amount of chloroplast suspension added. Reaction mixture is described in text.

Results

CO_2 Fixation and Oxygen Evolution.—The fixation of CO_2 by isolated spinach chloroplasts is illustrated in Fig. 1. It is evident that the reaction was strictly light-dependent and proceeded at an almost constant rate for an hour. As shown in Fig. 2, the rate of the reaction was directly proportional to the amount of whole chloroplast material added, up to 1.5 mg. of chlorophyll, where the reaction probably became light-limited. This CO_2 fixation was accompanied by the evolution of oxygen. As shown in Table I, for each micromole of CO_2 fixed there was approximately one micromole of oxygen evolved. CO_2 uptake was measured with radiocarbon, while oxygen evolution was measured manometrically in parallel reaction vessels (see Methods). The evolved gas was identified as oxygen with luminous bacteria. We consider that the agreement between oxygen evolved and CO_2 fixed is satisfactory considering the small amounts involved and the use of different methods for measuring each gas. The observed CO_2/O_2 ratio of approximately 1/1 is in agreement with the well-known photosynthetic quotient of intact cells, and suggests that the over-all reaction of photosynthesis by isolated chloroplasts may tentatively be represented by the general photosynthetic equation[3]

$$CO_2 + 2H_2O \longrightarrow (CH_2O) + H_2O + O_2$$

TABLE I

CO_2 FIXATION AND OXYGEN PRODUCTION BY ISOLATED CHLOROPLASTS

(See text for experimental conditions.)

Expt. no.	979	1010	1011	1050
μmole $C^{14}O_2$ fixed	1.0	0.81	0.38	1.04
μmole O_2 produced	1.5	0.83	0.50	1.00

Products of CO_2 Fixation.—Both soluble and insoluble products resulted from the fixation of radiocarbon by chloroplasts. The distribution of C^{14} between soluble and insoluble compounds after various periods of illumination is shown in Table II. A relatively large amount of radioactivity appeared in the insoluble fraction after a short period of illumination (30 sec.) and the percentage of radiocarbon entering insoluble products did not vary appreciably with time, at least up to one hour. As will be shown below, this insoluble radioactive material has been identified as starch.

TABLE II

DISTRIBUTION OF RADIOACTIVITY BETWEEN SOLUBLE AND INSOLUBLE FRACTIONS

(See text for experimental conditions)

Expt. no.	Illumination time, min.	Total C^{14} fixed, c.p.m.	% radioactivity Sol. fraction	% radioactivity Insol. fraction
975	0.5	6,822	65.0	35.0
	1.0	8,940	78.0	22.0
	5.0	73,520	75.0	25.0
930	30	252,875	80.0	20.0
	60	457,000	69.4	31.6

A typical radioautogram of the compounds found in the soluble fraction is shown in Fig. 3. The

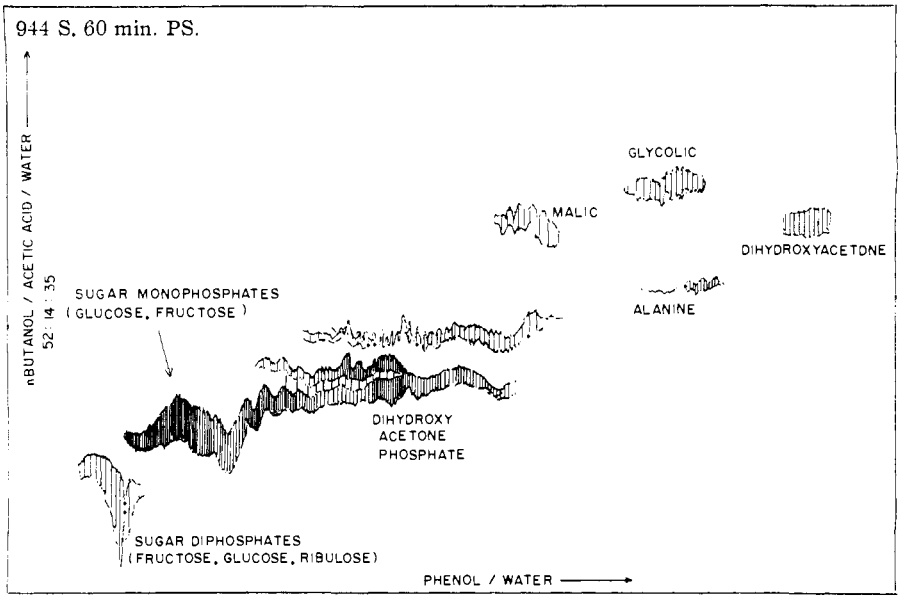

Fig. 3.—Typical radioautogram of the soluble fraction. For details see text.

