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CYANIDE EFFECTS ON CARBON DIOXIDE FIXATION IN CHLORELLA

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Abstract

Green algae have been treated with radioactive KCN in an investigation of the effect of cyanide on photosynthesis. A multitude of products have been found to be formed in very short exposures (10 to 15 sec). One of these products has been identified with the product formed when the algae are given radioactive CO_2 and nonradioactive KCN. The same product has been synthesized by a nonenzymatic cyanohydrin addition reaction on ribulose-1, 5-diphosphate. It has been shown to be a 2-carboxy-pentitol (probably mostly ribitol)-1, 5-diphosphate. Upon hydrolysis it gives an hydroxy acid (or mixture of isomers) closely related to hamamelonic acid. The significance of this and the other as yet unidentified products of cyanide interraction with a biological system is discussed with respect to the use of cyanide as an inhibitor.

CYANIDE EFFECTS ON CARBON DIOXIDE FIXATION IN CHLORELLA[1]

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Introduction

The effects of cyanide on biochemical systems, both in vivo and in vitro, have been studied under a large variety of conditions. Thus, the use of cyanide as an inhibitor for enzymes such as tyrosinase[5] and catalase[6] is well know. The mode of action appears to be due to metal inactivation.[7] The possibility that cyanide acts as a carbonyl trap by cyanohydrin formation has been suggested in a limited way.[8]

Applications of cyanide to the problem of photosynthesis are also quite numerous. For example, in 1919 Warburg found that HCN, administered at very low concentrations, decreased the rate of photosynthesis (O_2 evolution) and increased the rate of respiration in Chlorella.[9] The experiment was done in the presence of strong light and abundant carbon dioxide. A similar result was observed by Ruben, et al. when cyanide was added to Chlorella at high concentrations (10^{-2} M KCN); both the photosynthesis rate (CO_2 uptake) and the carbon dioxide dark-fixation rate were reduced to 0.3% of the normal values, but its respiration remained essentially unchanged.[10] On the other hand, Gaffron noted that cyanide affected Scenedesmus D1 in the opposite manner.[11] Thus, an addition of 2×10^{-4} M KCN inhibited respiration nearly completely while

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[4]King George VI Memorial Fellow for New Zealand, 1956-1957.

[5]L. E. Tenebaum and H. Jensen, J. Biol. Chem. 147, 27 (1943).

[6]K. Zeile and H. Hellström, Z. physiol. Chem. 192, 171 (1930).

[7]Neilands, Stumpf, and Stanier, Outlines of Enzyme Chemistry (Wiley, New York, 1955), p. 91.

[8]E. A. Zeller, The Enzymes, J. B. Sumner and K. Myrbäck, Eds., Vol. II, part 1 (Academic, New York 1951), p. 552.

[9]O. Warburg, Biochem. Z. 100, 230 (1919).

[10]Ruben, Kamen, and Hassid, J. Am. Chem. Soc. 62, 3443 (1940).

[11]H. Gaffron, Biol. Zentr. 59, 302 (1939).

photosynthesis (O_2 evolution) remained untouched. In a later work it was found that the cyanide-suppressed CO_2 dark fixation was a function of the preillumination period.[12] That is, the longer the KCN is allowed to stand in the light with the plant, before the introduction of $C^{14}O_2$ and darkening, the greater the inhibition of CO_2 dark fixation. A marked effect on the C^{14} content of the water-soluble fraction was also observed. Meanwhile, in this laboratory, we had examined the effect of cyanide on $C^{14}O_2$ fixation by Scenedesmus (Gaffron D-3 strain) during photosynthesis.[13] The algae were preilluminated aerobically without CO_2 for 30 minutes. Cyanide (3×10^{-4} M) was added 1 minute before the addition of $C^{14}O_2$ followed by 1 minute photosynthesis. A 95% inhibition of $C^{14}O_2$ fixation was found. It is interesting to note that the ratio of 3-phosphoglycaric acid to hexose and triose phosphates varied from 1:4.5 under noninhibited conditions to 3.2:1 under cyanide-inhibited conditions. The conclusion from this experiment was that cyanide, under these conditions, does not prevent the initial carboxylation step and the subsequent formation of phosphoglyceric acid. Related to this work is the cyanide-inhibition experiment on the in vitro system, carboxydismutase: CO_2 fixation appeared to be completely suppressed at 10^{-2} M KCN while lower concentrations (10^{-3} M and 10^{-4} M) yielded about 50% and 2% inhibition, respectively.[14]

An extension of the examination of the effect of CN^- on the pattern of CO_2 fixation in the alcohol-water-soluble fractions of Chlorella was made in the early part of 1957 by Otto Kandler, in this laboratory.[15] The algae were subjected to normal photosynthesis (10 sec) with $C^{14}O_2$. To the algal suspension was added KCN (final concentration M/80) followed by a 10-second light period, and finally hot alcohol was injected (to a final concentration of 80%) to stop all the enzyme reactions. The essential features of these experiments were the large decrease of C^{14} activity in phosphoglyceric acid and concurrent increase of C^{14} activity in the diphosphates. The latter, when treated with acid phosphatase, gave two new spots plus other known sugars. The new spots were characterized by means of paper electrophoresis and rechromatography. These spots were found to exhibit an acid-lactone behavior.

It is the purpose of this paper to establish in more detail the role of cyanide in the formation of this new compound in Chlorella. Beyond this it seemed useful to examine the fate of $C^{14}N^-$ in biological systems in order to try to understand its widespread biological effects.

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[12] Gaffron, Fager, and Rosenberg, Symposia Soc. Exptl. Biol. 5, 262 (1951).

[13] Calvin, Bassham, Benson, Lynch, Ouellet, Schou, Stepka, and Tolbert, Sumposia Soc. Exptl. Biol. 5, 284 (1951).

[14] H. L. Kornberg, J. R. Quayle, and M Calvin, Studies on the Carboxylation Reaction of Photosynthesis, UCRL-2885, May 1955.

[15] Otto Kandler, in Chemistry Division Quarterly Report, UCRL-3710, March 1957, p. 8, also Arch. Biochem. and Biophys., in press.

Experimental Procedures

Photosynthesis Experiment

The cultures of Chlorella pyrenoidosa used in the photosynthesis experiments were grown in the medium described by Holm-Hansen et al.[16] using a continuous-culture apparatus with constant fluorescent lighting of approximately 1,000 ft-c. After harvesting, the algae were centrifuged, washed, and resuspended in distilled water to give a 1% suspension. Aliquots of 10 ml were used in the photosynthesis experiments, which were performed in the vessel illustrated in Fig. 1.

Illumination was provided by reflector spotlight, which gave a light intensity of approximately 7,000 ft-c on either side of the vessel; excessive heat production was avoided by use of water-cooled infrared filters.

The algae were preilluminated for 20 minutes; a continuous stream of 1% CO_2 in air was bubbled through during this period. At the commencement of the experiment the air stream was shut off and sodium bicarbonate (1 ml of 0.0013 M) was added. After 2 minutes' photosynthesis with occasional stirring, KCN (1 ml of 0.24 M) was added, and after a further 15 seconds the algae were killed by the addition of boiling ethanol (40 ml) to produce an 80% alcoholic mixture. The experiments were of two types:

(a) Using a combination of $NaHC^{14}O_3$ (20 μC/ml) and KCN; designated as $C^{14}O_2$, CN^-;

(b) Using a combination of $NaHCO_3$ and $KC^{14}N$ (3.6 mC/ml)[17]; designated as CO_2, $C^{14}N^-$.

Extracts for chromatography were prepared by centrifuging the mixture; the supernatant liquid was retained and the residue was extracted successively with 10 ml of 20% ethanol and 10 ml of boiling water. All the extracts were combined, concentrated to approximately 3 ml in a rotating evaporator,[18] and chromatographed in two dimensions (phenol-water first dimension, butanol-propionic acid-water second dimension).[19] The diphosphate was eluted with water and either used as such or hydrolyzed. Two methods of hydrolysis were employed: (a) HCl (1 N) hydrolysis in a sealed tube at 120°C for 16 hours, and (b) enzymic hydrolysis at pH 5.0 (0.2 M in acetate buffer and 0.01 M Mg^{++}) using purified Polidase-S[20] (Schwarz Laboratories, Inc.) or acid prostatic phosphatase.[21]

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[16] O HOlm-Hansen, P. Hayes, and P. Smith, in Chemistry Division Quarterly Report, UCRL-3595, Oct. 1956, p. 56.

[17] R. Lemmon, B. Fingerman and H. Simon, in Chemistry Division Quarterly Report, UCRL-3836, June 1957, p. 18. It was shown that the amount of $C^{14}O_2$ present in $KC^{14}N$ could not be greater than 0.001%.

[18] Rinco Instrument Co., Greenville, Illinois.

