STUDIES ON PHOTOSYNTHESIS

PART I: Biosynthesis of Sucrose from Glycolate

PART II: Bicarbonate Utilization by Washed Algae

Thesis for the Degree of Ph. D.
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ABSTRACT

STUDIES ON PHOTOSYNTHESIS

PART I: BIOSYNTHESIS OF SUCROSE FROM GLYCOLATE
PART II: BICARBONATE UTILIZATION BY WASHED ALGAE

by Eduardo Jimenez Saenz

Two different aspects of photosynthesis were considered in this work. First, the role of glycolate as a precursor of sucrose was investigated, and secondly, the inhibitory effect of washing algae with distilled water was studied.

Glycolate-1- or -2-C^{14} and serine-3-C^{14} were fed to leaves of different plants in the light for short time periods. Labeled sucrose was isolated by chromatography, hydrolyzed and the distribution of C^{14} in each carbon atom was determined. Glycolate-1-C^{14} was converted into 3, 4 labeled hexoses, while glycolate-2-C^{14} was changed into 1, 2, 5 and 6-C^{14} hexoses. Serine-3-C^{14} was metabolized into 1, 6 labeled glucose and fructose. These data contributed to an understanding of a Glycolate Pathway for metabolizing photosynthetic carbon products. Glycolate was converted to serine, serine to glycerate, and glycerate to sugar by reversal of the Embden-Meyerhof Pathway. The contribution of the Glycolate Pathway was discussed for the randomization of label in the hexose molecules.

Upon washing algae three times with distilled water, the photosynthetic rate of fixation of $NaHC^{14}O_3$ at pH 8.2 or above was inhibited 80 to 90 percent. This inhibition was reversed

when the pH was lowered to 7 or below by buffers such as phosphate, phosphoglycolate or amino acids. This phenomenon was best explained by the assumption that $C^{14}O_2$ entered the cells many times faster than the bicarbonate. Two additional facts which supported this hypothesis were that the buffers need not enter the cells to be effective, and that the products of photosynthesis were not altered by three washings or by pH changes of the medium. At high bicarbonate concentrations, diffusion of the anion also supported photosynthesis. For unexplained reasons the increased photosynthetic rates induced by buffers at pH 7 were prevented by uranyl or arsenate ions.

STUDIES ON PHOTOSYNTHESIS

PART I: Biosynthesis of Sucrose from Glycolate

PART II: Bicarbonate Utilization by Washed Algae

By

Eduardo Jimenez Saenz

A THESIS

Submitted to the School for Advanced Graduate Studies
of Michigan State University in partial
fulfillment of the requirements for
the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1962

To

My wife

And children

ACKNOWLEDGMENTS

I wish to express my appreciation to the members of my Academic Advisory Committee, and in particular to Dr. N. E. Tolbert of the Department of Biochemistry for his constant aid and encouragement throughout the course of the investigation and during the preparation of this manuscript.

It is with gratification that I again acknowledge the generous assistance of the Rockefeller Foundation, whose sponsorship so greatly aided me in pursuing this study.

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INTRODUCTION

Glycolic acid metabolism has been investigated in microbial, animal and plant systems. The significance of organic acids in plant metabolism was only partially understood by the earlier physiologists and biochemists who considered them, as a general rule, only as products of the oxidative processes connected with the respiration of carbohydrates and fats (49, 63). Among the early investigators, only a few entertained different ideas concerning the participation of organic acids in other vital processes. Sachs (63), for example, stated that Hugo de Vries believed that the vegetable acids played an important part in the turgescence of the cells and hence in growth. However, Liebig (42) proposed the most controversial theory, namely that the formation of these acids was the first reaction of photosynthesis. Liebig's theory had no experimental basis (45) and therefore was severely criticized by other scientists. Pfeffer (49) argued that, even if the chloroplasts do actually produce carbohydrates directly from organic acids, this still would not support Liebig's contention that the formation of organic acids was the first step in the assimilation of carbon dioxide. Pfeffer thought that the free organic acids (malic, isomalic and oxalic), which were produced during respiration, were further oxidized under the action of light and that the carbon dioxide thus produced was then immediately assimilated.

Today it is not difficult to realize that even though Liebig's theory was too general in its conception, it was not completely wrong, since the organic acids indeed play an essential role in photosynthesis. The work by Calvin and his associates (16) has clearly demonstrated that the compound 3-phosphoglyceric acid is the first detectable product of the fixation of carbon dioxide in photosynthesis.

The objectives of the present work were twofold. First, to determine if glycolic acid, which can be considered as a by-product of the photosynthetic carbon cycle (76, 83), is utilized by higher plants in the formation of hexoses as proposed by the Glycolate Pathway (60). Second, to attempt to explain a behavior exhibited by washed algae during photosynthesis.

REVIEW OF LITERATURE

Glycolic and phosphoglycolic acids have been identified as early products of photosynthesis. This suggests the importance of these compounds in the metabolism of the plant (2, 7, 13, 16, 69).

Origin of Glycolate in Plants

The origin of glycolate in plants has not been definitely established, although the experimental evidence obtained by several workers seems to indicate that this compound is formed via a side oxidation pathway from the photosynthetic carbon cycle (60, 76).

According to the work by Tolbert and Zill (76) and Wilson and Calvin (83) free glycolic acid is formed and accumulates in cells photosynthesizing under conditions of low carbon dioxide pressure. Under these circumstances there may be only a small reservoir of phosphoglyceraldehyde which normally acts as the acceptor for the glycolyl fragment derivable from a pentose or hexose phosphate during sugar metabolism. The transfer of the glycolyl fragment to phosphoglyceraldehyde is mediated by transketolase. This enzyme was purified by Horecker et. al. (33) who also described several mechanisms for the reaction involved. In all cases, the participation of an "active glycolaldehyde" (glycolyl fragment) is required. The 2-carbon piece is bound to the enzyme through thiamine pyrophosphate which has

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been identified as the coenzyme for transketolase (33). One hypothesis has been that if the concentration of acceptor is reduced, the 2-carbon moiety is liberated giving rise to glycolate (83). This proposal has not been substantiated with adequate data.