principal products identified in the chromatogram were mono- and diphosphates of hexoses and pentoses, and dihydroxyacetone phosphate. Glycolic and malic acids, alanine and free dihydroxyacetone also were found.

Since chromatograms of the whole chloroplast extract had some spots distorted by the presence of salt, additional chromatograms were prepared after desalting the extract by passing it through a column of Amberlite IR-100. Chromatograms of the "acid plus neutral" (not adsorbed on the column) and "basic" (adsorbed on the column and eluted with aqueous ammonia) fractions obtained in this manner are shown in Figs. 4a and 4b, respectively. In the "acid plus neutral" fraction (Fig. 4a) the spots corresponding to the phosphorylated sugars, malic and glycolic acids, and dihydroxyacetone were clearly defined. In the "basic fraction" (Fig. 4b) aspartic acid, glycine and alanine were identified. The other spots on this chromatogram are probably the result of contamination of this fraction by acid and neutral compounds adhering to the column. Exploration of the sugar phosphate areas by elution from the paper, followed by phosphatase treatment and co-chromatography with authentic sugars revealed the presence of the following compounds: from the "sugar diphosphate" area—fructose, glucose and a little ribulose; from the "sugar monophosphate" area—fructose and glucose; and from another phosphate spot—dihydroxyacetone.

Among the non-phosphorylated compounds the following have been identified by elution from the paper and co-chromatography with authentic carriers: glycolic and malic acids, aspartic acid, alanine, glycine and free dihydroxyacetone. Identity of the amino acids was confirmed by chromatography of the hydroxy acids resulting from deamination with nitrous acid.

The identification of the phosphorylated sugars and other compounds is obviously not complete. It is possible that further work will reveal the presence of phosphoglyceric acid[9] and of other sugars such as sedoheptulose.[10]

Treatment of the insoluble fraction of the chloroplast extracts with salivary amylase[11] resulted in the formation of radioactive maltose, accompanied by a smaller amount of radioactive glucose (Fig. 5). Since the formation of maltose by the action of salivary amylase is considered to be a specific test for starch[11] we concluded that starch was present in the insoluble fraction. Evidence that starch is the principal, if not the only, insoluble compound formed during photosynthesis by isolated chloroplasts was provided by the results shown in Fig. 6. To obtain this chromatogram, the insoluble fraction, after extraction with ethanol, was treated with acid as used for the hydrolysis of proteins into their constituent amino acids.[12] Hydrolysis was carried out by refluxing for 5.5 hours with a mixture of equal parts of constant boiling (77.5%) formic acid and 5 *N* HCl. The hydrolysate was then chromatographed as described for the soluble fraction. The amino acids resulting from the chloroplast protein, represented as outlined areas in Fig. 6, were identified by spraying the paper with ninhydrin. The colored areas were found to contain no radioactivity, hence no protein synthesis from CO_2 had occurred. The radiocarbon of the insoluble fraction was localized in a single compound identical with that formed when maltose was subjected to the same HCl–formic acid treatment. This compound has been identified as levulinic acid, which is known to be formed when starch and sugars are subjected to drastic acid hydrolysis.[13]

(9) A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and W. Stepka, THIS JOURNAL, **72**, 1710 (1950).

(10) A. A. Benson, J. A. Bassham and M. Calvin, *ibid.*, **73**, 2970 (1951).

(11) W. Z. Hassid, R. M. McCready and R. S. Rosenfels, *Ind. Eng. Chem., Anal. Ed.*, **12**, 142 (1940).

