methoxyfluoboric acid ionizes to form methoxyfluoborate ions and protons solvated by one molecule of undissociated acid. However, Greenwood[6] has cautioned that ionic dissociation schemes postulated for pure liquids do not necessarily represent the behavior of complexes in reaction media.

The formation of hydroxyfluoborates in the present research suggests the possibility of ionization during chemical reaction as

$$2CH_3OHBF_3 \rightleftarrows (CH_3)_2OBF_3H^+ + HOBF_3^-$$

In support of this proposed equation, exploratory experiments have shown that the dimethyl ether boron trifluoride complex does solvate the proton. Results of these investigations will be reported at a later date.

(6) N. N. Greenwood and R. L. Martin, *Quart. Rev., (London)*, **8**, 1 (1954).

Metatheses of tetramethylammonium hydroxide with monohydroxyfluoboric acid is described by

$$(CH_3)_4NOH + HBF_3OH \longrightarrow (CH_3)_4NBF_3OH + H_2O$$

Experimental

Methoxyfluoboric acid and hydroxyfluoboric acid were prepared by saturating anhydrous methanol and water with boron trifluoride, after which the compounds were purified by fractional recrystallization.[5,7]

The X-ray powder diffraction photographs were made with copper radiation, filtered by nickel, using a Philips 57.3 mm. powder camera. The samples were mounted on Pyrex fibers. Intensities were estimated visually by comparison with a film of known intensity.

(7) J. C. McGrath, G. G Stack and P. A. McCusker, THIS JOURNAL, **66**, 1263 (1944).

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Photosynthesis by Isolated Chloroplasts. II. Photosynthetic Phosphorylation, the Conversion of Light into Phosphate Bond Energy

BY DANIEL I. ARNON, F. R. WHATLEY AND M. B. ALLEN*

RECEIVED JUNE 4, 1954

Whole chloroplasts removed from green cells were found able to convert light into the chemical energy of pyrophosphate bonds of adenosine triphosphate (ATP). This photochemical esterification of inorganic phosphate outside the living cell, termed photosynthetic phosphorylation, is independent of CO_2 fixation and of the dark reactions of respiration. Photosynthetic phosphorylation has been demonstrated in a system consisting of washed spinach chloroplasts, NaCl, ascorbic acid and P^{32}-labeled orthophosphate, in the presence of a phosphate acceptor system (adenylic acid or hexokinase and glucose). ATP was identified as the product of the reaction (a) directly, by adsorption on norite followed by hydrolysis of labile phosphate and (b) indirectly, using hexokinase and glucose as an ATP acceptor system either during the progress of photosynthetic phosphorylation or following the termination of the reaction and the chemical isolation of ATP. In both cases the product of the hexokinase reaction, glucose-6-phosphate, was chromatographically identified.

Chloroplasts removed from living cells retain for an appreciable period of time that part of the photosynthetic apparatus which is responsible for the evolution of oxygen under the influence of light (Hill reaction)[1] in accordance with equation 1

$$A + H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} H_2A + {}^1/_2O_2 \qquad (1)$$

in which A represents an electron or hydrogen acceptor other than carbon dioxide. Evidence has now been obtained that isolated whole chloroplasts, unaided by other cellular particles or enzyme systems, are also able to convert light energy into chemical energy in the form of the high energy pyrophosphate bonds of adenosine triphosphate (ATP). The following equations[2] represent a tentative scheme of the course of the reaction, in accordance with experimental facts now known.

$$H_2O \xrightarrow[\text{light}]{\text{chloroplasts}} 2e + 2H^+ + [O] \qquad (2)$$

$$2e + 2H^+ + [O] + AMP + 2Pi \longrightarrow ATP + H_2O \qquad (3)$$

$$\text{Sum: } 2Pi + AMP \xrightarrow[\text{chloroplasts}]{\text{light}} ATP \qquad (4)$$

AMP and Pi represent adenosine-5-phosphate and orthophosphate. Adenosine diphosphate (ADP) was found to be as effective as AMP. This photochemical esterification of inorganic phosphate by chloroplasts, henceforth referred to as photosynthetic phosphorylation, provides a mechanism for converting light energy into ATP, independent of energy released by reoxidation of partly or wholly reduced products of photosynthetic CO_2 fixation.[3]