[19] A. A. Benson, Moderne Methoden der Pflanzenanalyse II, K. Paech and M. V. Tracey, Eds. (Springer, Berlin. 1955), p. 113.

[20] S. S. Cohen, J. Biol. Chem. 201, 71 (1953).

[21] G. Schmidt, Methods of Enzymology, S. P. Colowick and N. O. Kaplan, Vol. II (Academic New York, 1955), p. 523.

Chromatography and Electrophoresis

Whatman No. 4 paper was used throughout, except where otherwise noted. For routine chromatography the paper was washed according to Benson;[19] for chromatography in ethanol–water–formic acid–sodium molybdate, it was washed according to Trudinger.[22] (In the latter case Whatman No. 1 was used.) Unwashed paper was used for electrophoresis and for chromatograms which were subsequently sprayed with Tollen's reagent. ($AgNO_3$ dissolved in a little water, concentrated NH_4OH added until the precipitate dissolves, then diluted with methanol to 5% $AgNO_3$ w/v). The apparatus routinely used in this laboratory for chromatography was employed. Electrophoresis was carried out in an apparatus similar to that described by A. B. Foster.[23] Glycine buffer (0.1 M) pH 9.75 was used and the experiment was run at 600 v (10 to 20 ma) for 3 hr. at room temperature. For chromatography with carrier hamamelonic acid diphosphate, the ethanol–water–formic acid–sodium molybdate system was used.

Phosphate esters were detected on the paper by spraying with the reagent discribed by Hanes and Isherwood,[24] drying in an oven with a forced air draft at about 60° C, and exposing to sunlight. Free sugar and hamamelonic acid were detected by spraying the paper, after thorough steaming, with Tollen's reagent. The paper was washed with 5% ammonia to remove the excess of silver salts and to reduce the background color. Both hamamelonic acid and the lactone react poorly with the spray, and prolonged heating (several minutes at 100° C) is required for satisfactory results.

Radioactive areas on paper chromatograms were detected by exposing the paper to DuPont X-ray film type 507. For plate counting, Mylar[25] end-window, Q gas GM tubes were employed; the planchets were prepared in 2N acetic acid and dried under an infrared lamp.

Preparation and Assay of Phosphoriboisomerase-Phosphoribulokinase Mixture

The preparative steps are illustrated in the flow diagram, Fig. 2.[26, 27] It is essential that all operations be carried out at 0° C.

The isomerase-kinase mixture was assayed by measuring the rate of acid production by means of a pH-stat.[28] The test solution contained 11 μmoles adenosine triphosphate (ATP),[29] 10 μM ribose-5-phosphate, 10 μM $MgCl_2$, and 5 μM cysteine in 10 ml. The pH was adjusted to 7.40 and held constant during the reaction by addition of 0.1 M NaOH. The reaction was started by the addition of the isomerase-kinase mixture (150 μl). In a typical assay 7.14 μmoles

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[22] P. A. Trudinger, Biochem. J. 64, 274 (1956).

[23] A. B. Foster, Chem. and Ind. (London) 1050 (1952).

[24] C. S. Hanes and F. A. Isherwood, Nature 164, 1107 (1949).

[25] Mylar is DuPont's registered trademark for its polyester film. Paul Hayes, in Chemistry Division Quarterly Report, UCRL-2932, March 1955, p. 48.

[26] Horecker, Hurwitz, and Weissbach, J. Biol. Chem. 218, 785 (1956).

[27] J. R. Quayle, Preparation and Some Properties of Ribulose-1, 5-Diphosphate, UCRL-3017, Jan. 1956.

[28] J. B. Neilands and M. D. Cannon, Anal. Chem. 27, 29 (1955).

[29] The following abbreviations are used throughout this paper: ATP, adenosine triphosphate; RMP, ribose-5-phosphate; RuDP, ribulose-1, 5-diphosphate; PGA, Phosphoglyceric acid; PGADP, [illegible] diphosphate; RuMP, ribulose-5-phosphate.

NaOH were consumed in 25 minutes; the alkali uptake slowed down considerably after 10 minutes. A control without ribose-5-phosphate showed that the preparation contained very little ATPase activity (alkali consumption ceased after 10 minutes; a total of 1.5 μmoles was consumed).

Preparation of RuDP

The method depends on the conversion of RMP to RuDP in the presence of ATP and the isomerase-kinase mixture. The barium salt of ribose-5-phosphate (4 μmoles, 1.83 g) was dissolved in water (40 ml) and potassium sulfate (40 ml of 0.15 M) added. The mixture was centrifuged and the residue washed with water (20 ml). To the combined washings and supernatant liquid 6 μmoles ATP, 0.4 μmole cysteine and 4 μmoles $MgCl_2$ were added and the volume made up to 300 ml. The reaction was carried out in the pH-stat at room temperature under an atmosphere of N_2 gas; the pH was adjusted to 7.40 and held constant by the addition of CO_2-free NaOH (1.8 N). The reaction was started by the addition of the isomerase-kinase preparation (5 ml) and stopped, after 37 minutes when the uptake of alkali (1.5 ml) had slowed down, by the addition of acid-washed Norite A (120 g). The solution was centrifuged (2000 rpm for 10 minutes) and the residue washed successively with four 200-ml increments of water. Barium acetate (4 ml of 1 M) was added to the combined supernatant liquid and washings, and the pH adjusted to 6.4 with saturated barium hydroxide. The precipitate was removed by centrifugation and washed with water (50 ml). The supernatant liquid and washings were combined and an equal volume of ethanol was added. RuDP precipitates in a gelatinous form; it was collected by centrifugation, washed with 80% ethanol and dried *in vacuo* over P_2O_5; yield: 1.75 g (crude).

Analysis of the preparation showed it to contain 0.4 μmole of inorganic phosphate and 2.31 μmoles of organic phosphate per mg. These analytical figures must be regarded as approximate only. If as assumed, the only phosphate ester present was the dibarium salt of RuDP, the product was 69% pure. In the experiments with cyanide, this preparation was used without further purification.

Purity of RuDP as Determined by Column Chromatography

The column employed was 0.8 cm (diameter) by 27 cm. The resin used was Dowex-1 formate; it was freed from fines by decantation and converted from the chloride to the formate form with 2 N formic acid.

The elution apparatus consisted of two vessels of equal size and shape. One, the mixing vessel, was provided with a stirrer and had an outlet to the top of the column; water (100 ml) was placed in this vessel. Ammonium formate (100 ml, 2 M) was placed in the other vessel and the two vessels connected together by means of a siphon tube. This system provides a linear gradient of ammonium formate at the outlet of the mixing vessel.

RuDP (100 mg Ba salt) was dissolved in water (30 ml) with the aid of a little Dowex-50 (H^+ form) and the pH adjusted to 6.0 with KOH. The solution was centrifuged and run through the column. The column was washed with water and subjected to gradient elution; fractions were collected every 5 minutes by means of an automatic device. The flow rate was 0.44 ml/min.

The fractions found to contain phosphorus by qualitative test[24] were assayed for total phosphate[30] and RuDP. The method used for the determination of RuDP was based on the fixation of $C^{14}O_2$ by RuDP in the presence of carboxydismutase. The reagent solution was prepared by mixing $NaHC^{14}O_3$ (200 µl of 0.036 N, 400 µC/ml), $MgCl_2$ (80 µl. of 0.01 M in 0.04 N HCl), tris[31] buffer (20 µl, pH 7.83, 1 M with respect to tris), and carboxydismutase (50 µl of a spinach preparation fractionated between 35% and 39% saturated ammonium sulfate and containing 6 mg of protein per ml). An aliquot from each column fraction (5 µl) was placed on a planchet and the reagent solution (15 µl) added. The planchet was placed on moist filter paper in a covered vessel, to reduce evaporation, and allowed to incubate at room temperature. After 2 hours the reaction was stopped by the addition of acetic acid (10 µl of 6 N) and the planchet prepared for counting in the usual manner. The elution pattern obtained is shown in Fig. 3.

Two well-separated phosphorus-containing peaks are observed. The first to emerge contains inorganic phosphate; the second peak is in the position expected for a diphosphate ester and the enzyme assay shows it to contain essentially all the RuDP emerging from the column. It is noteworthy that the RuDP and phosphate peaks do not coincide in the second peak, which suggests the presence of at least one phosphate ester in addition to RuDP; this impurity emerges from the column slightly ahead of RuDP. From the ratio RuDP/organic phosphate, the quantity of this contaminant can be calculated. This constitutes the shaded area on the graph, Fig. 3, and represents about 20% of the material in the second peak. The fractions emerging between A and B (Sample a), and B and C (Sample b) were bulked. To each of them barium acetate (500 µl of 1 M) and an equal volume of 95% ethanol were added. The solutions were cooled in ice and the precipitate collected by centrifugation. The precipitates were washed with 50% ethanol (20 ml), 95% ethanol (10 ml), and absolute ethanol (10 ml), and dried *in vacuo* over P_2O_5. Material recovered: Sample a, 22 mg.; Sample b, 23 mg.