That pentose phosphate can serve as the precursor of glycolate was suggested by Weissbach and Horecker (82) who reported the _in vitro_ formation of glycine from 1-C^{14}-ribose-5-phosphate in spinach extracts. According to these investigators, the incorporation of carbon-1 of ribose into the methylene carbon atom of glycine could be explained by a sequence of reactions which involve the formation of glycolate from the first two carbon atoms of the pentose phosphate. _In vivo_ evidence that a pentose may be the precursor of glycolate has been presented by Griffith and Byerrum (29). These investigators fed D-ribose-1-C^{14} to tobacco leaves (_Nicotiana rustica_) and found that the C^{14} content was highest in the alpha carbon of glycine and glycolate, and the alpha and beta carbons of serine. Their conclusion also was that carbon atoms 1 and 2 of the pentose gave rise to glycolate.

Tolbert and Zill (76) summarized the data from several sources concerning the origin of glycolate. The same distribution of C^{14} is found in glycolate as in the alpha and beta carbons of phosphoglycerate formed during short time photosynthesis experiments (16). _In vitro_ enzymatic studies (26, 82), and the ease of air oxidation of ribulose diphosphate to phosphoglycolate (28) substantiated these results. More recently, Rabson, Tolbert and Kearney (60) established the position of phosphoglycolate as the immediate precursor of glycolate. Another significant observation is the activation of a pathway for glycolate metabolism which takes place only after the establishment of the photosynthetic carbon cycle in green plants (3, 73). Tanner, Brown and Eyester (67) indicated that glycolate is the first product from the conversion of carbon dioxide in photosynthesis and that the only possible intermediate in such a reaction is the radical (CHO.). However, the possibility that glycolate arises from the condensation of two 1-carbon pieces formed directly from carbon dioxide was discarded long ago by Calvin's group (65) who based their conclusion on the fact that glycolate accumulates even in the absence of carbon dioxide.

Physiological Importance of Glycolate

In spite of the fact that plants may accumulate large amounts of glycolate during photosynthesis (7, 50, 80, 84), precise knowledge of its physiological value has only been gained recently. In 1949 Tolbert, Clagett and Burris (72) stated that nothing was known about the role of glycolate in the physiology of plants. In fact, it could be said that the beginning of the study of glycolate metabolism in plants was marked by this group of investigators with the report of an enzyme system which catalyzes the oxidation of L-alpha-hydroxymonocarboxylic acids (18, 72).

Glycolic Acid Oxidase

The enzyme found by Clagett *et. al.* (18) oxidizes glycolic acid at a faster rate than L-lactic acid. It is of particular interest because of its high activity, wide distribution among green plants, low activity in etiolated plants, and because it may function as a terminal oxidase in plants (72). Activation of the apoenzyme found in etiolated tissues was shown to be a case of substrate activation (41) and not light activation in a physical sense, as first proposed (71, 73). The conditions for activation of glycolic acid oxidase, both *in vitro* and *in vivo*, have been studied by Kuczmak (41). He found that this enzyme is indeed activated by feeding etiolated plants glycolate, lactate, alpha-hydroxybutyrate and even glyoxylate, as proposed by Tolbert and Cohan (73). Kuczmak also found that the apoenzyme, as present in etiolated leaves, becomes active upon addition of an excess of FMN to the tissue homogenate. Since green plants contain more FMN than etiolated ones, it can be interpreted that the activation of the apoenzyme during greening results from the increase in formation of flavin mononucleotide. However, Kuczmak indicated that the content of FMN in plants is 10^{-8}M which is well below the 10^{-4}M concentration necessary to activate the apoenzyme *in vitro* (41).

Glycolic acid oxidase is a flavoprotein and its prosthetic group has been repeatedly reported to be riboflavin-5-phosphate, or FMN (17, 34, 37, 41, 85). Flavin adeni

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adenine dinucleotide, or FAD, cannot substitute for FMN in partially purified enzyme preparations. Any activity of FAD with crude sap preparations can be explained by the enzymatic conversion of this substance to FMN (41). This enzyme is predominantly located in the cytoplasm (17, 41, 60).

The Glycolate Pathway

The series of biochemical reactions which start with the formation of phosphoglycolate as a by-product of the photosynthetic carbon cycle and end up in the synthesis of hexoses has been called the Glycolate Pathway by Rabson, Tolbert and Kearney (60), and it is summarized in Figure 1. Based on studies relative to the unique loss of C^{14}-labeled glycolate from isolated chloroplasts (36, 69), the products of the Glycolate Pathway should be localized in the cytoplasm (60). The importance of this system resides in the fact that it makes possible the accumulation of reservoirs in the cytoplasm of compounds from newly fixed carbon dioxide, which are produced in the chloroplasts during photosynthesis.

The hydrolysis of phosphoglycolate is catalyzed by a specific enzyme, phosphoglycolic acid phosphatase, which has been obtained in partially purified form from tobacco leaves by Richardson and Tolbert (61). The glycolate thus formed is subsequently oxidized to glyoxylate, as mentioned above, by glycolic acid oxidase. Transamination of glyoxylate yields glycine which in turn can be further metabolized into serine (74). Tolbert and Cohan (73) presented data to show