Photosynthetic phosphorylation was found to proceed unimpaired when the partial pressure of CO_2 was maintained at an extremely low level. The nature of the electron acceptor(s) in reaction 2, of the intermediate electron carriers involved, and the number of high-energy phosphate bonds generated in reaction 3 are being investigated. As shown in equation 4, photosynthetic phosphorylation is not accompanied by the evolution of oxygen. The brackets around the oxygen atom in reactions 2 and 3 are intended to denote that it has not yet been determined whether: (a) oxygen is released in reaction 2 and immediately consumed in reaction 3, thus giving no manometrically measurable pressure change, or (b) the oxygen in reaction 2 represents the formation of some oxidized substance within the chloroplast which is reduced in accordance with reaction 3. In this connection the recent observation by Lundegårdh[4] of the oxidation of cyto-

* This investigation was supported in part by a research grant RG-2034 of the National Institutes of Health, Public Health Service. We wish to thank Dr. Hardin B. Jones for gifts of radioactive phosphorus.

(1) Hill, R., *Symp. Soc. Exp. Biol.* **5**, 222 (1951).

(2) This formulation is not intended to exclude the possibility that ATP is formed by the addition of pyrophosphate to AMP.

(3) J. Franck, *Arch. Biochem. Biophys.*, **45**, 190 (1953).

(4) H. Lundegårdh, *Physiologia Plantarum*, **7**, 375 (1954).

chrome-f in green cells on exposure to light is of interest.

The over-all reaction of photosynthetic phosphorylation (reaction 4) differs from the conventional Hill reaction (reaction 1) in that there is no net O_2 evolution; moreover, the reactants, phosphate and adenylate, and the product, ATP, are known cellular constituents rather than non-physiological oxidants such as ferricyanide or quinone. The photosynthetic phosphorylation system also differs from the model system of oxidative phosphorylation with illuminated chloroplast fragments studied by Vishniac and Ochoa.[5] In their system illuminated chloroplast fragments served a limited function of reducing added diphosphopyridine nucleotide (DPN). The reduced DPN was then reoxidized by mitochondria from non-photosynthetic tissue, namely, rat liver or mung beans. (We consider it doubtful that mitochondria from non-green portions of plants have greater significance for photosynthetic events than mitochondria from animal tissues.) In the absence of mitochondria their system was inactive; the omission of DPN halved the rate of the reaction. By contrast, under our experimental conditions no effect was observed from adding DPN or TPN (triphosphopyridine nucleotide) nor were any cellular particles other than washed whole chloroplasts required for photosynthetic phosphorylation. Chloroplasts were separated by differential centrifugation from other particles of smaller size such as mitochondria. The addition of these smaller particles to washed whole chloroplasts gave no increase in esterification of inorganic phosphate. Photosynthetic phosphorylation also differs from oxidative phosphorylation of the type investigated by Lehninger.[5a] In oxidative phosphorylation oxygen is consumed, while in photosynthetic phosphorylation, there is no evidence of utilization of atmospheric oxygen nor would any be expected from equations 2, 3 and 4.

Photosynthetic phosphorylation has been demonstrated in a system consisting of washed spinach chloroplasts, NaCl, ascorbic acid and P^{32}-labeled orthophosphate in the presence of a phosphate acceptor system: adenylic acid (AMP) or hexokinase plus glucose (hexokinase system). ATP synthesis was measured by the incorporation of P^{32} into organic phospnate. ATP was identified as the product of reaction 4 (a) directly, by adsorption on norite[6] followed by hydrolysis of labile phosphate and (b) indirectly, using hexokinase and glucose as an ATP acceptor system *either* during the progress of photosynthetic phosphorylation *or* following the termination of the reaction and the chemical isolation of ATP. In both cases the product of the hexokinase reaction, glucose-6-phosphate (G-6-P) was chromatographically identified.

Results

Light Dependence.—Esterification of inorganic phosphate by isolated chloroplasts was compared in light and darkness. Whole chloroplasts were prepared in cold 0.5 *M* glucose, and placed in vessels with phosphate, ascorbate and hexokinase. The "dark" vessel was covered with aluminum foil to exclude light. Several vessels were illuminated for different periods of time, to provide data for a time curve. After the reaction was terminated the reaction mixtures were analyzed for esterified phosphate. Figure 1 shows that phosphorylation by chloroplasts is a light-dependent reaction. It also demonstrates that phosphorylation proceeds for at least 1 hour at an almost constant rate.