Both samples contained small quantities of inorganic phosphate, though very much less than the starting material. By treatment with acid phosphatase the only sugar found to be present in Sample b was ribulose; Sample a appeared to contain a small quantity of ribose in addition to ribulose. Sample b is considered to be a highly purified dibarium salt of ribulose diphosphate.

Reaction of RuDP with $KC^{14}N$

RuDP (12 mg Ba salt, crude) was triturated with water (1 ml) and Na_2SO_4 (40 µmole). The residue of barium sulfate was removed by centrifugation and washed with water (200 µl). The combined supernatant liquid and washings were allowed to react with 1.3 mg of $KC^{14}N$ (1 µC/µmole) at room temperature for 16 hours. An aliquot was plated in 6 N acetic acid and counted; 21,000 dpm/µl was fixed as an acid-stable compound.[32] This represents approximately 100% reaction, when the impurities present in RuDP are taken into account.

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[30] R. J. L. Allen, Biochem. J. 34, 858 (1940).

[31] Tris (hydroxymethyl) aminomethane.

[32] The symbol HaDP is used in this manuscript to designate the product of the addition of KCN to RuDP. It is derived from the name hamamelonic acid diphosphate. As will appear later, the compound upon hydrolysis produces a mixture of stereoisomeric hexulose phosphates including hamamelonic acid.

Two-dimensional chromatography in the usual solvent system indicated that 87% of the activity was in the diphosphate area, as shown in Fig. 4. One-dimensional chromatography of the unhydrolyzed reaction product in butanol–propionic acid–water for 48 hours indicated the presence of one main spot, with the same R_f value as HmDP with a small amount of other impurities, as shown in Fig. 5.

An aliquot of the above material (25 µl.) plus 100 µg of carrier authentic hamamelonic acid[33] was hydrolyzed with 1 N HCl by heating for 16 hours in a sealed tube at 120° C. The resulting solution was chromatographed in two dimensions (butanol–propionic acid–water followed by phenol-water), and the chromatograph is shown in Fig. 6. It was found that the radio-activity and the silver spray did not coincide exactly. This is almost certainly due to the probability that the radioactive material consists of both epimers at the α-carbon atoms. A similar spot in the lactone position was produced by the action of acid phosphatase on the material; this indicates that the reaction product contains a carboxyl group and is not the cyanohydrin, which is presumably formed first, but is subsequently hydrolyzed.

Preparation of Hamamelonic Acid Diphosphate (2-carboxy-ribitol-1, 5-diphosphate)

RuDP (117 mg Ba Salt, crude) was triturated with water (10 ml) containing K_2SO_4 (75 mg). The insoluble material was removed by centrifugation and KCN (900 µl of 0.24 M) added. After 18 hours at room temperature the solution was centrifuged and the small amount of residue discarded. The supernatant liquid was cooled in ice and barium acetate (600 µl of 1 M) added. The copious precipitate was centrifuged off, washed with 5-ml portions of 80% and 90% ethanol, and dried in vacuo over P_2O_5; yield: 120 mg A further 15 mg of hamamelonic acid diphosphate Ba salt could be recovered by the addition of ethanol (2 ml) to the supernatant liquid. The material (120 mg plus 15 mg) was contaminated with $BaSO_4$, which was removed by trituration with HCl (10 ml of 0.1 N) and centrifuged. The residue was washed with water (5 ml); it weighed 42 mg and consisted mainly of $BaSO_4$.

The solution was cooled in an ice bath and the precipitate of hamamelonic acid diphosphate, Ba salt, was centrifuged off, washed with 50% ethanol (7.5 ml) and 90% ethanol (5 ml) and dried in vacuo over P_2O_5; yield 26 mg (A). A further crop was obtained by adding equal volumes of ethanol to the supernatant liquid. This was centrifuged, washed with 50% ethanol (4 ml) and 90% ethanol (5 ml) and dried in vacuo over P_2O_5; yield 22 mg of hamemelonic acid diphosphate Ba salt (B).

Both A and B were co-chromatographed with the products of the reaction of $KC^{14}N$ on RuDP, and coincidence of radioactivity and the phosphomolybdate color was obtained.

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[33] We are grateful to Prof. O. Th. Schmidt for a gift of several mg of that stereoisomer which he has established to be hamamelonic acid. O. Th. Schmidt and K. Heintz, Ann. 515, 77 (1935).

Chromatography in the solvent system of Cowgill, as modified by Trudinger, showed a sharp separation from RuDP.

Preparation of Carrier Hamamelonic Acid.

Hamamelonic acid diphosphase A (5 mg) was dissolved in HCl (1 ml of 1 N) and heated for 16 hours in a sealed tube at 110° C. For carrier 100 µl of this solution was used. Before use it was treated with Na_2SO_4 solution (50 µl of 0.05 M) and centrifuged.

Results

A radioauthograph of a typical $C^{14}O_2$, CN^- experiment is shown in Fig. 7. The general picture is rather similar to that obtained in the absence of cyanide. From a cursory examination it might be concluded that cyanide is acting only as an enzyme poison. That this is far from the truth is illustrated in Fig. 8, which is the radioautograph of a typical experiment, CO_2, $C^{14}N^-$. The sole source of radioactivity here is cyanide; hence, all the spots are the products of the reaction of cyanide with materials present in the algae.

Radioautographs of dephosphorylated diphosphate areas of the above experiments are shown in Figs. 9 and 10 (enzymic hydrolysis) and in Figs. 11 and 12 (acid hydrolysis). Both of these radioautographs show large amounts of a material not observed before in experiments without cyanide. Since it has been demonstrated that cyanide participated in the formation of this material, it is probable that this substance is formed by a cyanohydrin reaction. As this substance originates in the diphosphate area, the possibility was investigated that it was produced by a cyanohydrin reaction on ribulose diphosphate. This reaction has been shown to yield hamamelonic acid diphosphate (together, possibly, with some epimer). A comparison of the electrophoretic behavior of the diphosphate area of the experiment, $C^{14}O_2$, CN^-, with synthetic hamamelonic acid diphosphate (produced by the non-enzymatic action of $C^{14}N^-$ on RuDP) is illustrated in Fig. 13. It can be seen that the two materials are electrophoretically indistinguishable under these conditions.

The diphosphate areas obtained using the material from experiments $C^{14}O_2$, CN^- and CO_2, $C^{14}N^-$ were chromatographed with RuDP and HmDP-C^{14} by means of the solvent system of Trudinger. The radioactive areas coincided with the areas reacting to the phosphate spray. In a control in which RuDP was added as a carrier, no such coincidence was obtained. This is evidence that the new material produced in experiments with cyanide on the algae is hamamelonic acid diphosphate, produced by the action of the cyanide on RuDP, either inside the algae or during the extraction procedure. A photograph of the sprayed paper was not included as it was not possible to photograph it before it disintegrated.

Further confirmation was obtained by hydrolyzing the diphosphate area in 1 N HCl for 16 hours. The material obtained from experiments using $C^{14}O_2$, CN^- and CO_2, $C^{14}N^-$ was shown to be identical with synthetic hamamelonic acid (made from HmDP) by co-chromatography in one dimension in butanol–propionic acid–water.

Conclusions

It has been shown that the new material which appears in photosynthesis experiments with algae by the use of high concentrations of cyanide is hamamelonic acid diphosphate together with some epimer. This compound has not been detected in normal photosynthesis experiments without cyanide addition. A substance that is chromatographically rather similar but not identical has been detected by Moses[34] in normal photosynthesis experiments. This material on dephosphorylation and reduction by KBH_4 yields hamamelonic acid and its isomers.

The source of hamamelonic acid diphosphate is clearly a cyanohydrin reaction on RuDP. In order to form appreciable amounts of HmDP it is essential to have a light period between the cyanide addition and the injection of boiling alcohol to kill the aglae.[15] Furthermore, the net fixation of radiocarbon as PGA is diminished when the algae are illuminated after the addition of cyanide. The amount of radiocarbon fixed as hamamelonic acid diphosphate is consistent with the amount disappearing from the pentose phosphates and the PGA pools. This can be interpreted if it is assumed that the addition of cyanide has little effect on the ability of the algae to reduce PGA, at least for an initial short period of time. Then in the light period after the cyanide addition, PGA is reduced to triose phosphate, which cycles into RuDP and is trapped by the cyanide. The principal effect of the cyanide must then be to block the uptake of CO_2, probably by inhibition of the carboxydismutase system,[14] and trap the RuDP as it accumulates from other intermediates of the photosynthetic cycle. In addition, it can be deduced that the reaction involving the phosphorylation of RuMP is also occurring during the light period after the addition of cyanide. This is consistent with the finding of Kandler,[35] who has taken his results on cyanide inhibition to indicate that a light phosphorylation is not inhibited by high concentrations of cyanide in the first few minutes. However, the validity of this method of determining phosphorylation rates must be re-examined in the light of the broad reactivity of CN^- here demonstrated.