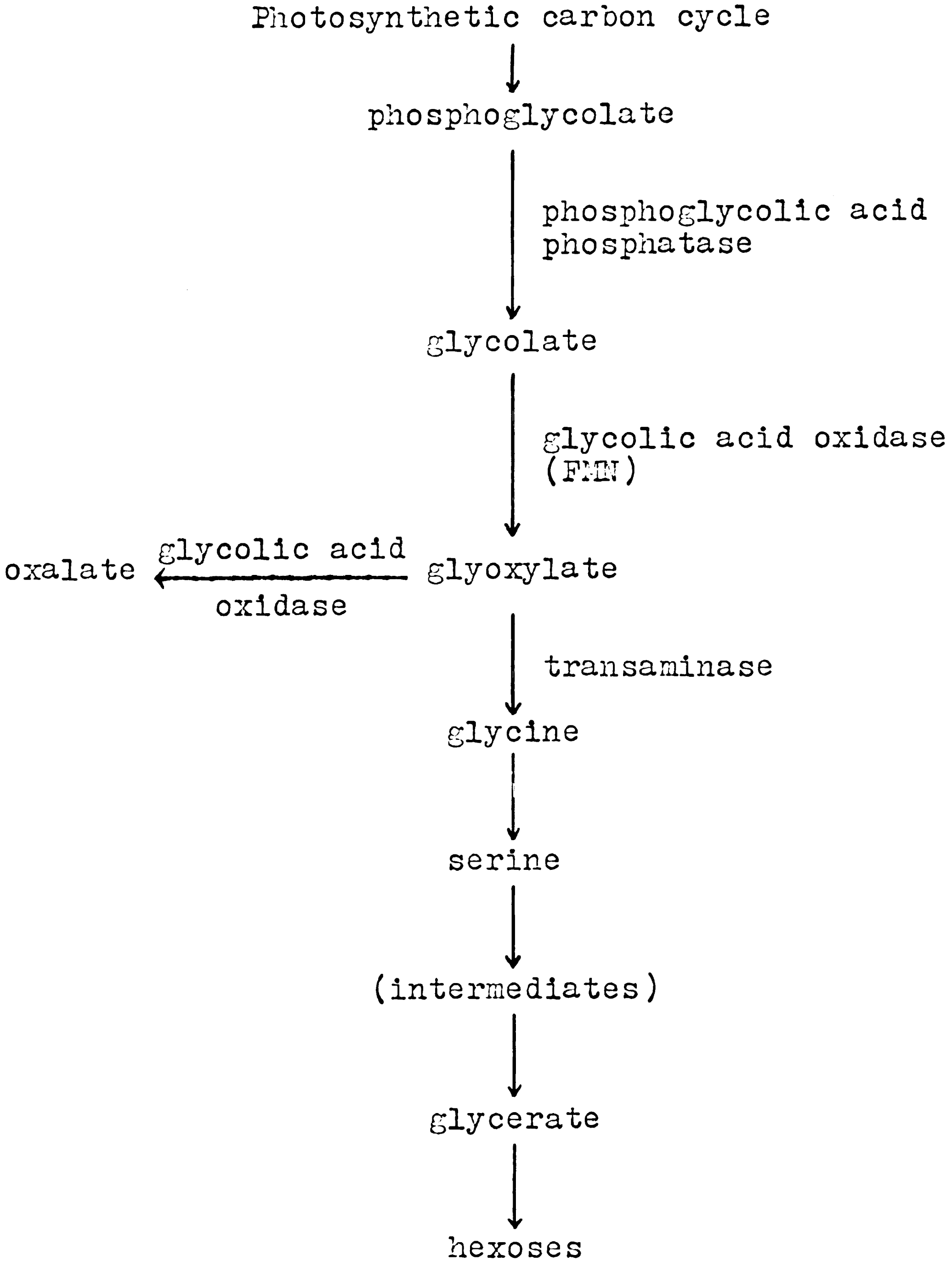
that the 2-carbon skeleton of glycolate goes unchanged to glycine and to the carboxy and alpha carbons of serine, while the beta carbon of this amino acid arises primarily from the alpha carbon of glycolate.

Evidence that glycolate and serine can be metabolized into glycerate by plants has been obtained from feeding experiments performed by Rabson _et_. _al_. (60). Glycolate-2-C^{14} was given to leaves of various kinds of plants, and the degradation data indicated that similar patterns of labeling were observed in serine and glycerate when glycolate-2-C^{14} had been fed to corn during 15 or 30 minutes in the light. Carbon atoms 2 and 3 of both compounds were equally labeled, and there was little randomization of C^{14} into the carboxyl group. This was taken as evidence that serine was directly converted into glycerate. Further evidence was obtained when the glycerate derived from serine-3-C^{14} was degraded. In this case it was found that the substrate was almost entirely converted to glycerate-3-C^{14}. In addition, Rabson _et_. _al_. found that when glycolate-2-C^{14} or serine-3-C^{14} were fed to leaves in the light, hexose phosphates and sucrose also became labeled. Therefore, the labeling in glycerate from either glycolate or serine, as well as the formation of labeled sugar phosphate and sucrose are in agreement with the Glycolate Pathway shown in Figure 1.

It was mentioned above that the beta carbon of serine arises primarily from the alpha carbon of glycolate. This implies that glycolate may also serve as a source of 1-carbon oxalate

FIGURE 1

GLYCOLATE PATHWAY



units. That such may be the case is suggested by the non-enzymatic formation of formate and hydrogen peroxide by the oxidative decarboxylation of glyoxylate derived from glycolate (72, 85). The direct incorporation of formate by plants into the beta carbon of serine, into choline and betaine, as well as into many compounds including sucrose, has been reported by Tolbert (68). Other examples in which formate can serve as a source of l-carbon units are found in the work by Krotkov, Vittorio and Reed (40) who observed that 24 hours after radioactive formate was fed to tobacco leaves, labeling could be detected in glucose. Likewise, Byerrum _et_. _al_. (14, 15) reported on the role of formate in the biosynthesis of methionine and lignin.

The metabolism of glycine as a precursor of sugars has been reported in an abstract by Wang (78) who fed labeled glycine to leaves of Khapli wheat and found that a considerable amount of the C^{14} was present in sugars. Both the methylene and the carboxyl carbon of glycine were effectively incorporated into carbohydrates in the light. However, when a supply of non-radioactive glycolic or glyoxylic acids was given to the leaves, the rate of transformation increased but the glyoxylic acid that was isolated from them as the phenylhydrazone did not show any higher radioactivity than the control. This data may be interpreted to indicate that the carbon chain of glycine was not first converted to glyoxylic or glycolic acids in the formation of sugars.