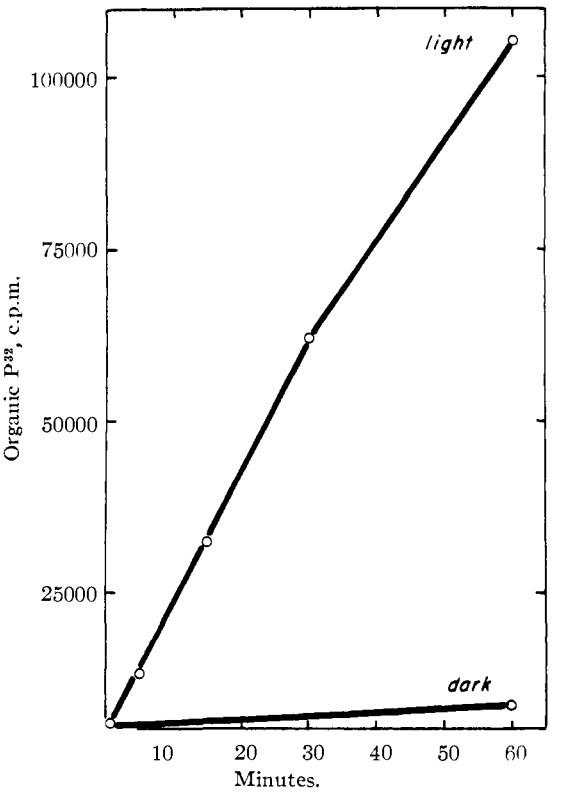

Fig. 1.—Effect of light on photosynthetic phosphorylation; chloroplasts prepared in 0.5 *M* glucose, 20 μmoles P_i added containing 450,000 c.p.m. P^{32}.

Photosynthetic Phosphorylation in the Absence of Carbon Dioxide.—Photosynthetic phosphorylation does not appear to require the presence of CO_2 for its operation.[7] A number of experiments were performed in which CO_2 was excluded from the system by making up fresh solutions in CO_2-free water and by having KOH in the center wells of the manometer vessels. In all cases phosphorylation proceeded at good rates when chloroplasts were illuminated with ascorbate and a phosphate accep-

TABLE I

PHOTOSYNTHETIC PHOSPHORYLATION IN THE ABSENCE OF CO_2

Exp. no.	Phosphate acceptor	Pi esterified, μmoles
906	Hexokinase system	3.4
920	AMP	1.5
922	AMP	2.7
924	AMP	1.5

(5) W. Vishniac and S. Ochoa, *J. Biol. Chem.*, **198**, 501 (1952).
(5a) A. L. Lehninger, *ibid.*, **190**, 345 (1951).
(6) R. K. Crane and F. Lipmann, *ibid.*, **201**, 235 (1953).
(7) D. I. Arnon, M. B. Allen and F. R. Whatley, *Nature*, **174**, 394 (1954).

tor system, either hexokinase plus glucose, or 10 μmoles of AMP. The results of several experiments are summarized in Table I.

Requirement for Oxygen.—When air in the manometer vessels was replaced by nitrogen and residual oxygen removed by having an Oxsorbent solution (chromous chloride) in the vessel it was found that photosynthetic phosphorylation was greatly diminished, as shown in Fig. 2. Aerobically the phosphorylation continued undiminished for an hour, but in the absence of oxygen phosphorylation proceeded at a comparable rate for only 5 minutes, followed by an insignificant increase during the next 55 minutes.[7a]