It has also been demonstrated that a facil reaction occurs between RuDP and KCN to produce HmDP. It is feasible that this reaction occurs readily because the intermediate cyanohydrin can cyclize to give an iminolactone, which should undergo rapid hydrolysis.

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[34] V. Moses, UCRL, unpublished work.

[35] O. Kandler, Naturwiss. 42, 390 (1955).

Figure Captions

Fig. 1. Glass vessel for KCN-algae work.

Fig. 2. Flow diagram for the preparation of phosphoriboisomerase-phosphoribulokinase.

Fig. 3. Elution pattern of RuDP. Klett reading of 230 corresponds to 0.5 μmole phosphate.

Fig. 4. Radioautograph: chromatography of the nonenzymatic reaction product of $KC^{14}N$ and RuDP.

Fig. 5. Radioautograph of chromatogram of ribulose diphosphate and hamamelonic acid diphosphate ($KC^{14}N$) in butanol propionic acid (48 hours).

Fig. 6. Co-chromatography of authentic hamamelonic acid lactone with the HCl hydrolysate of the nonenzymatic reaction product of $KC^{14}N$ and RuDP. (Note that origin is at lower left.)

Fig. 7. Radioautograph of a chromatogram of the hot alcohol-water extract of Chlorella treated as follows: Normal photosynthesis for 2 min with $C^{14}O_2$ followed by addition of KCN (final concentration 0.02 M) along with a further 15 sec light period and injection of hot alcohol (end concentration 80%) to kill the algae ($C^{14}O_2$, CN^- experiment).

Fig. 8. Radioautograph of a chromatogram of the hot alcohol-water extract of Chlorella treated as follows: Normal photosynthesis for 2 min with CO_2 followed by addition of $KC^{14}N$ (final conc. 0.02 M) along with a further 15 sec light period and finally, injection of hot alcohol (end concentration 80%) to kill the alge (CO_2, $C^{14}N^-$ experiment).

Fig. 9. Radioautograph: chromatography of the enzymatically hydrolyzed diphosphate area of Fig. 7 ($C^{14}O_2$, CN^- experiment).

Fig. 10. Radioautograph: chromatography of the enzymatically hydrolyzed diphosphate area of Fig. 8 (CO_2, $C^{14}N^-$ experiment).

Fig. 11. Radioautograph: Chromatography of the HCl-hydrolyzed diphosphate area of Fig. 7 ($C^{14}O_2$, CN^- experiment).

Fig. 12. Radioautograph: Chromatography of the HCl-hydrolyzed diphosphate area of Fig. 8 (CO_2, $C^{14}N^-$ experiment).

Fig. 13. Radioautograph: Parallel paper electrophoresis of the diphosphate area of Fig. 7 ($C^{14}O_2$, CN^- experiment) and the nonenzymic reaction product of $KC^{14}N$ and RuDP.

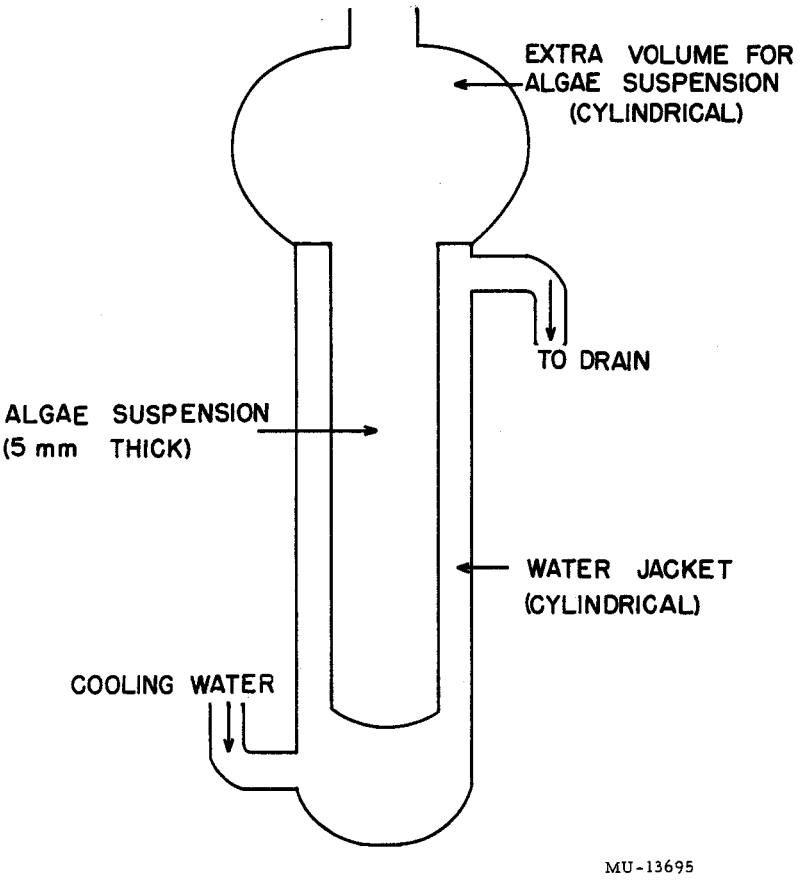

EXTRA VOLUME FOR
ALGAE SUSPENSION
(CYLINDRICAL)
TO DRAIN
ALGAE SUSPENSION
(5 mm THICK)
WATER JACKET
(CYLINDRICAL)
COOLING WATER
MU-13695

Fig. 1.