(12) R. J. Block and D. Bolling, "The Amino Acid Composition of Proteins and Foods," C. C. Thomas, Publ., Springfield, Ill., 1945.

(13) W. W. Pigman and R. W. Goepp, Jr., "Chemistry of the Carbohydrates," Academic Press, Inc., New York, N. Y., 1948.

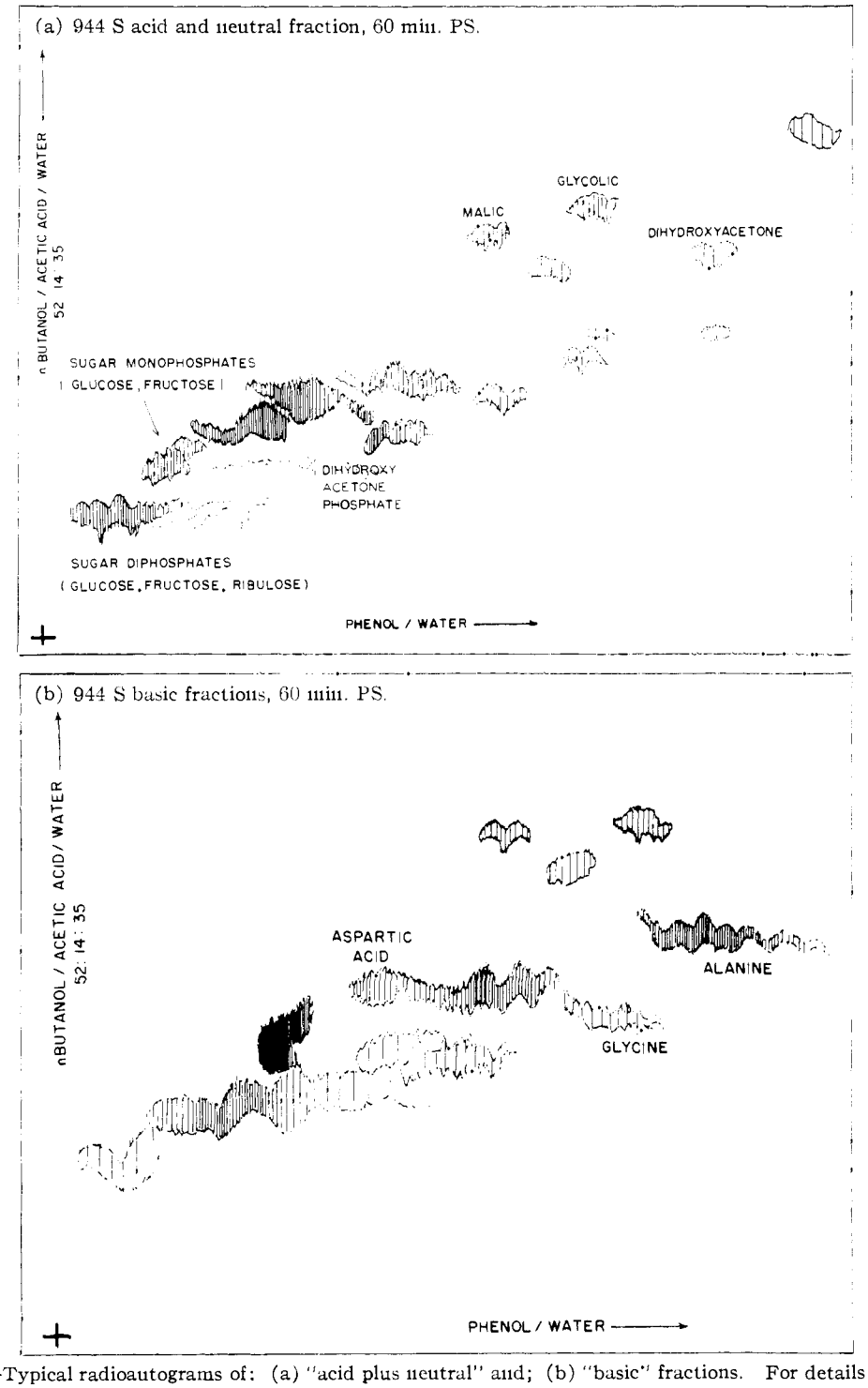

Fig. 4.—Typical radioautograms of: (a) "acid plus neutral" and; (b) "basic" fractions. For details see text.