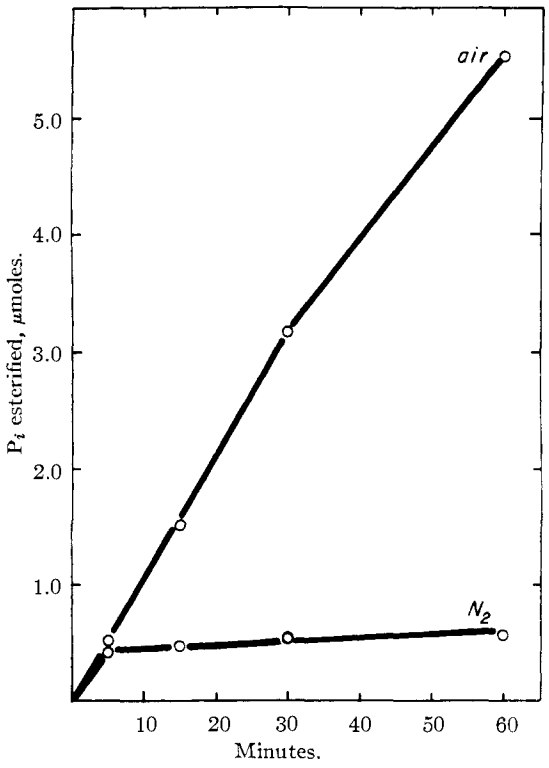

Fig. 2.—Photosynthetic phosphorylation under aerobic and anaerobic conditions; chloroplasts prepared in 0.5 *M* glucose, *cf.*[7a]

(7a) NOTE ADDED IN PROOF.—Further investigation of cofactors of photosynthetic phosphorylation has clearly shown that molecular oxygen is not required for photosynthetic phosphorylation. In fact, in the presence of the proper combination of cofactors, photosynthetic phosphorylation was found to proceed much more rapidly anaerobically than aerobically.

In addition to ascorbic acid, the inclusion of the following cofactors markedly increased photosynthetic phosphorylation: Mg^{++} (replaceable, but less effectively, by Mn^{++} or Co^{++}), riboflavin phosphate (FMN) and the vitamin K compound menadione (2-methyl-1,4-naphthoquinone). Mg^{++} was required under all conditions. The effect of the other cofactors was influenced by the presence of oxygen. Under aerobic conditions a marked increase in photosynthetic phosphorylation was obtained on the addition of *either* FMN plus ascorbate (*ca.* 7-fold increase) *or* menadione (*ca.* 3-fold increase). However, in the presence of FMN plus ascorbate no effect of added menadione was observed under aerobic conditions. Under anaerobic conditions the addition of either FMN plus ascorbate or menadione caused a *ca.* 16-fold increase in photosynthetic phosphorylation. Moreover, anaerobically the effects of FMN plus ascorbate and of menadione were additive (*ca.* 27-fold increase with all three) and under these conditions we obtained an esterification of 16.7 μmoles of Pi (out of 20 added) per 0.5 mg. chlorophyll per hour.

The stimulating effect of menadione was inhibited by dicoumarol and "antivitamin K" substituted naphthoquinones such as 2-hydroxy-3-[3'-(4-*trans*-hydroxycyclohexyl)-propyl]-1,4-napthoquinone (kindly supplied by Dr. W. G. Dauben).

Effect of Ascorbate.—Illuminated chloroplasts when supplied only with inorganic phosphate and the hexokinase acceptor system carried out some esterification of phosphate. On the addition of ascorbate there was a marked increase in phosphorylation, as shown in Table II. The addition of 0.5 μmole of ascorbate gave a marked increase in phosphorylation; higher concentrations of ascorbate gave only a small additional increase. The chloroplast preparations do not oxidize ascorbate in the dark. Lipmann[8] considered the possibility that ascorbic acid may be involved in oxidative phosphorylation. Friedkin and Lehninger[9] observed that the reduction of cytochrome-c by ascorbate, as distinct from several other reductants, was coupled with some esterification of inorganic phosphate. The role of ascorbate in photosynthetic phosphorylation is being further investigated.[7a]

TABLE II

EFFECT OF ASCORBATE ON PHOTOSYNTHETIC PHOSPHORYLATION

Chloroplasts prepared in 0.5 *M* glucose. Hexokinase system used as phosphate acceptor.

	Phosphate esterified, μmoles							
Ascorbate added, μmoles →	0	0.5	1.0	1.5	2	5	10	20
Exp. 908	0.54	..	..	..	0.96	1.2	..	1.3
909	0.86	..	..	..	3.3	3.5	4.7	..
910	1.8	3.0	3.0	3.2	3.5	..	3.7	..