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Utilization of Bicarbonate by Algae

The role of bicarbonate ions as a substrate for photosynthesis has been a controversial subject for a long time. Until about 1947 it was considered that bicarbonate did not participate directly in photosynthesis. According to Rabinowitch (55), evidence for the participation of bicarbonate was supplied in particular by Osterlind and by Steemann-Nielsen. The capacity of certain higher aquatic plants and algae to utilize bicarbonate ions seems to be mainly a matter of adaptation to the natural habitat. Such adaptation was probably due to a change in the permeability of the cell membrane, rather than to an alteration in the mechanism of carboxylation during photosynthesis (56).

Of particular concern to the present study of bicarbonate uptake by washed or unwashed algae, is Rabinowitch's summary of the work by Osterlind (56). It is stated that Scenedesmus quadricauda differs from Chlorella pyrenoidosa with respect to the influence of bicarbonate on their rate of growth. Although the two species contained different amounts of carbonic anhydrase, the difference was not great enough to account for the wide discrepancy in their apparent capacity to utilize bicarbonate. Hence, variations in membrane permeability were adduced as the most likely explanation. When the rate of photosynthesis of these two species was measured manometrically, it was found that ten-day old Scenedesmus cultures, and all the Chlorella cultures studied, showed no evidence of bicarbonate

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utilization. However, five-day old cultures of Scenedesmus proved able to photosynthesize at a maximum rate in a solution of sodium bicarbonate (10^{-5}M, pH 8.1) containing only insignificant amounts of free carbon dioxide (2×10^{-7} mole/1). At pH 4 to 4.6, the same cells produced oxygen at a rate proportional to the concentration of carbon dioxide (between 0.9 and 9×10^{-5}M). In the latter case, the bicarbonate concentration was too low (about 2×10^{-6}M) to be significant. Based on this peculiar behavior of Scenedesmus, Osterlind suggested that the capacity of this alga to use bicarbonate ions depends on an enzyme that requires photoactivation. He proposed that either HCO_3^- ions undergo activation before they can enter the cell, or their activation occurs inside the cell, in which case, activation may simply mean dehydration and the enzyme requiring activation may be carbonic anhydrase (57). However, Osterlind could not determine for certain whether bicarbonate was utilized as such or whether it was first converted to CO_2 inside the cell.

Carbonic anhydrase is widely distributed in plants, and is predominantly localed in the cytoplasm (81). The movement of CO_2 as bicarbonate across cell membranes, inside the cell, and into the chloroplast has been postulated as a function for this enzyme. The non-enzymatic movement of bicarbonate ions into cells suspended in water or nutrient solution has been postulated by Tolbert and Zill (76) in terms of an anionic shift involving bicarbonate and glycolate ic

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ions. This shift, first demonstrated in Chlorella, may also function in higher plants and it would provide for rapid movement of the bicarbonate ion without a corresponding obligated cationic shift (70, 76). The secretion of glycolate by isolated chloroplasts has also been demonstrated (36, 69). This phenomenon might function as an anion shift inside the cell, across the chloroplast membrane.

The Washing Effect

Algae used in the photosynthesis experiments are generally centrifuged and resuspended in water one or more times in order to remove undesirable substances present in the original medium. However, such washing procedure often results in decreased rates of carbon dioxide fixation and this phenomenon has been attributed to several causes. Thus, Warburg, Burk and Schade (79) believed that Chlorella cells are damaged by strong centrifugation and that the cells may be partially destroyed during resuspension of tightly packed sediments. Clendenning and Brown (19) studied photosynthesis in heavily centrifuged Nostoc and Chlorella and came to the conclusion that no damage such as cell rupture or intracellular displacements could be detected after 3 hours of centrifugation at 145,000 x g which could be associated with damage to the photosynthetic capacity of the algae. In this case, the cells were kept in a complete nutrient medium at about 0° C. The photosynthetic ability, however, can be impaired when use is made of unrefrigerated centrifuges operated at uncontrolled room temperatures. Overheating and oxygen exhaustion seem to be the main factors responsible for the decline in photosynthetic activity after lengthy and inadequate centrifugations (19).

The removal of cations in particular from the suspending medium has been recognized as another major cause for the lower rates of carbon dioxide fixation upon illumination of washed algae (10, 43, 48). The fact that washing may affect under certain circumstances some metabolic system or system of the algae, has been suggested by Clendenning, Brown and Eyester (20). They stated that the injury to Nostoc by washing with neutral potassium-free media probably involves altered permeability and loss of cellular constituents. Schmidt[1] indicated that washing with water containing calcium ions should protect algae against photosynthetic inactivation. Loss of intracellular nitrogenous compounds should be prevented by calcium ions (66). The alternative exists that leaching out of cellular components may be a result rather than a cause of changes in cell metabolism. Barber and Russell (4) concluded after studying the relationships between metabolism and the exchangeability of ions in plant tissues, that an appreciable part of the capacity of carrot parenchyma to hold ions by exchange is dependent on concurrent metabolism.

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[1] Personal communication. Dr. R. R. Schmidt, Assistant Professor of Biochemistry and Nutrition. Virginia Polytechnic Institute. Blacksburg, Virginia.

The effect of various mineral salts, both singly or in combination, on the uptake and distribution of photosynthetically incorporated $C^{14}O_2$ in Chlorella, was investigated by Holm-Hansen et. al. (31). They reported that when magnesium sulfate, potassium nitrate and potassium phosphate were combined in the same concentrations as are found in the normal nutrient solution, the uptake of radioactive carbon was increased and more C^{14} was incorporated into the amino acids. The percentage of radioactivity in the sugar phosphates, on the other hand, decreased when compared to the controls in distilled water. The most striking effect of the addition of just one salt was obtained particularly with ammonium chloride. This increased the total uptake of $C^{14}O_2$ by a factor of 3, and raised the amount of radioactivity found in the amino acids from 9.9 to 57 per cent, while the label in the sugar phosphates fell from 64 to 7 per cent. These results indicate that some metabolic pathways may be influenced by the addition of salts, as suggested by Clendenning et. al. (20), without greatly affecting others. Holm-Hansen et. al. further indicated that according to their experiments, the capacity of Chlorella cells to store such basic raw materials as nitrogen and phosphorus compounds, appears to be quite small. Within 30 minutes after the removal of the algae from complete nutrient medium signs of nitrogen and phosphorus deficiency could be observed when the cells were subsequently placed under conditions for rapid photosynthetic assimilation of CO_2 (32). In contrast to this concept is the report by Al Kholy (1) that algae are able to store phosphorus and continue growing for some time after complete depletion of this element in the external medium. In agreement with the latter is evidence gathered by Schmidt (66) from cytochemical and physiological studies indicating that Chlorella pyrenoidosa cells accumulate polyphosphate which in turn can be used for growth and division.