Effect of Oxygen and Added Cofactors.—As previously reported[4] a marked improvement in CO_2 fixation was found when the reaction was carried out under nitrogen, rather than in air. This effect is illustrated in Table III. Thus CO_2 fixation, like photosynthetic phosphorylation,[5-7] was favored by anaerobic conditions.

Various metal ions, vitamins and coenzymes were tested for their effect on carbon dioxide fixation by chloroplasts. The addition of di- or triphosphopyridine nucleotide, adenosine mono- or triphosphate, uridine mono- or triphosphate, or

TABLE III

EFFECT OF OXYGEN ON CO_2 FIXATION

(Experimental conditions as in text except that gassing with N_2 was omitted for aerobic vessels.)

Expt. no.		929	931	932
$C^{14}O_2$ fixed, c.p.m.	aerobic	276,700	417,800	431,900
	anaerobic	736,800	875,200	1,217,900

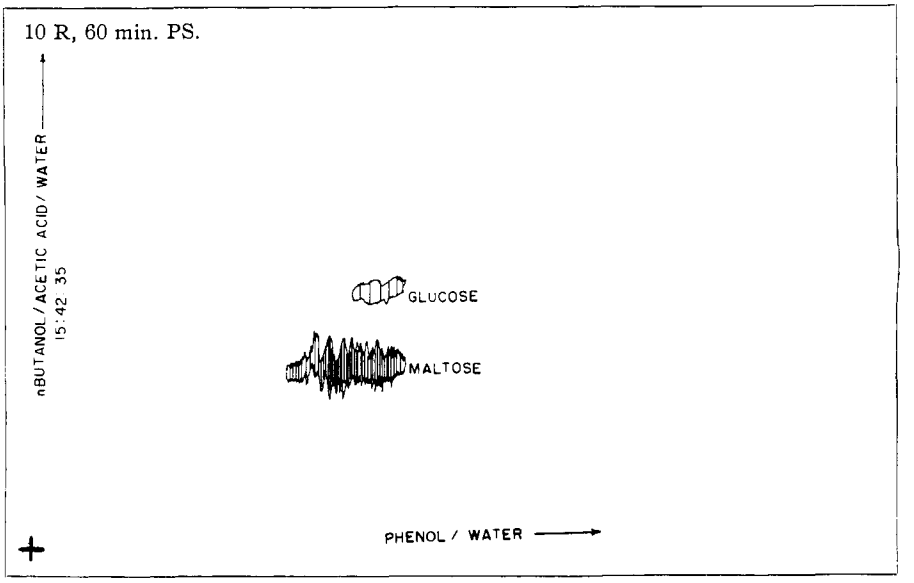

Fig. 5.—Products of hydrolysis of insoluble fraction by salivary amylase. For details see text.