Photosynthetic Phosphorylation as Measured by the Hexokinase System.—Hexokinase[10] which catalyzes reaction 5

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP} \quad (5)$$

is frequently used as a "trapping" system for newly-formed ATP. This enzyme was used to measure photosynthetic phosphorylation by trapping ATP as it was formed during illumination. As shown in Table III, organic phosphate was produced when whole chloroplasts were illuminated together with phosphate, ascorbate, AMP, hexokinase and glucose. AMP (ADP was almost equally effective) served as the acceptor for inorganic phosphate,

TABLE III

PHOTOSYNTHETIC PHOSPHORYLATION AS MEASURED BY THE HEXOKINASE + GLUCOSE SYSTEM

Chloroplasts prepared in 0.5 *M* glucose. 20 μmoles of Pi containing 700,000 and 561,000 c.p.m. added in experiments 907 and 910, respectively.

Exp. no.	Treatment	PO_4 esterified C.p.m.	μmoles
907	Complete	70,000	2.0
907	AMP omitted	22,800	0.66
907	Hexokinase omitted	21,700	0.62
910	Complete	104,300	3.7
910	AMP replaced by ADP	83,500	3.0

(8) F. Lipmann, in "Currents in Biochemical Research," D. E. Green, Ed., Interscience Publishers, Inc., New York, N. Y., 1946, p. 137.

(9) M. Friedkin and A. L. Lehninger, *J. Biol. Chem.*, **178**, 611 (1949).

(10) M. Kunitz and M. R. McDonald, *J. Gen. Physiol.*, **29**, 393 (1946).

forming ATP which was used by hexokinase in accordance with reaction 5. The omission of either AMP or hexokinase diminished phosphorylation. Nevertheless, an appreciable amount of phosphate was esterified without added AMP or hexokinase, suggesting the presence of some phosphate acceptor system within the chloroplasts.

Glucose-6-phosphate was shown by chromatographic separation (Table IV) to be the product of photosynthetic phosphorylation in the presence of the hexokinase system, as would be expected from equation 5. After stopping the reaction with trichloroacetic acid (TCA), aliquots of the TCA extract were chromatographed on Whatman No. 4 filter paper, previously washed with 0.5% oxalic acid. Authentic glucose-6-phosphate was used as a marker and as a carrier. The chromatograms were developed in the ethyl acetate–acetic acid–water solvent of Mortimer.[11] After drying, the papers were sprayed with the Hanes–Isherwood reagent[12] for phosphate compounds. The radioactivity of the colored areas corresponding to the separated glucose-6-phosphate and inorganic phosphate was then measured.

TABLE IV

IDENTIFICATION OF GLUCOSE-6-PHOSPHATE PRODUCED DURING PHOTOSYNTHETIC PHOSPHORYLATION IN THE PRESENCE OF HEXOKINASE

Chloroplasts prepared in 0.5 *M* glucose.

Treatment	Pi esterified (Mg method), μmoles	Radioactive G-6-P (chromatographically isolated), μmoles
Complete system	2.0	3.2
AMP omitted	0.66	1.2
Ascorbate omitted	.80	1.1
Hexokinase omitted	.62	0.03

By determining the c.p.m. of the glucose-6-phosphate and inorganic phosphate spots on paper, and knowing the total amount of labeled inorganic phosphate added to the reaction mixture, it was possible to compute the number of micromoles of inorganic phosphate converted to glucose-6-phosphate. The results of such a computation are given in Table IV, which shows the importance of the individual components of the complete reaction mixture. A comparison between the esterified phosphate as measured by the magnesia mixture method and the chromatographic method confirms that the organic phosphate formed was glucose-6-phosphate. The consistently lower results for organic phosphate obtained by the magnesia mixture were probably due to adsorption of some of the glucose-6-phosphate on the $MgNH_4PO_4$ precipitate. In the absence of added hexokinase no formation of glucose-6-phosphate was observed.

Adenylic Acid (AMP) as a Phosphate Acceptor System in Photosynthetic Phosphorylation.—In the hexokinase system for measuring phosphorylation there is no accumulation of ATP, since as soon as it is formed it is used up in the phosphorylation of glucose in accordance with reaction 5. It was desirable to demonstrate directly the formation of ATP in photosynthetic phosphorylation. This was done by using AMP as the phosphate acceptor. Chloroplasts prepared in 0.35 *M* NaCl were illuminated in the presence of different amounts of AMP, without added hexokinase and glucose. The photosynthetic phosphorylation under these conditions was compared with that in the presence of hexokinase and glucose. The results are summarized in Table V. Chloroplasts alone, in the absence of any phosphate acceptor system, showed only a trace of photosynthetic phosphorylation. One micromole of AMP was clearly insufficient as a phosphate acceptor in the absence of hexokinase; with hexokinase, however, this level of supply of AMP was compatible with vigorous phosphorylation. In the presence of 10 or 20 micromoles of AMP appreciable quantities of inorganic phosphate, up to 25% of the total, were esterified. Following separation of the photochemically synthesized ATP from the reaction mixture, the ester phosphate was identified as ATP, chemically, by adsorption on norite and acid hydrolysis of labile phosphate and, enzymatically, with the hexokinase system.