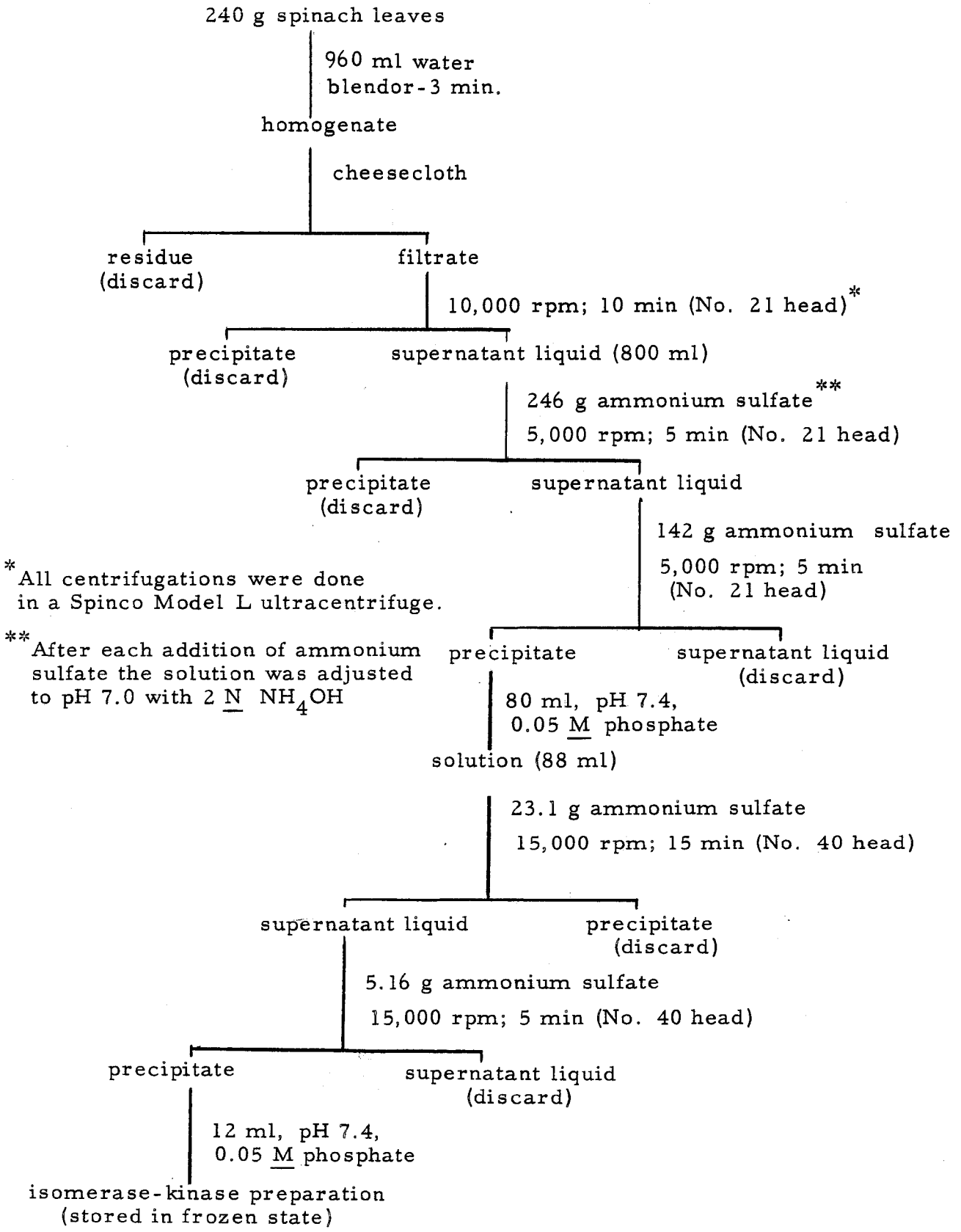

240 g spinach leaves
960 ml water
blendor-3 min.
homogenate
cheesecloth
residue (discard)
filtrate
10,000 rpm; 10 min (No. 21 head)*
precipitate (discard)
supernatant liquid (800 ml)
246 g ammonium sulfate**
5,000 rpm; 5 min (No. 21 head)
precipitate (discard)
supernatant liquid
142 g ammonium sulfate
5,000 rpm; 5 min (No. 21 head)
precipitate
supernatant liquid (discard)
80 ml, pH 7.4, 0.05 M phosphate
solution (88 ml)
23.1 g ammonium sulfate
15,000 rpm; 15 min (No. 40 head)
supernatant liquid
precipitate (discard)
5.16 g ammonium sulfate
15,000 rpm; 5 min (No. 40 head)
precipitate
supernatant liquid (discard)
12 ml, pH 7.4, 0.05 M phosphate
isomerase-kinase preparation (stored in frozen state)
*All centrifugations were done in a Spinco Model L ultracentrifuge.
**After each addition of ammonium sulfate the solution was adjusted to pH 7.0 with 2 N NH_4OH

Fig. 2.

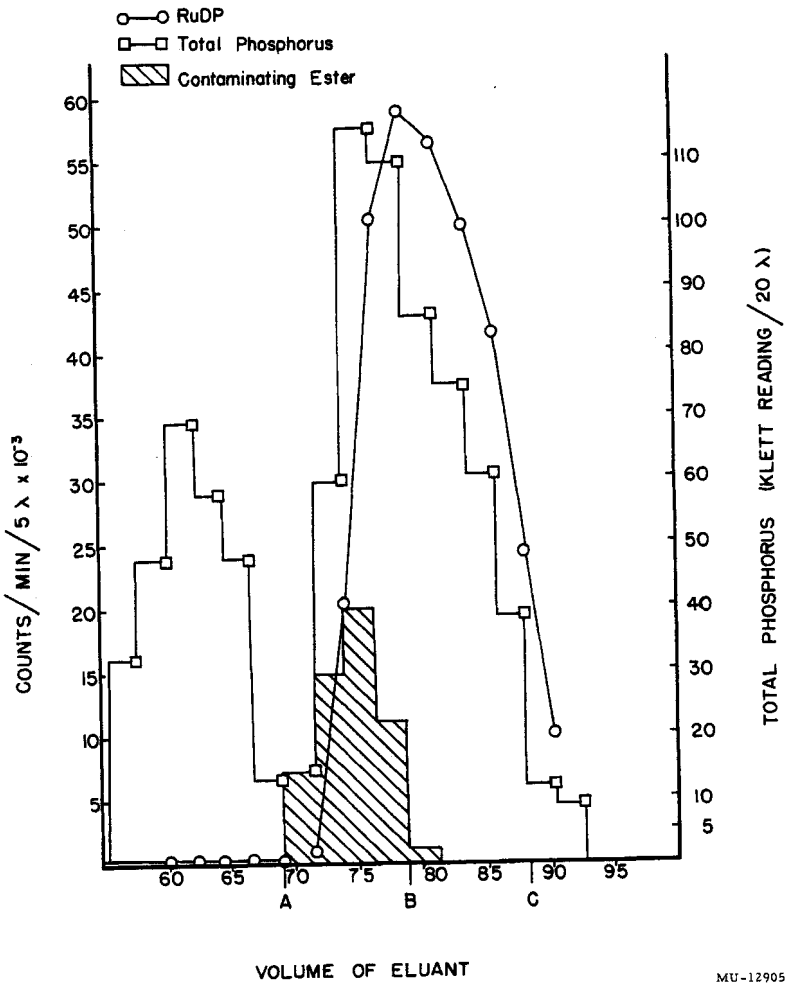
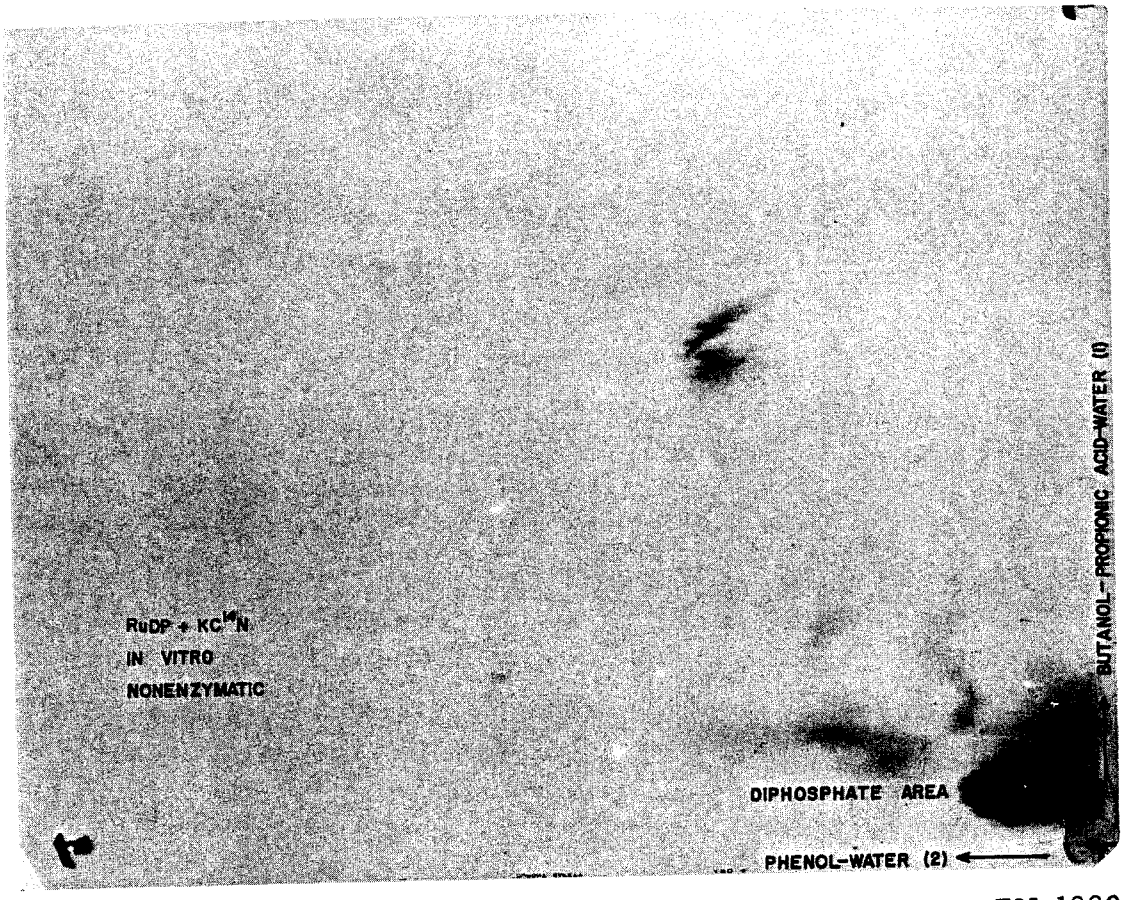
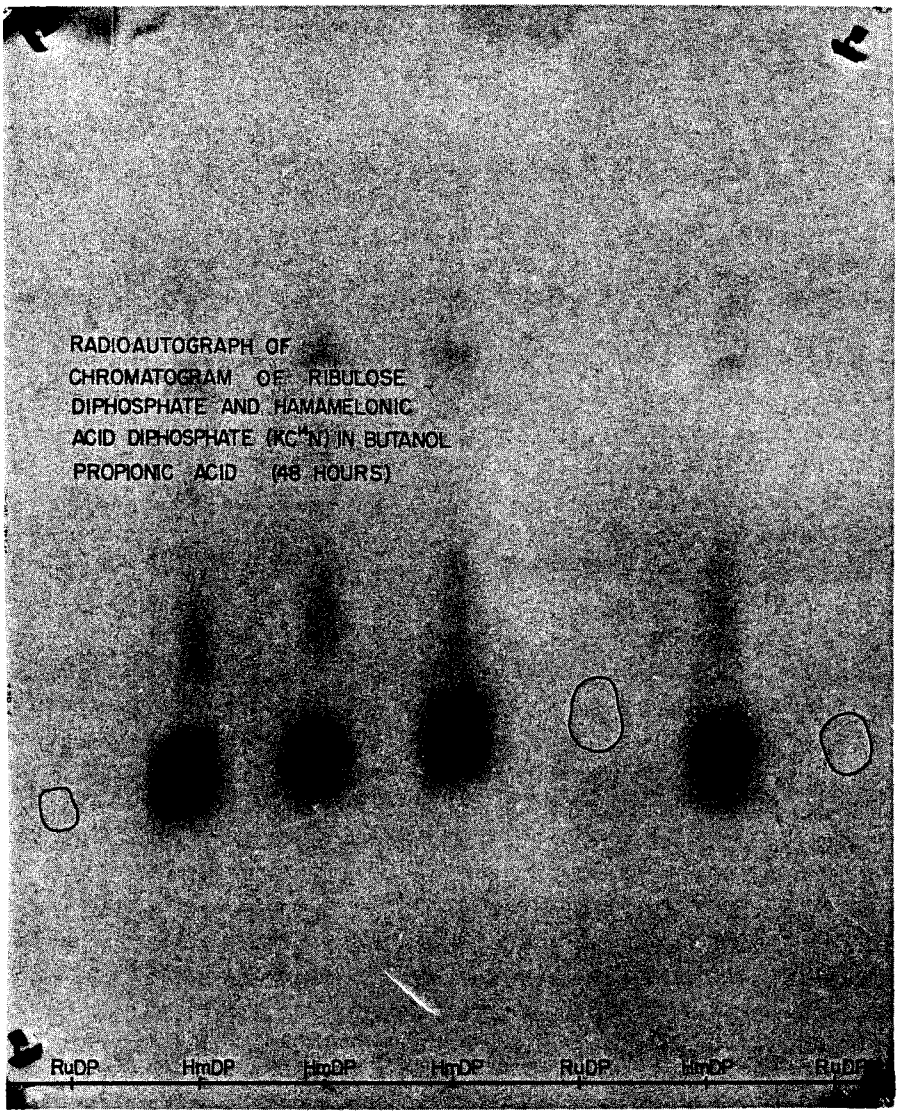