Bassham and Calvin (5) have stated that addition of a small quantity of phosphate buffer (2.5×10^{-5}M K_2HPO_4-KH_2PO_4) to an algal suspension of Chlorella or Scenedesmus which had been washed twice with distilled water, improves the subsequent chromatography of the cell extracts. This addition of phosphate is a common practice by algalogists who study photosynthesis. The stimulation of photosynthetic rates obtained with phosphate and an explanation for the effect, will be the subject of Part II of this dissertation.

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MATERIALS AND METHODS

Higher Plants

Ten-day old Tatcher wheat and Hawkeye soybean seedlings were grown on standard greenhouse soil mixture in a growth chamber with a light intensity of 2,500 f.c., a day temperature of about 24°C and a night temperature of about 12°C. Coffee leaves were obtained from a shrub in the collection of Economic Plants on this Campus[1]. This plant appeared healthy and its age was estimated to be about 7 years.

Algae

The unicellular algae Chlorella pyrenoidosa, Chlamydomonas eugametos and Chlamydomonas reinhardti were grown in sterilized nutrient media in growth tubes at 20°C. The composition of the nutrient medium for Chlorella was as follows: KNO_3, 5 x 10^{-3} M; K_2HPO_4, 5 x 10^{-4} M; KH_2PO_4, 5 x 10^{-4} M; $MgSO_4$, 2 x 10^{-3} M; $Ca(NO_3)_2$, 2.5 x 10^{-4} M; Hoagland's trace nutrients (77), 2 ml/l; Fe (as the ferric-EDTA complex), 5 p.p.m. The pH was adjusted to 7 before sterilization. The iron solution was sterilized separately and added to the main solution when both were cool. This procedure was necessary in order to prevent severe flocculation of salts after sterilization of the medium. Even then,

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[1] With the kind permission of Dr. W. B. Drew, Head of the Botany and Plant Pathology Department. Michigan State University. East Lansing.

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The composition of the medium for Chlamydomonas was: NH_4NO_3, 2.5 x 10^{-2} M; $MgSO_4$, 8 x 10^{-4} M; $CaCl_2$, 2.3 x 10^{-4} M; K_2HPO_4, 8.2 x 10^{-2} M; KH_2PO_4, 5.3 x 10^{-2} M; Hoagland's trace nutrients and Fe as above. The pH was adjusted to 6.9 before sterilization.

Continuous artificial illumination was provided by means of a bank of fluorescent lamps with an intensity of about 1,000 f.c. The cultures were constantly aerated with a mixture of air containing about 1 percent of CO_2. Both algae and growth tubes were washed thoroughly at least once a week to prevent deterioration of the cultures. The need for the occasional washings is an experimentally derived fact. Excretion of inhibitory substances will be discussed in the section pertinent to results. Presumably this effect eventually will inhibit an algae culture unless the medium is changed.

Short-time Photosynthesis Experiments

Selected wheat seedlings were cut off about 1 cm above the soil level. The remnants of the coleoptiles were carefully removed and the leaves rapidly placed in a glass tube containing a small volume of water. This tube was placed in a photosynthetic chamber, which was similar to that described by Towers and Mortimer (77). The photosynthesis apparatus consisted of two reflector lamps, which illuminated the leaves from opposite directions with an intensity of about 2,000 f.c. Two large flattened pyrex jars filled with water served as heat filters. In addition, the closed photosynthetic chamber was immersed in a water bath so that the temperature of the system could be maintained at about 20°C. Usually, the leaves were allowed a period of 3 to 5 minutes for adjustment to the new environment before the radioactive carbon dioxide was released. The $C^{14}O_2$ was generated inside the chamber by injecting excess acid (about 0.25 ml of 5 N lactic acid) onto a calculated amount of $BaC^{14}O_3$ containing approximately 20 μc of the isotope.

Two different types of photosynthesis experiments were conducted: one which lasted only for 2 minutes, and another in which the period of illumination was prolonged for 10 minutes. The objective was to determine the shortest length of time which would permit the leaves to photosynthetically produce sufficient labeled sucrose for degradation purposes, yet keep the randomization of C^{14} in the molecule at the lowest possible level. At the end of each experiment the leaves were quickly removed from the chamber and dropped into boiling 80 percent ethanol. The alcoholic extraction was prolonged for 10 minutes and was followed by two more extractions with boiling distilled water. The extracts were then combined and assayed for total fixation of $C^{14}O_2$, before they were prepared for chromatography according to the methods of Benson *et*. *al*. (6).

The sucrose area on the chromatogram was identified by its R_f values and by chromatography of its hydrolyzate, which indicated that only two new radioactive spots corresponding to glucose and fructose were formed. All the sucrose-C^{14} obtained from a given experiment was eluted from the corresponding chromatograms and combined into a single sample. This sample was then reduced by evaporation with the aid of a Rotary Evapo Mix, acidified with HCl to a final concentration of 1 N, and heated for 20 minutes in a boiling water bath to effect hydrolysis. When cool, the solution was treated with Amberlite IR 45-OH until it was neutral to paper indicator. The hydrolyzate was recovered by filtration, following which the resin was thoroughly washed with water. The radioactive solutions thus obtained were then stored in the deep freezer until used for degradation.