TABLE V

ADENYLIC ACID AS A PHOSPHATE ACCEPTOR SYSTEM IN PHOTOSYNTHETIC PHOSPHORYLATION

Chloroplasts were isolated in 0.35 *M* NaCl. 20 μmoles of Pi containing 414,600 c.p.m. added in each case.

Treatment	Phosphate esterified: C.p.m.	μmoles	% of added Pi
Chloroplasts only	2,100	0.10	0.5
Chloroplasts + 1 μmole AMP	7,350	0.36	1.8
Chloroplasts + 10 μmoles AMP	105,800	5.1	25.6
Chloroplasts + 20 μmoles AMP	90,500	4.4	22.1
Chloroplasts + hexokinase system (1 μmole AMP)	130,200	6.3	31.4

Identification of ATP Formed by Photosynthetic Phosphorylation. (a) Adsorption on Norite.—Crane and Lipmann[6] have shown that norite adsorbs ATP (and other nucleotides) but not inorganic phosphate or phosphorylated sugars. These observations, which were confirmed in our laboratory, were used to distinguish the products of photosynthetic phosphorylation. With the hexokinase system the product of photosynthetic phosphorylation was glucose-6-phosphate, which is not adsorbed on norite. With AMP as the sole phosphate acceptor, ATP, which is adsorbed by norite, would be the expected product of the reaction. Table VI shows that the products formed in the two cases behaved toward norite as predicted. Prior to treatment with norite, inorganic phosphate was removed from the

TABLE VI

SEPARATION OF ATP BY ADSORPTION ON NORITE

	AMP system[a]	Hexokinase system[b]
	C.p.m.	
Organic phosphate (Mg filtrate)	90,500	130,000
Adsorbed on norite	99,000	7,400
Eluted with pyridine	69,000	.....
Total phosphate (TCA extract)	277,500	
Adsorbed on norite	123,700	
Released by acid hydrolysis	95,350	

[a] AMP used to accept phosphate. [b] Hexokinase system used to accept phosphate.

(11) D. C. Mortimer, *Can. J. Chem.*, **30**, 653 (1952).

(12) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

TCA extracts of the reaction mixture by precipitation with magnesia mixture. The filtrates were acidified with HCl. One-ml. aliquots were each treated with *ca.* 50 mg. norite, which was then filtered off and washed with water. The radioactivity of the magnesia filtrate (total organic phosphate) and of the washed norite was measured.

Table VI also shows that most of the ATP adsorbed on norite could be eluted with pyridine, as expected for a nucleotide.

(b) **Acid Hydrolysis of Labile Phosphate.**—In another experiment in which AMP was the sole phosphate acceptor, norite was added directly to the TCA extract of the reaction mixture, without prior treatment with magnesia mixture. The norite was filtered off, washed with glass-distilled water and suspended in *N* H_2SO_4. The suspension was placed in a water-bath at 100° for 10 minutes.[6] This treatment hydrolyzed ATP, releasing most of the radioactive phosphate from the norite, as shown in Table VI.

(c) **Enzymatic Identification of Isolated ATP.**—In previous experiments (Tables III and IV) the hexokinase system was present in the reaction mixture during the course of photosynthetic phosphorylation. We have also isolated ATP formed when AMP was the sole phosphate acceptor in photosynthetic phosphorylation and used the hexokinase system in the dark to synthesize glucose-6-phosphate. Following illumination inorganic phosphate was removed from the TCA extract by precipitation at *p*H 8.5 as $MgNH_4PO_4$. ATP was precipitated as the barium salt after the addition of 50 micromoles of non-radioactive ATP as carrier. The barium salt was converted to the sodium salt of ATP, and the solution neutralized to *p*H 7.3. Hexokinase and 1% glucose were then added and the mixture was incubated for 40 minutes at 30°. At the end of this period TCA was added to precipitate the proteins and the nucleotides were removed by adsorption on norite. Aliquots of the remaining filtrate, containing glucose-6-phosphate, were then chromatographed as described above. The results are shown in Fig. 3.