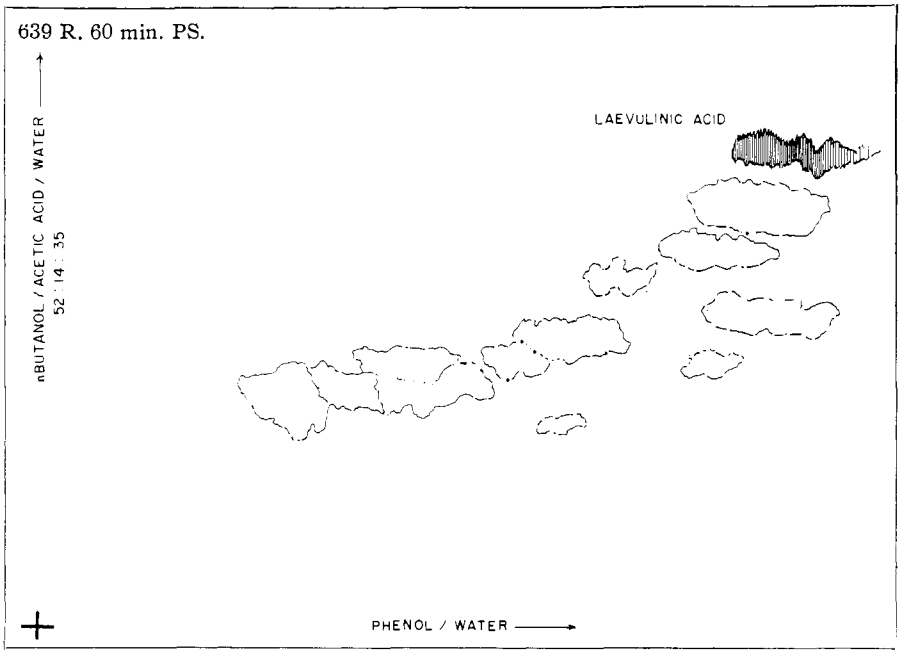

Fig. 6.--Products of drastic acid hydrolysis of insoluble fraction. For details see text.

thioctic (lipoic) acid (kindly supplied by Drs. L. J. Reed and T. H. Jukes) was without effect on CO_2 fixation. Addition of cocarboxylase or of coenzyme A stimulated CO_2 fixation to a small extent in some, but not in all, chloroplast preparations. Interestingly enough, the addition of the cofactors of photosynthetic phosphorylation[6,7]: magnesium ion, riboflavin or riboflavin phosphate, and vitamin K compounds such as menadione, was inhibitory. Inorganic phosphate was found to have a similar inhibitory effect.[4] These observations support the view[4] that there may be a competitive relation between photosynthetic phosphorylation and CO_2 fixation by isolated chloroplasts.

Since the chloroplasts themselves contain inorganic and organic phosphate, the observation of CO_2 fixation in the absence of added phosphate carried no implications regarding the participation of phosphate in CO_2 fixation. Such participation would be expected on energetic grounds, and this expectation was confirmed by the finding of phosphorylated compounds among the products of carbon dioxide reduction.

The only substances so far found to stimulate CO_2 fixation by chloroplasts are ascorbate and manganous ion, as shown in Table IV. The response to added ascorbate, though usually appreciable, varied in magnitude with different chloroplast preparations. This variability may be accounted for by the variation in the ascorbate content of chloroplasts. Analyses showed that

chloroplast preparations contained from 0.05 to 0.3 μmole ascorbate per mg. chlorophyll. Unlike the other cofactors, ascorbic acid and manganese stimulate both photosynthetic phosphorylation and CO_2 fixation by isolated chloroplasts. A further discussion of the relations between photosynthetic phosphorylation and CO_2 fixation is given elsewhere.[8]

TABLE IV

STIMULATION OF CO_2 FIXATION BY ASCORBATE AND MANGANESE

Control included: chloroplast suspension in 0.35 *M* NaCl, containing 0.5 mg. chlorophyll, 40 μmole tris-(hydroxymethyl)-aminomethane buffer, *p*H 7.2, and 0.35 *M* KCl to a final volume of 3.0 ml. Mn added as $MnCl_2$, ascorbate as a solution of the sodium salt. Other experimental conditions as in text.

Treatment	C.p.m. $C^{14}O_2$ fixed Expt. 1004	Expt. 1009
Control	43,400	120,350
+ 2 μmole Mn	37,650	119,100
+ 10 μmole ascorbate	74,250	141,400
+ Mn + ascorbate	93,400	185,000

Methods

Preparation of Chloroplasts.—Suspensions of intact chloroplasts were prepared by grinding spinach leaves with sand in 0.35 *M* NaCl, removing the coarse debris, centrifuging at low speed (1000 × *g*) to bring down whole chloroplasts, washing these with NaCl solution, and resuspending in NaCl. Full details are described elsewhere[8]. Unless otherwise noted, a quantity of this suspension containing 2.0 mg. of chlorophyll was used in each reaction vessel.

Under the conditions so far investigated, carbon dioxide fixation has been found to be strictly dependent on the integrity of the chloroplasts. Treatment of the chloroplast preparations with water, which was without effect on their capacity for photolysis or photosynthetic phosphorylation,[8] completely destroyed their ability to fix CO_2. The capacity of chloroplasts for CO_2 fixation diminished rapidly at room temperature. At low temperatures the chloroplasts were more stable under conditions which permitted CO_2 fixation. 50–75% of the CO_2 fixing capacity of spinach chloroplasts was lost during an hour's storage in ice in the dark, but on 30–40% during incubation for a similar period in the light.