Glycolate-C^{14} Feeding Experiments

Vacuum Infiltration Technique. The leaves of wheat and soybean seedlings were fed either glycolate-1-C^{14} or -2-C^{14} by vacuum infiltration. The wheat plants were handled in a way already described in the photosynthesis section. In the case of soybean, only the youngest and partially expanded leaves were utilized. Both kinds of leaves were cut into small pieces and placed at the bottom of a Thunberg tube containing a labeled glycolate solution (specific activity, 100 μc/ml). Care was taken that the leafy material was completely covered by the solution. Then, the tube was attached to the suction line and enough vacuum was created to achieve complete infiltration of the tissues when the vacuum was released. The tube was gently agitated while suction was applied to facilitate the removal of air bubbles. The infiltration was carried out at low light intensity, but afterwards the intensity was about 6,000 f.c. A period of 3 minutes in the bright light was allowed for metabolism of the substrates. Subsequently, the samples were killed and extracted as previously described. The extracts were chromatographed according to the techniques mentioned above.

The glycolate-1-C^{14} or -2-C^{14} solutions were prepared as follows. The calculated amounts of either calcium salt were transferred to small conical tubes containing about 0.25 ml of distilled water. Then, enough Dowex-50-Na resin was added and mixed until the insoluble salts went into solution. The sodium glycolate solutions were subsequently removed from the resin by means of a medicine dropper, and the resin was washed several times with water until a final volume of 1 ml of solution had been collected.

<u>Selection of Plants for Labeling of Sucrose</u>. Glycolate-1-C^{14} or -2-C^{14} were fed to leaves of wheat, soybean and coffee in the light for a period of 10 minutes, in an attempt to find out which was capable of forming the largest amount of sucrose in this time period. Feeding was accomplished by immersing the petioles into the radioactive solutions, which were contained in short pointed test tubes. The rate of transpiration and hence the uptake of the solutions

by the leaves was enhanced by blowing a mild air current on them. After the feeding period, the leaves were removed from the solution, and the excess glycolate-C^{14} was washed off by placing the petioles under running tap water for a few seconds. The leaves were then extracted with 80 percent ethanol and assayed by paper chromatography.

Serine-3-C^{14} Feeding Experiment

The base of several young wheat leaves were placed in a radioactive serine solution (100 µc/ml) for 10 minutes in the light. At the end of this period, the leaves were extracted and assayed by chromatography.

Use of Algae Cultures for Photosynthesis

Approximately 100 ml samples of 1-day old algae of a given species were drawn off the continuous culture into precooled tubes and centrifuged for 5 minutes at 900 x g in a refrigerated centrifuge at 2°C. The supernatant was discarded and the algal pellet resuspended in cool distilled water. This washing procedure was repeated three times. Then, the algae were resuspended in a small volume of water, transferred to a precooled graduated pointed test tube and further centrifuged for about 5 minutes at full speed in a clinical centrifuge. The volume of packed cells was recorded and the cells were diluted to a final density of 1 ml of packed cells per 100 ml of suspension.

Prior to the photosynthesis studies, the algal suspension was aerated for at least 10 minutes in the cold. Then, 3-ml aliquots were pipetted into flattened test tubes and further adapted for 10 minutes to designated light and temperature conditions. Except for the flattened test tubes, the rest of the photosynthesis apparatus was the same as described above. After the equilibration period, small volumes of solutions under investigation were added to the algal suspension, followed by 20 µl of 0.1 N $NaHC^{14}O_3$ (0.5 mc/ml). The fixation period was usually extended for 10 minutes, unless otherwise specified. At the end of each time period, 1-ml aliquots were transferred to test tubes containing about 5 ml of hot absolute methanol. The volume of the alcoholic extracts was then adjusted to 10 ml with water and 0.5-ml aliquots were counted for total fixation of C^{14}. It was necessary to add about 3 drops of 2 N acetic acid to release the excess radioactive bicarbonate, while drying the aliquot in the counting dish. A Nuclear Chicago scaler, model 161 A, with a thin end window Geiger-Mueller tube was used. The counts were not corrected for self absorption. Total fixation was calculated on the basis of counts per second per milliliter of algal suspension. The distribution of radioactivity among the soluble products of photosynthesis was determined by counting the spots on the paper chromatograms.

Uptake of P^{32}-labeled Phosphate or Serine-3-C^{14} by Washed Algae

The alga *Chlorella pyrenoidosa* was washed three times,

resuspended and adapted according to the procedure described for the photosynthesis experiments. Two different kinds of Chlorella were used: one which was grown in its own medium (basal phosphorus content), and another which was grown in the presence of excess of phosphorus (Chlamydomonas medium). For simplicity, these two conditions will be designated respectively as "low-P" and "high-P".

After the period of adaptation, the control sample received only 20 ul of $NaHC^{14}O_3$ (0.5 mc/ml). Other samples received either 10 μmoles of nonradioactive phosphate-HCl buffer (pH 7) plus the same amount of $NaHC^{14}O_3$, or 10 μl of radioactive phosphate (1 mc/ml) plus 10 μmoles of phosphate carrier. At the end of the 10 minute period, the C^{14}-treated samples were handled as described above. Samples tagged with P^{32} were analyzed as follows: (a) 0.1-ml aliquots were taken out and counted for total activity in the whole sample. (b) 2-ml aliquots were pipetted out and filtered with the aid of a millipore filter whose pore size was 0.45 μ. The cells were washed once with 3 ml of 0.01 N HCl and then killed with 1 ml of 1 N HCl[1]. Further extraction of the algae was achieved with methanol and distilled water at room temperature. The radioactivity in the supernatant, washing and combined extracted fractions was counted as described

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[1] According to personal communication with Professor R. C. Ball of the Fisheries and Wildlife Department, Michigan State University, washing with 0.01 N HCl should remove any adsorbed P^{32} on the surface of the cells without injuring the algae. Stronger concentrations, on the other hand (e.g., 1 N), should kill and extract the cells.

previously. The counts were expressed as counts per second per milliliter. The filters with the cell residues were also counted.