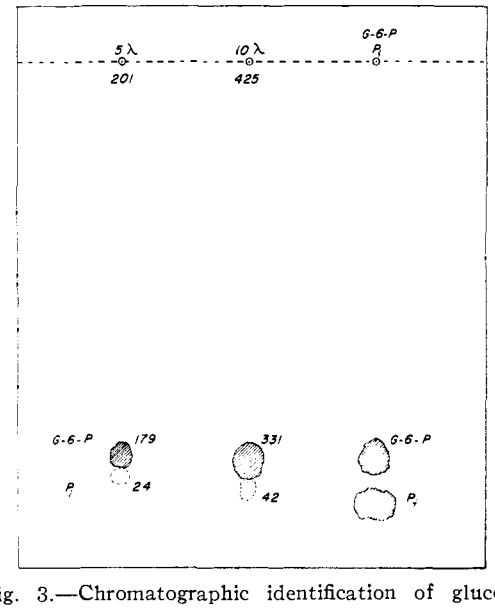

Fig. 3.—Chromatographic identification of glucose-6-phosphate (G-6-P) formed from ATP synthesized during photosynthetic phosphorylation. Volume of solution chromatographed was 5 or 10 λ applied on dotted line as shown. 1 μmole each of G-6-P and P_i applied as markers. Chromatogram was run in ethyl acetate:acetic acid:water solvent, as described in text. Numbers adjacent to spots refer to c.p.m. P^{32}. Numbers below dotted line refer to c.p.m. P^{32} applied.

Role of Other Factors.—Of the several compounds tested so far the only one which consistently increased photosynthetic phosphorylation was ascorbic acid, as previously discussed. The addition of DPN, TPN, uridine triphosphate, cocarboxylase, thioctic acid and a mixture of 6-thioctic acid and cocarboxylase was without effect on photosynthetic phosphorylation. (The thioctic acid was kindly supplied by Drs. T. H. Jukes and E. L. R. Stokstad.) The role of other cofactors is under investigation.[7a]

Experimental

Separation of Chloroplasts.—All operations were carried out in the cold. Spinach leaves were ground in 0.35 *M* NaCl or 0.5 *M* glucose, and whole chloroplasts were separated by centrifugation at 1000 *g* for 7 minutes. The chloroplasts were washed once and resuspended in NaCl or glucose solution. A more detailed discussion of the preparation of chloroplasts will be presented elsewhere.

Measurement of Photosynthetic Phosphorylation.—The reaction was carried out in Warburg manometer vessels of conventional design. To the main compartment were added 15 μmoles of a mixture of potassium and sodium phosphate, *p*H 7.1, 10 μmoles of sodium ascorbate, except as otherwise noted, and an acceptor for the newly esterified phosphate, consisting of either 10 μmoles of AMP, or 1 μmole of AMP with 1% glucose and 75 units of hexokinase.[10] In the side-arm was placed 5 μmoles of phosphate, *p*H 7.1, containing P^{32}. The vessels were then chilled in ice. A suspension of chloroplasts in 0.5 *M* glucose or 0.35 *M* sodium chloride was pipetted into the main compartment and the volume brought to 3 ml. by the addition of cold glucose or sodium chloride solution. Unless otherwise stated, an aliquot of chloroplasts was taken to give 2 mg. of chlorophyll per vessel; determinations of chloropylll were made as previously described.[13] The vessels were attached to manometers and shaken at 15° in a refrigerated water-bath adapted for photosynthesis work.[14] The reaction was started by tipping in the P^{32} solution and turning on the light.

The reaction was terminated by turning off the light and adding 0.3 ml. of 20% trichloroacetic acid to each vessel. Chloroplast debris was removed by centrifugation, the precipitate washed with 2% trichloroacetic acid, and the supernatant liquid and washings were made up to 5 ml. These operations were carried out in the cold.

"Organic phosphate" was measured by the radioactivity which remained after the removal of the inorganic phosphate as magnesium ammonium phosphate. Two ml. of trichloroacetic acid extract was mixed with 2 ml. of magnesia mixture,[15] to which was added a drop of phenolphthalein solution to check that the *p*H remained at 8.5. The radioactivity of the inorganic phosphate was diluted by the subsequent addition of 0.2 ml. of 0.1 *M* K_2HPO_4, and the mixture allowed to stand in the cold for 1 hour. The precipitate of $MgNH_4PO_4$ was then filtered off and washed twice with dilute magnesia mixture. Blank runs showed that insignificant amounts of radioactive inorganic phosphate remained in solution after this procedure.