RuDP
Total Phosphorus
Contaminating Ester
COUNTS/MIN/5 λ x 10⁻³
TOTAL PHOSPHORUS (KLETT READING/20 λ)
VOLUME OF ELUANT
A
B
C
MU-12905

Fig. 3.


RuDP + KC^{14}N
IN VITRO
NONENZYMATIC
DIPHOSPHATE AREA
PHENOL-WATER (2)
BUTANOL-PROPIONIC ACID-WATER (1)

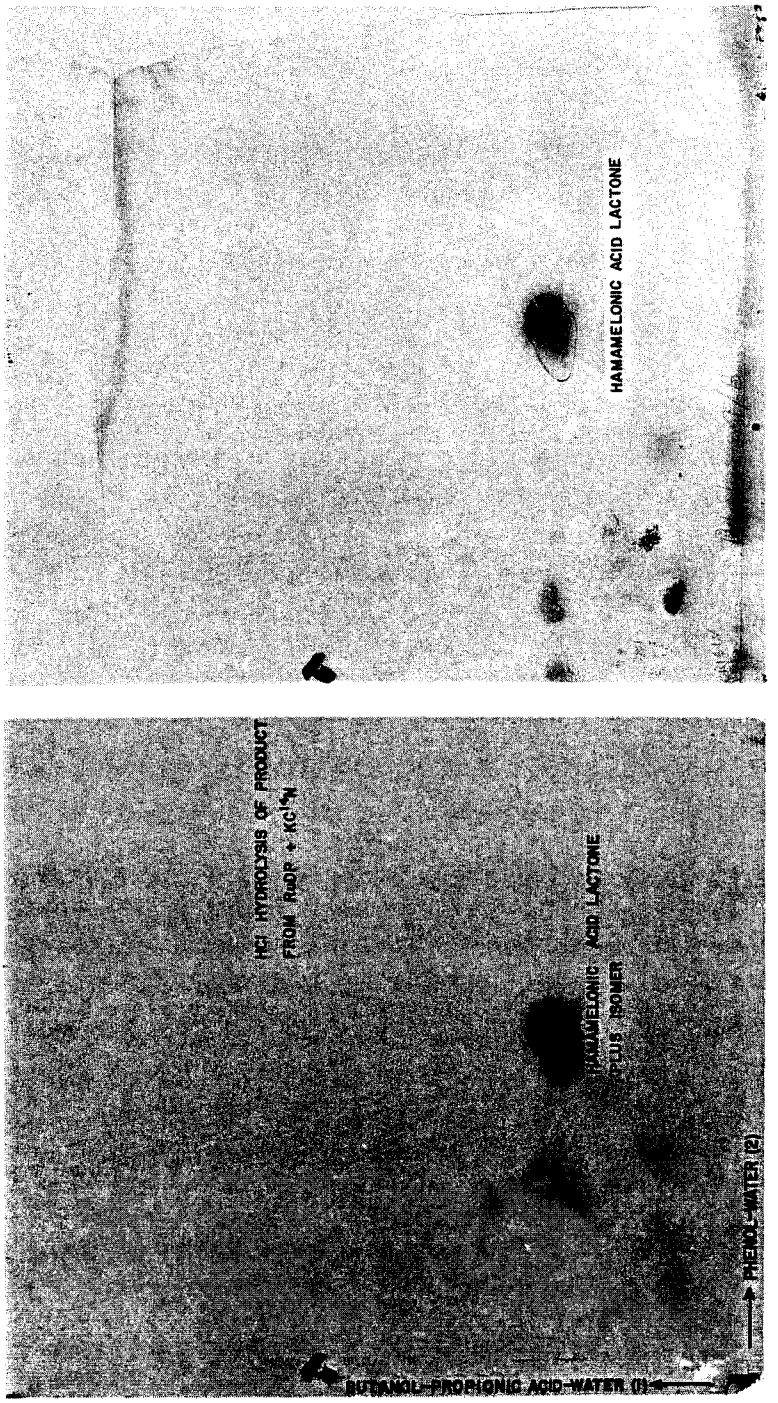
ZN-1880

Fig. 4.


RADIOAUTOGRAPH OF
CHROMATOGRAM OF RIBULOSE
DIPHOSPHATE AND HAMAMELONIC
ACID DIPHOSPHATE (KC14N) IN BUTANOL
PROPIONIC ACID (48 HOURS)
RuDP
HmDP
HmDP
HmDP
RuDP
HmDP
RuDP

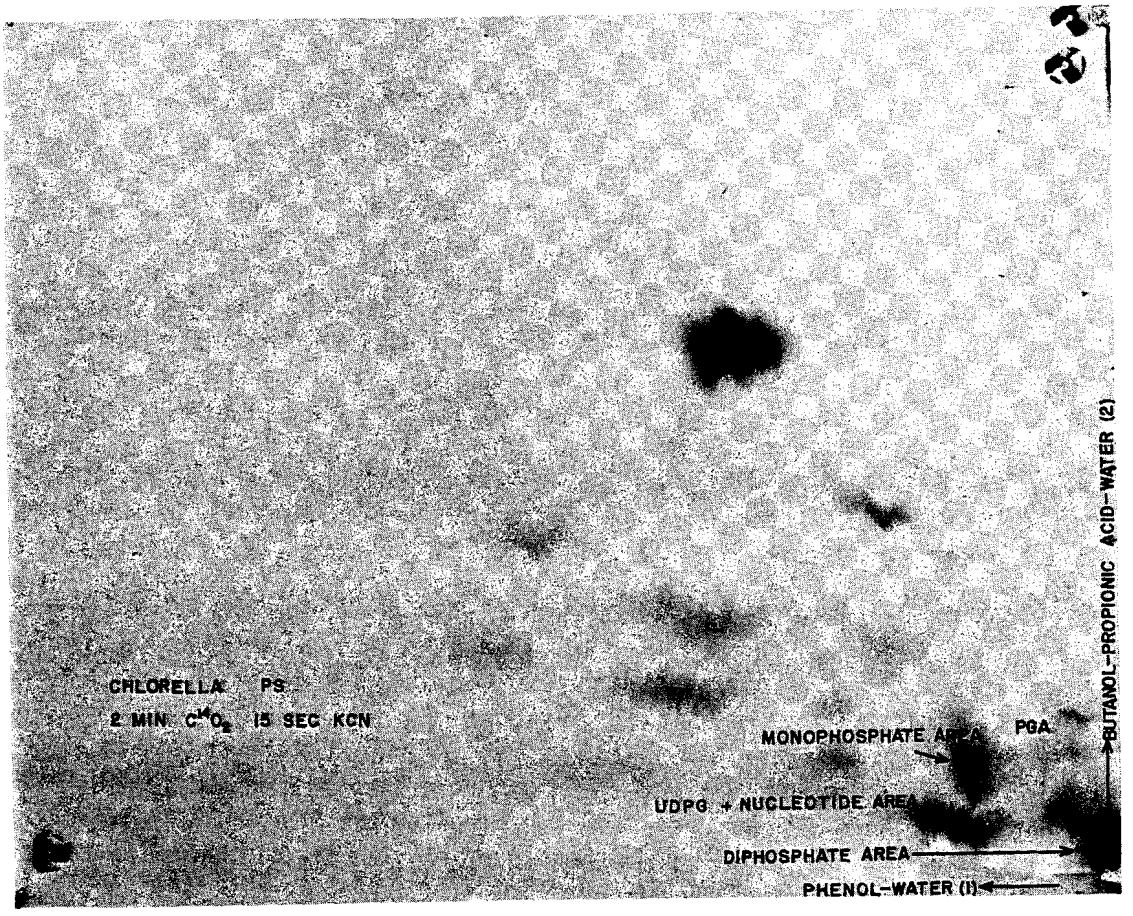
ZN-1879

Fig. 5.