Measurement of Oxygen Evolution.—For measurement of oxygen evolution the chloroplasts were suspended in the same reaction mixture that was used for CO_2 fixation experiments except that radiocarbon was omitted. The partial pressure of CO_2 in the reaction vessels was held constant by means of 0.4 ml. of Warburg #9 buffer distributed on filter paper in the center well and side arm of the vessels. The manometer vessels were gassed with nitrogen which had been equilibrated with Warburg #9 buffer by bubbling through a series of flasks containing the buffer solution. Since the pressure changes were small, three controls, two in the light and one in the dark, from which no oxygen evolution could be expected were run to make sure that the manometric effects observed resulted from oxygen evolution by illuminated chloroplasts. The two controls in light were: (1) reaction mixture and buffer (no chloroplasts) and (2) action mixture + buffer + chloroplasts + 0.2 ml. Oxsorbent (chromous chloride) to absorb the evolved oxygen. The dark control consisted of the reaction mixture, chloroplasts and buffer. All determinations of gas exchange were runs in duplicate. All three controls gave similar small positive pressure changes. The average of these was taken as a blank. In computing oxygen evolution by illuminated chloroplasts, the blank was subtracted from the positive pressure changes observed in the vessels containing chloroplasts, reaction mixture and buffer.

Confirmatory evidence that chloroplasts evolve oxygen while fixing CO_2 was obtained with luminous bacteria, which are known to require oxygen for light emission.[14] The manometer assembly in which chloroplasts had been fixing CO_2 under an atmosphere of nitrogen (freed of traces of oxygen by bubbling through alkaline pyrogallol) was transferred to a dark room and a suspension of *Photobacterium phosphoreum* in 3% NaCl (kindly supplied by Dr. B. J. Bachman) was introduced into a sidearm of the reaction vessel. This sidearm was previously closed with a serum bottle cap. The oxygen-free bacterial suspension was injected through the cap with a syringe. A distinct glow, indicating the presence of oxygen, was visible in the vessel in which chloroplasts had previously been illuminated. No luminescence was observed in vessels not previously illuminated or in those previously illuminated but containing no chloroplasts. The luminous bacteria were grown in a medium of the following composition: NaCl, 3 g.; Peptone, 0.5 g.; yeast extract 0.5 g.; glycerol 0.5 g.; $CaCO_3$ 2 g.; agar 2 g.; *M* KH_2PO_4 1 ml.; *M* K_2HPO_4 1 ml.; and H_2O 100 ml.

Measurement of CO_2 Fixation.—The CO_2 fixation experiments were carried out in a refrigerated constant temperature bath at 15°, illuminated either with Mazda projector flood lamps[15] or with a bank of three 12 inch cerise fluorescent tubes,[16] which provided an illumination of approximately 6500 Lux at the bottom of the reaction vessel. The reaction was carried out in Warburg reaction flasks, chilled in ice prior to immersion in the bath. The flasks were equilibrated in the bath and flushed with nitrogen gas for 5 min. prior to turning on the light. The reaction mixture contained, in addition to chloroplasts, 40 μmole of tris-(hydroxymethyl)-aminomethane buffer, *p*H 7.2, and 10 μmole of sodium ascorbate. 0.35 *M* KCl was added to bring the total volume to 3.0 ml. $C^{14}O_2$ was supplied from a sidearm containing 1.5 μmole of $Na_2C^{14}O_3$ (*ca.* 1.5 × 10^6 c.p.m.). In some experiments the sodium carbonate was added directly to the reaction vessel. In other experiments, vessels with twin "Siamese" sidearms were used. At zero time $C^{14}O_2$ was liberated by mixing 0.3 ml. of 0.5 *M* HCl, present in one of the twin sidearms, with the sodium carbonate present in the other. In experiments lasting an hour the same results were obtained with either method of supplying carbon dioxide to the chloroplasts. The reaction was stopped by pouring 0.3 ml. of 0.5 *M* HCl from the sidearm into the main compartment of the vessel. Control experiments in which the chloroplasts were inactivated by boiling water, hot 80% ethanol, or trichloroacetic acid instead of HCl showed no significant effect of the killing method on the distribution of radioactivity between soluble and insoluble products, or on the pattern of products found in the soluble fraction.