Procedure for the Degradation of Hexoses

Carbon by carbon degradation[1] of glucose-C^{14} and fructose-C^{14} was accomplished by the combined bacterial and chemical method originally described by Berstein et. al. (9). Alterations of these procedures are also explained in some detail in another manuscript on this work by Jimenez et. al. (35). Essentially, glucose and fructose were converted to lactate, ethanol and carbon dioxide by *Leuconostoc mesenteroides*, strain 549B (44), which were grown at 30°C and harvested in the gassing phase. The cells were washed three times and resuspended in distilled water. The fermentations with labeled sugars were carried out in the apparatus described by Krichevsky and Wood (39). Anaerobiosis and CO_2 removal were accomplished by continuously flushing with high purity N_2. Usually, two hours were sufficient to ferment 95 percent of the hexoses. Carbon dioxide was trapped in 0.4 M NaOH and quantitatively determined by titration of the remaining hydroxide ions. The ethanol was recovered by distillation from a neutral solution and the lactate by ether extraction at pH 3. The lactate

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[1] The degradations were performed by Mr. R. L. Baldwin in Dr. W. A. Wood's laboratory of the Department of Biochemistry, Michigan State University.

was subjected to 11 volume distillation to remove contaminating organic acids. Then, it was oxidatively decarboxylated by the MnO_2 method. The ethanol and the acetaldehyde were converted to acetate with chromic acid, and the acetate was then degraded by the Schmidt azide procedure (64).

To determine the accuracy of the procedures, glucose-1-C^{14}, -2-C^{14} from New England Nuclear Corp., were degraded. The yields of CO_2, ethanol and lactate were the same whether glucose alone or glucose plus fructose served as the carrier substrates (Table I). This indicated that the conversion of hexoses to the expected products was nearly complete. The cross contamination between carbon atoms was less than 1 percent in all cases. The inclusion of fructose did not alter the specific activities of the products of degradation. Consequently, it was deemed satisfactory to use _L. mesenteroides_, strain 549B, for fermenting the mixture of glucose and fructose obtained from the hydrolysis of the sucrose-C^{14} produced in the plant metabolism experiments.

In a recent paper by Busse _et_. _al_. (12), data has been presented pertaining the degradation of fructose-C^{14} with _L. mesenteroides_, strain 39. It was found by these workers that this strain converted 31% of the fructose to mannitol, but the other fermentative products were nearly the same as those obtained from glucose. It was indicated, however, that a greater randomization of labeling occured among the other products. Such was not the case under the present experimental conditions, judging by the fact that better yields of CO_2 were obtained with strain 549B than were reported with the strain 39. Hence, it could be expected that mannitol production was much lower. Further more, the degradation of glucose-C^{14} standards in the presence of fructose indicated that the influence of the latter upon C^{14} distribution and the specific activity of the degradative products was within the limits of fluctuation expected from this procedure.

Since the labeled substrate used in the present studies was nearly an equal mixture of glucose and fructose, the maximum error from spread of the tracer that could be attributed to poor fermentation of the fructose fraction would be only 50 percent of that produced by fructose alone. Even if L. mesenteroides, strain 549B, fermented fructose as reported by Busse et. al., a maximum error of 10 to 12 per cent would then result which is not sufficient to affect the conclusion derivable from the present data.

TABLE I. Yield and distribution of C^{14} in fermentation products formed from glucose-1-C^{14}, -2-C^{14} and -6-C^{14} with and without added fructose.*

	Products					
Substrate	CO_2	CH_3----CH_2OH		COOH-CHOH-CH_3		
	(umoles)	(umoles)		(umoles)		
Glucose + Fructose	955	685	800	400	346	336
	(dpm/umoles)	(dpm/umoles)		(dpm/umoles)		
Glucose-1-C^{14} +	630	2.2		4.1		
Glucose + Fructose	638	1.3		1.1		
Glucose-6-C^{14} +	7.1	4.6		3.1	2.6	633
Glucose + Fructose	3.6	4.1		2.1	1.8	620
Glucose-2-C^{14} + Glucose	3.1	730	5.2	2.3		

* For each degradation a total of 1 mmole of hexose was fermented. Each flask contained 500 umoles of glucose-C^{14} and 500 umoles of either glucose or fructose. The specific activities of the glucose-1-C^{14}, -2-C^{14} and -6-C^{14} without carrier were 1250, 1484 and 1294 respectively. The fermentations were run and the products isolated and degraded as described in the text.

PART I

RESULTS AND DISCUSSION

Photosynthesis Experiments with Higher Plants

The photosynthetic products formed by young wheat leaves after 2 or 10 minutes of exposure to $C^{14}O_2$ are shown in Table II. The same pattern of distribution of C^{14} among the products was found in these experiments as has been repeatedly reported by other workers (6, 8, 75).

With 2 minute photosynthesis, approximately 50 per cent of the soluble radioactivity was found among the sugar phosphate esters. Substantial amounts of sucrose-C^{14} were also produced during this short period of illumination. Serine, glycine and glyceric acid were also labeled. After 10 minutes in the light, the percentage of the total radioactivity in sugar phosphates had considerably decreased due to the accumulation of sucrose. Thus, the proportion of total C^{14} in sucrose rose to a level at least 4 times higher. The relative percentage of C^{14} in serine, glycine and glycerate also diminished after the longer exposure to light. Alanine, aspartate and malate could only be detected on the chromatograms after 10 minutes of photosynthesis. Glycolate-C^{14} was present but only in trace amounts. Besides these compounds there were several others which together accounted for only a few percent of the total soluble C^{14} fixed after 2 or 10 minutes of illumination.

The distribution of radioactivity in the hexoses

TABLE II. Formation of labeled compounds by young wheat leaves after two or ten minutes of $C^{14}O_2$ photosynthesis.

Compound	Period of Illumination			
	2 minutes		10 minutes	
	c/s*	%	c/s	%
Sugar phosphate esters	100.8	55.8	175.2	12.7
Sucrose	32.5	17.1	1000.0	71.5
Serine	14.5	7.9	66.5	4.8
Glycine	18.7	9.8	30.9	2.3
Glycerate	9.7	5.2	29.2	2.0
Aspartate	-	-	31.0	2.1
Alanine	-	-	24.0	1.7
Malate	-	-	15.4	1.1
Glycolate**	-	-	4.2	0.2
Others	8.7	4.2	22.5	1.6

* Counts per second, directly obtained from the paper chromatograms.

** Uncorrected for loss due to sublimation.

TABLE III. Percentage distribution of C^{14} in hexoses of sucrose.