HAMAMELONIC ACID LACTONE
HCl HYDROLYSIS OF PRODUCT
FROM RuDP + KCl^{14}N
HAMAMELONIC ACID LACTONE
PLUS ISOMER
PHENOL-WATER (2)
BUTANOL-PROPIONIC ACID-WATER (1)

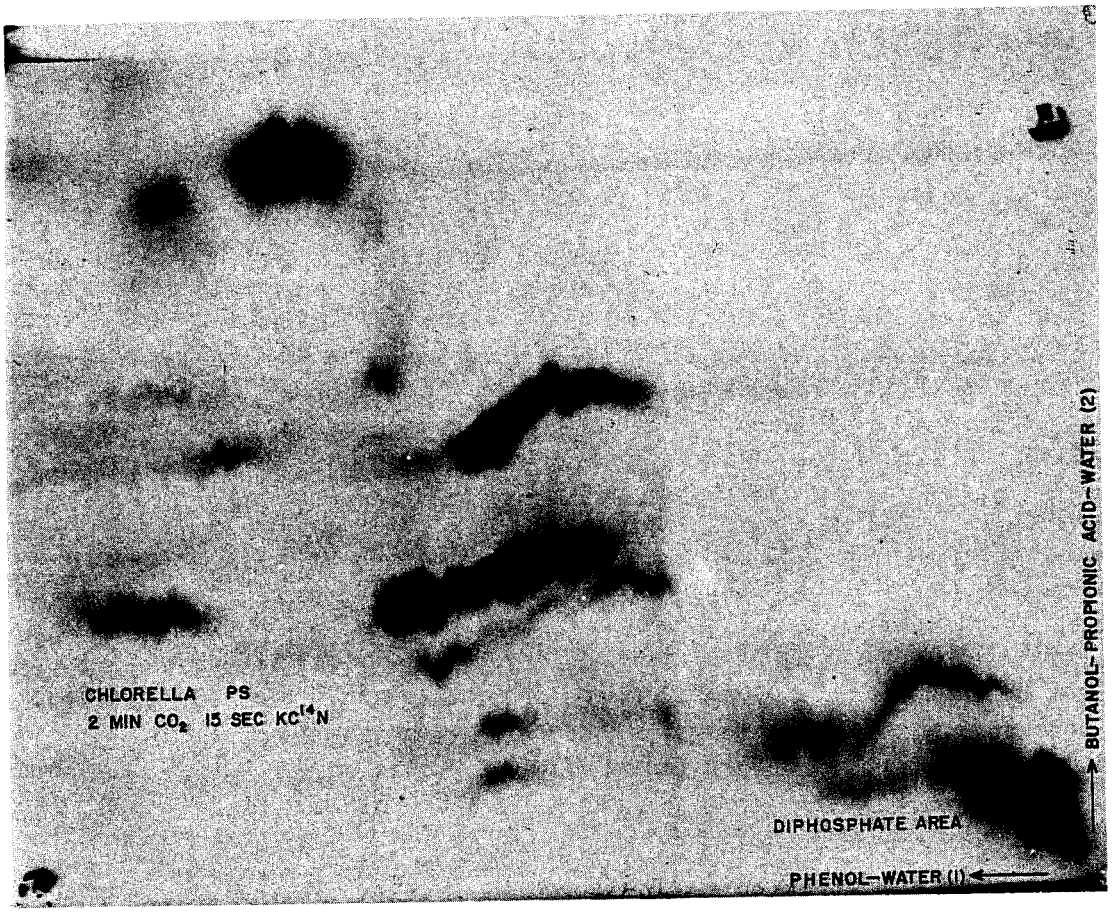
ZN-1871

Fig. 6.


CHLORELLA PS
2 MIN $C^{14}O_2$ 15 SEC KCN
MONOPHOSPHATE AREA
PGA
UDPG + NUCLEOTIDE AREA
DIPHOSPHATE AREA
PHENOL-WATER (1)
BUTANOL-PROPIONIC ACID-WATER (2)

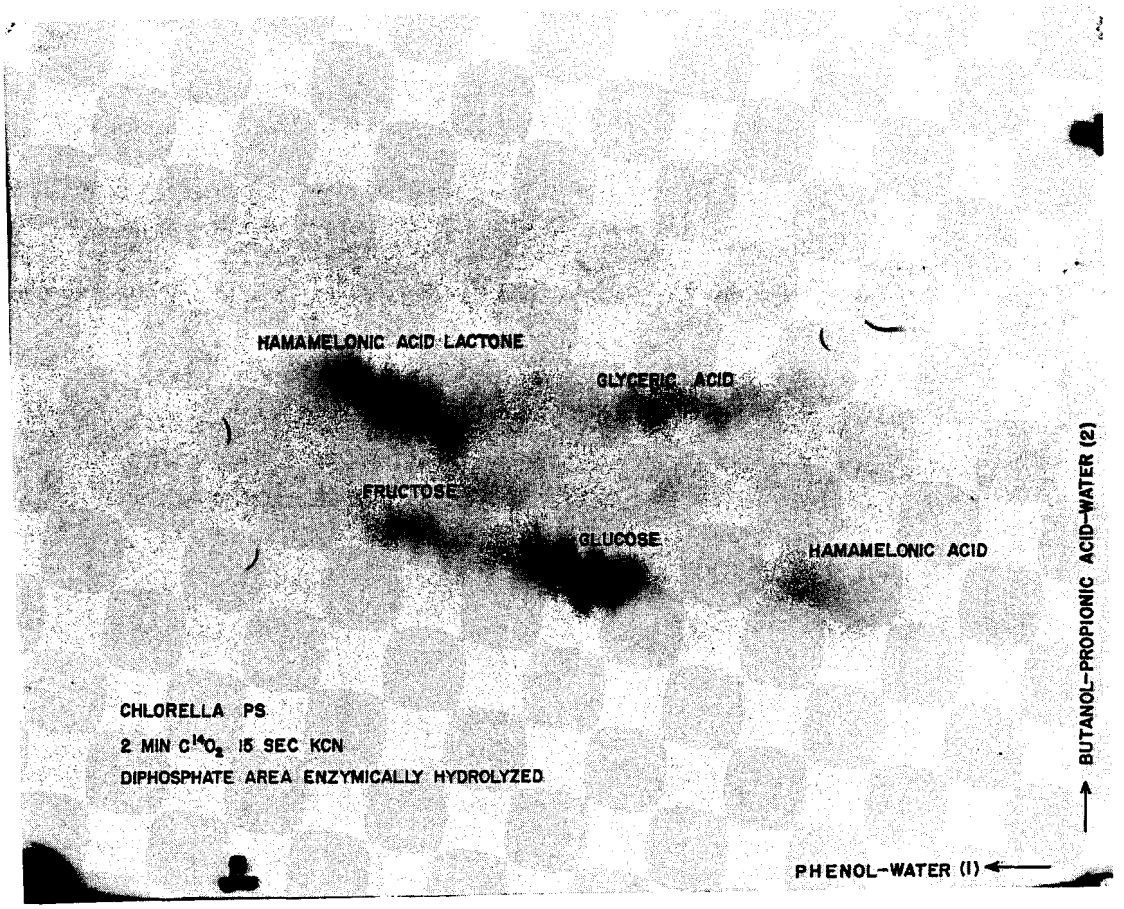
ZN-1876

Fig. 7.