For measurement of the total CO_2 fixed, aliquots of the vessel contents were pipetted onto stainless steel planchets, mixed with a drop of 0.5 *M* HCl, and evaporated to dryness under an infrared lamp. The dry samples were measured with a thin window counter of conventional design. When necessary, counts were corrected to zero sample thickness. Omission of the HCl treatment had no effect on the number of counts measured, but this treatment was routinely included as a precaution against any bicarbonate remaining in the sample.

Identification of Products.—The contents of the Warburg vessels were centrifuged, the residue washed with water, and aliquots of the supernatant liquid subjected to two-dimensional paper chromatography using as solvents (a) phenol saturated with water[17] at 20° and (b) a mixture of 52 parts *n*-butanol, 14 parts glacial acetic acid and 35 parts water.[18] Generally about 0.05 ml. of the whole supernatant liquid was applied in several successive applications on Whatman No. 4 paper (22½″ × 18¼″) previously washed with 0.5% oxalic acid.[19] In the desalted samples, the size of the aliquot put on the paper was usually adjusted to give *ca.* 6000 c.p.m. at the origin.

The distribution of radiocarbon among the various compounds was determined by the technique of radioautography used previously in the study of phosphorus metabolism in

(14) M. W. Beijerinck, *Konin. Akad. Wetens. (Amsterdam)*, **4**, 45 (1901).

(15) D. I. Arnon and F. R. Whatley, *Arch. Biochem.*, **23**, 141 (1949).

(16) The design of this lighting system was based on the suggestions of Drs. Jack Myers and W. A. Arnold.

(17) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **38**, 224 (1944).

(18) S. M. Partridge, *ibid.*, **42**, 238 (1948).

(19) A. A. Benson, S. Kawaguchi, P. M. Hayes and M. Calvin, THIS JOURNAL, **74**, 4477 (1952).

plants[20] and first applied to paper chromatography by Fink, *et al.*[21] This technique was adapted and extensively used by Benson, *et al.*,[9] for the identification of photosynthetic products. Since the salt necessary to maintain the chloroplasts in an active state interfered with the chromatograms, the extracts were further treated by passing them through a column of Amberlite IR-100 (hydrogen form). The column, about 15 cm. long and 2 cm. in diameter, was prepared by gravity sedimentation of an aqueous suspension of the resin.

The sample to be desalted (about $^9/_{10}$ of the soluble fraction) was diluted with water to 50 ml. and passed through the column at a rate of 2–3 ml. per min. The column was then washed with water (150 ml.) at a similar rate of flow, and the combined eluate and washings, representing the "acid plus neutral" fraction, was evaporated to a small volume (0.7 ml.) at 30° under reduced pressure. The "basic" fraction, which was adsorbed on the resin, was eluted with aqueous ammonia (approx. 2 *N*). The elution was continued until ammonia could be detected in the eluate. The eluate of the "basic" fraction was reduced in volume in the same way as the "acid plus neutral" fraction. Aliquots of the desalted fractions were used for paper chromatography. The "acid plus neutral" fraction contained phosphorylated sugars and organic acids and the "basic" fraction contained the amino acids.

Dihydroxyacetone, alanine, glycine, aspartic acid, malic acid and glycolic acid were identified by cutting out spots from the paper and co-chromatographing with authentic samples of these compounds in the phenol water and butanol–acetic acid mixtures. Identity of the unknown with the authentic compound was evidenced by the appearance of a single spot. Additional evidence for the identity of the amino acids was obtained after deaminating a sample of radioactive material, mixed with carrier, by treatment with a mixture of KNO_2 and glacial acetic acid. The resulting hydroxy acids were then identified by co-chromatography on paper.