Radioactive phosphorus was estimated by pipetting 1-ml. samples into shallow plastic cups, evaporating to dry-

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

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

(15) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques and Tissue Metabolism." Burgess Publishing Co., Minneapolis, Minn., 1949.

ness under an infrared lamp with the aid of a current of warm air, and counting under a thin-window Geiger counter of conventional design.

The total amount of P^{32} added to each vessel was 500,000–1,000,000 c.p.m.

BERKELEY, CALIFORNIA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

A New Method for the Synthesis of Macrocyclic Peptides

BY JOHN C. SHEEHAN AND WALLACE L. RICHARDSON[1]

RECEIVED AUGUST 10, 1954

A synthesis of the crystalline macrocyclic peptide cyclotri-(glycyl) by a convenient new method is reported. Triglycine azide hydrochloride, prepared by selective diazotization of triglycine hydrazide, is cyclized in 42% yield by neutralization under dilution conditions.

Recently the syntheses of several cyclic tripeptides have been claimed. In two instances only amorphous products were obtained.[2,3] In a third case the cyclic peptide product was stated to be crystalline, although it consisted presumably of four stereoisomeric forms (two racemates), since it was obtained by heating glycyl-DL-alanyl-DL-phenylalanine methyl ester under reflux in a methanolic solution containing ammonia.[4] The cyclic peptide–hormone oxytocin, as prepared by du Vigneaud and co-workers,[5] constitutes a special case since the cyclization step involved formation of a disulfide linkage by the controlled oxidation of two sulfhydryl groups present as cysteine residues in the peptide chain.

This communication reports a new and convenient method for the synthesis of cyclic peptides containing more than two amino acid units (macrocyclic peptides). Triglycine azide hydrochloride is cyclized by neutralization in homogeneous aqueous solution at 0–4° under dilution conditions, producing 42% of crystalline cyclo-(triglycyl).

In 1949 Hofmann and Magee[6] reported that in acidic media triglycine hydrazide could be diazotized selectively (without appreciable deamination) to the corresponding azide, from which they obtained a polyglycine by basification in concentrated solution. Since our aim was to accomplish the opposite result, namely, to promote cyclization and to inhibit polymerization, we selected conditions of relatively high dilution for the neutralization. The reaction sequence employed is illustrated by the accompanying equations.

Since limited solubility in the common solvents handicaps molecular weight determinations based on colligative properties, X-ray unit cell measurements are being undertaken. Cyclotriglycyl samples are homogeneous as determined by paper chromatography, and the general behavior of the compound leaves little doubt that it is not a dimer or higher molecular weight compound.

The generality of this new peptide cyclization technique is being investigated, and extensions involving very slow addition of peptide azide salts to buffered solutions are being explored.

Phthaloyltriglycine ethyl ester, a precursor in this sequence, was prepared by a modification of the general method for the preparation of phthaloyl peptide esters as recommended by Sheehan, Chapman and Roth.[7] Best results were obtained when phthaloylglycyl chloride was added to a mixture of diglycine ethyl ester hydrochloride and triethylamine in methylene chloride at low temperature.

Cleavage of the phthaloyl group from phthaloyltriglycine ethyl ester was effected at room temperature and the product was isolated as triglycine ethyl ester hydrochloride. It was not feasible to convert the phthaloyl peptide ester directly to the corresponding peptide hydrazide by use of an excess of hydrazine hydrate.

Triglycine hydrazide was readily prepared by storing triglycine ethyl ester hydrochloride three days with excess hydrazine hydrate and then passing the reaction products through a column packed with an anion-exchange resin. On concentration, the effluent yielded the very hygroscopic triglycine hydrazide, which was selectively diazotized. The triglycine azide hydrochloride solution thus produced was diluted with a large volume of ice-water and several equivalents of sodium bicarbonate was added. After storage of the solution for two days at 0–4°, cyclo-(triglycyl) was isolated in 42% yield by crystallization from water as colorless rods. Analysis showed this material to be a hemihydrate.

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