CHLORELLA PS
2 MIN CO_2 15 SEC $KC^{14}N$
DIPHOSPHATE AREA
PHENOL-WATER (I)
BUTANOL-PROPIONIC ACID-WATER (2)

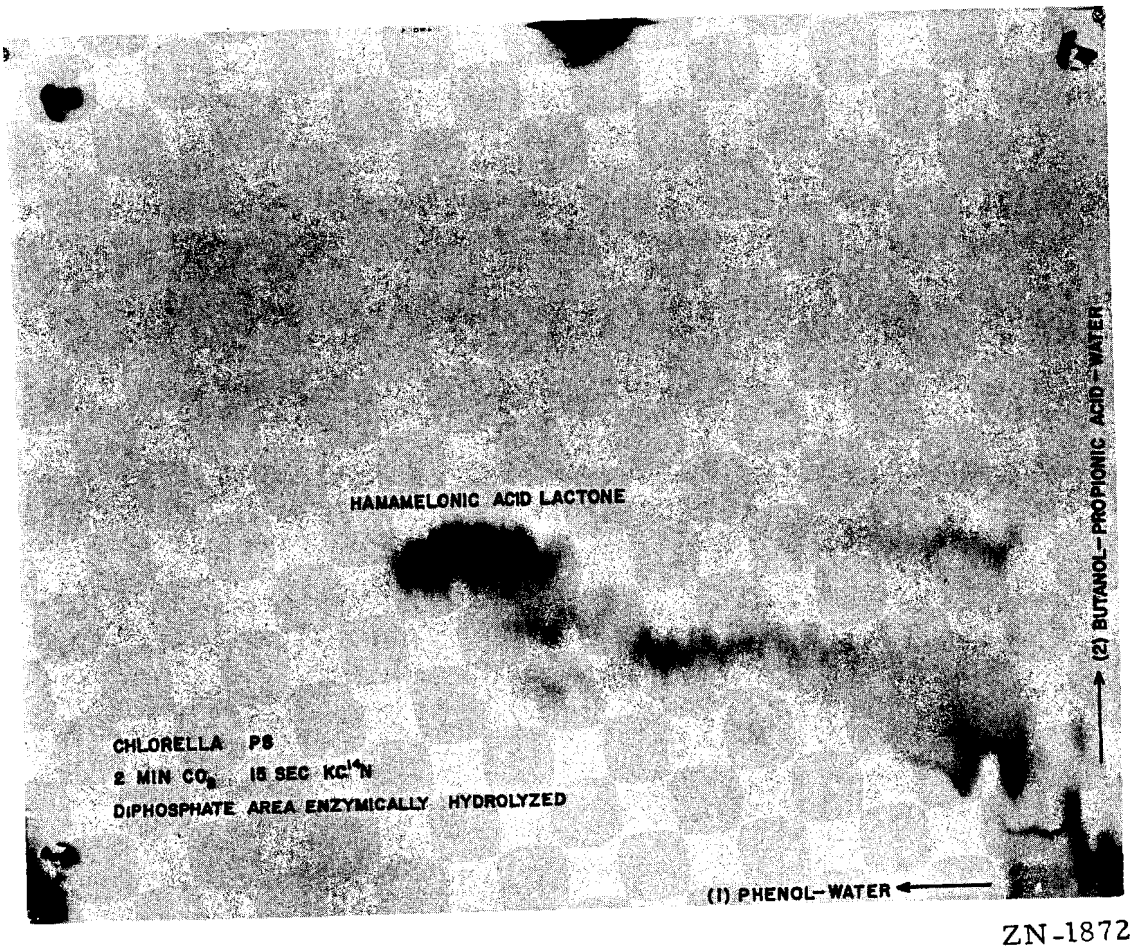
ZN-1873

Fig. 8.


HAMAMELONIC ACID LACTONE
GLYCERIC ACID
FRUCTOSE
GLUCOSE
HAMAMELONIC ACID
CHLORELLA PS
2 MIN $C^{14}O_2$ 15 SEC KCN
DIPHOSPHATE AREA ENZYMICALLY HYDROLYZED
BUTANOL-PROPIONIC ACID-WATER (2)
PHENOL-WATER (1)

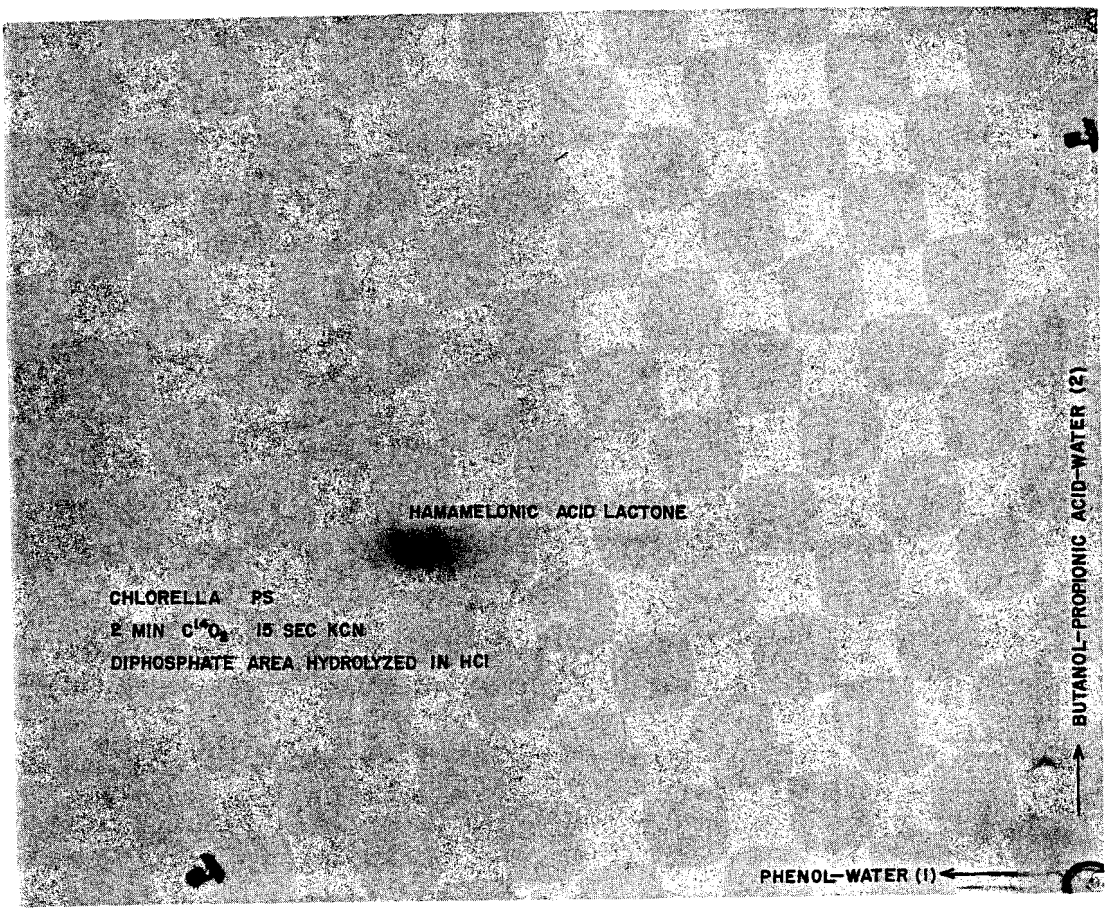
ZN-1875

Fig. 9.



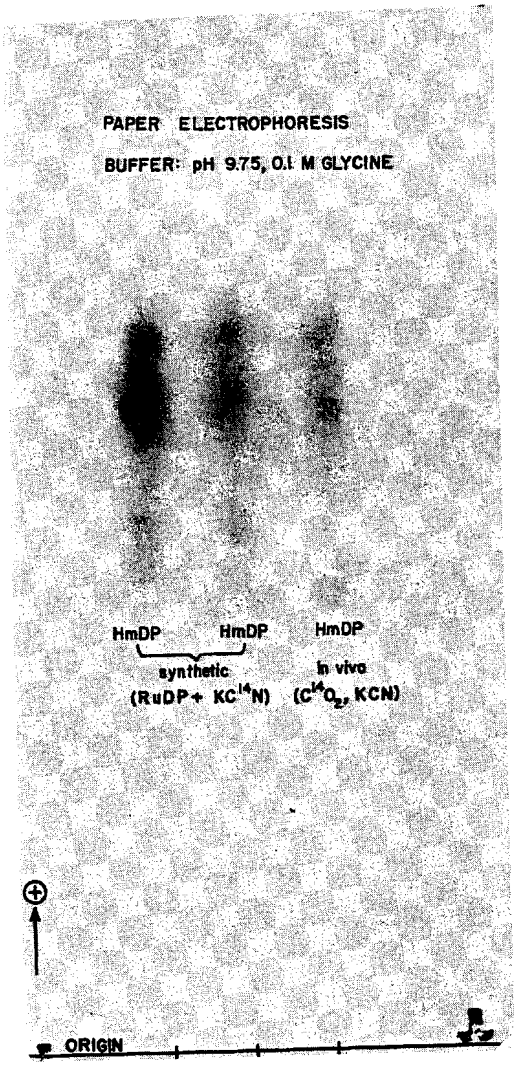
ZN-1872

Fig. 10.



ZN-1877

Fig. 11.



ZN-1878

Fig. 13.