Sugar phosphates were identified by eluting the radioactive material from the paper, adding to the eluate 0.05 ml. of 0.2 *M* tris-(hydroxymethyl)-aminomethane buffer, *p*H 8.8, and 0.2 ml. of a solution of alkaline phosphatase (General Biochemicals, 4 mg. per 10 ml.), then incubating at 38° for two to three hours. The phosphatase treated material was then co-chromatographed with authentic sugars.

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(20) D. I. Arnon, P. R. Stout and F. Sipos, *Amer. J. Bot.*, **27**, 791 (1940).

(21) R. M. Fink, D. E. Dent and K. Fink, *Nature*, **160**, 801 (1947).

NOTES

Unsaturated Phenols. II.[1] Attempted Syntheses of *o*-Vinylphenol

BY ALFRED R. BADER

RECEIVED MARCH 25, 1955

As part of a more extensive study of unsaturated phenols, small quantities of *o*-vinylphenol were required. At least five apparently convenient syntheses are described in the literature: (i) the reaction of phenol with ethylene oxide,[2] (ii) the sulfuric acid-catalyzed rearrangement and dehydration of β-phenoxyethanol,[2] (iii) the reaction of vinyl acetate with phenol,[3] (iv) the decarboxylation of *o*-hydroxycinnamic acid,[4] and (v) the thermal decomposition of the benzodioxin obtained from phenol and acetaldehyde.[5]

Smith and Niederl claimed that phenol and ethylene oxide react in the presence of sulfuric acid to give a 65% yield of *o*-vinylphenol, characterized by its tetrabromide and phenoxyacetic acid reported previously.[4b] Many attempts to repeat the work of Smith and Niederl were unsuccessful. The ultraviolet spectrum of the crude reaction product, mainly unreacted phenol, showed no conjugated unsaturation. The crude reaction product was brominated, and the bromophenols were separated by chromatography; no tetrabromide, easily obtained from *o*-vinylphenol, could be isolated.

Smith and Niederl[2] postulated β-phenoxyethanol as the intermediate in the formation of *o*-vinylphenol from phenol and ethylene oxide. Support for this was found in the alleged reaction of β-phenoxyethanol with sulfuric acid at room temperature to yield *o*-vinylphenol. Actually, β-phenoxyethanol is recovered unchanged from the reaction conditions described (identical infrared spectra and physical constants).[6]

The reaction of vinyl acetate with phenol in the presence of sulfuric acid has been reported[3] to yield a polymer from which *o*-vinylphenol has been alleged to be easily obtainable by thermal depolymerization. The product of the very vigorous reaction is a polymer, but no *o*-vinylphenol could be obtained therefrom.

The decarboxylation of *o*-hydroxycinnamic acid[4] provides a convenient method for the preparation of *o*-vinylphenol.

(1) For paper I see THIS JOURNAL, **75**, 5967 (1953).

(2) R. A. Smith and J. B. Niederl, *ibid.*, **53**, 806 (1931).

(3) J. B. Niederl, R. A. Smith and M. E. McGreal, *ibid.*, **53**, 3390 (1931).

(4) (a) H. Kunz-Krause and P. Manicke, *Arch. Pharm.*, **566**, 555 (1929); (b) K. Fries and G. Fickewirth, *Ber.*, **41**, 367 (1908).

(5) E. Adler, H. v. Euler and G. Gie, *Arkiv Kemi, Mineral., Geol.*, **16A**, No. 12, 1 (1943); *C. A.*, **38**, 5839 (1944).

(6) Adler, *et al.*,[5] also were unable to obtain *o*-vinylphenol from β-phenoxyethanol.

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Steroids. LXVIII.[1] 17-Ethylepitestosterone

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In view of the facile preparation of 17-methylepitestosterone from 17-methylene-Δ^4-androsten-3-one through preferential epoxidation of the exocyclic double bond, followed by lithium aluminum hydride reduction and manganese dioxide oxidation,[1] we decided to prepare the hitherto unknown 17-ethylepitestosterone (IV) by an analogous route. This substance was required for testing for possible ana-

(1) Paper LXVII, F. Sondheimer, O. Mancera, M. Urquiza and G. Rosenkranz, THIS JOURNAL, **77**, 4145 (1955).