Experiment	Substrate	C_1	C_2	C_3	C_4	C_5	C_6
1	$C^{14}O_2$	17.2	15.8	17.3	19.0	15.6	15.7
2*	$C^{14}O_2$	9.4	8.9	22.6	34.6	7.9	4.4
3	Serine -3-C^{14}	43.7	**	**	2.5	17.2	44.5
4	Glycolate -2-C^{14}	25.7	25.8	4.1	3.7	19.7	19.9
5	Glycolate -1-C^{14}	16.8	14.7	16.0	21.4	13.4	14.0
6+	Glycolate -1-C^{14}	8.6	12.6	22.1	27.0	8.5	9.4

* Except experiment #2 which was for 2 minutes, all others lasted 10 minutes.

\+ Wheat leaves were used in all experiments except #6, in which soybean leaves were utilized.

** Less than 2% of the C^{14} was found in carbon atoms 2 and 3 combined.

derived from the labeled sucrose formed during $C^{14}O_2$ fixation is shown in Table III (experiment 1 and 2). After 2 minute photosynthesis (experiment 2), the hexoses were labeled predominantly in the carbon atoms 3 and 4, with C_4 having a higher specific activity than C_3. These results agree with those reported by Gibbs and Kandler (27). The discrepancy observed in the amount of tracer shared by carbon atoms 5 and 6 with respect to 1 and 2 is not considered significant and can be explained by the limitations inherent to the degradation procedure. Within a period of 10 minutes (experiment 1), on the other hand, the sucrose molecule was uniformly labeled.

Glycolate-C^{14} Feeding Experiments

Vacuum Infiltration. The object of using this technique was to overcome the limitation imposed by the slow rate of penetration of labeled substrates into the leaf. However, this technique was not suitable for this purpose since neither glycolate-1-C^{14} nor glycolate-2-C^{14} were converted during the 3 minute period of exposure to light into enough labeled sucrose for degradation purposes (Table IV). Only traces of sucrose-C^{14} were formed by wheat leaves when glycolate-1-C^{14} was the substrate, and none was produced by soybean leaves. Small amounts of serine, glycine, glycerate and other compounds could be detected chromatographically in the extracts of both leaf samples, particularly when the substrate was glycolate-2-C^{14}. It was not determined whether

TABLE IV. Products of glycolate -1-C^{14} and glycolate -2-C^{14} metabolism during a period of three minutes in the light by vacuum-infiltrated wheat or soybean leaves.

A. Wheat

	Substrate			
	Glycolate -1-C^{14}		Glycolate -2-C^{14}	
Compound	c/s*	%	c/s*	%
Unmetabolized glycolate	200.0	94.8	220.0	92.1
Sucrose	2.5	1.2	--	--
Serine	traces	--	9.2	3.8
Glycine	traces	--	5.1	2.4
Glycerate	1.8	0.9	2.1	0.8
Others	6.0	3.0	2.3	0.9
B. Soybean				
Unmetabolized glycolate	615.0	98.5	210.0	85.7
Sucrose	--	--	--	--
Serine	2.4	0.3	9.1	3.7
Glycine	5.2	0.8	8.5	3.5
Glycerate	1.5	0.2	12.4	5.1
Others	1.3	0.2	5.0	2.0

* Values are based on C^{14} content of a paper chromatogram.

the impairment of the metabolic activity of the leaves by the vacuum infiltration was due to physical damage of the cell structure or to insufficient oxygen supply.

Labeling of Sucrose-C^{14} by Different Plants. Wheat, soybean and coffee leaves were fed glycolate-1-C^{14} or -2-C^{14} by transpiration for 10 minutes in the light to determine which could synthesize most efficiently labeled sucrose. It was found that wheat leaves converted a larger percentage of the labeled substrates to sucrose than either soybean or coffee leaves (Table V). Wheat not only formed more sucrose, but was also able to metabolize a larger portion of the added glycolate into other compounds. Soybean and coffee metabolized less of the substrate but converted a significantly larger percentage of the added glycolate-C^{14} into serine-C^{14}. This suggests that some of the enzymes of the Glycolate Pathway (p. 7), which catalyze the steps beyond serine may be relatively less active in these two plants than in wheat. Thus serine accumulated.

With respect to the percentage distribution of C^{14} in the hexoses of sucrose, it was found (Table III, experiments 4, 5 and 6) that feeding either glycolate-1-C^{14} or -2-C^{14} led to label in sucrose as predicted by the Glycolate Pathway. It has been shown that glycolate-1-C^{14} is converted to serine and glycerate labeled in the carboxyl group, while glycolate-2-C^{14} is converted to serine-2, 3-C^{14} and glycerate-2, 3-C^{14}. Hence, it was predicted that glycolate-1-C^{14} would label the hexoses in the 3 and 4 positions, and

TABLE V. Products of glycolate -1-C^{14} and glycolate -2-C^{14} metabolism during a period of ten minutes in the light by wheat, soybean or coffee leaves.

	Substrate			
	Glycolate -1-C^{14}		Glycolate -2-C^{14}	
Compound	c/s*	%	c/s*	%
A. Wheat				
Unmetabolized glycolate**	68.0	41.1	95.9	42.1
Sucrose	50.8	27.8	64.7	29.6
Serine	16.8	13.8	15.0	9.1
Glycine	6.8	7.4	17.6	8.0
Glycerate	5.0	3.1	9.4	4.3
Others	13.0	6.8	14.7	6.9
B. Soybean				
Unmetabolized glycolate**	21.2	45.1	66.7	50.0
Sucrose	3.3	6.6	3.1	2.3
Serine	11.0	23.0	25.2	19.0
Glycine	3.6	7.7	8.6	6.5
Glycerate	2.9	5.9	17.1	12.9
Others	5.8	11.7	12.4	9.3
C. Coffee				
Unmetabolized glycolate**	11.0	53.1	30.4	54.3
Sucrose	0.6	2.9	1.3	2.3
Serine	4.0	19.4	16.7	29.8
Glycine	0.7	3.4	5.1	9.1
Glycerate	--	--	1.9	3.4
Others	4.4	21.2	0.6	1.1

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* Values are based on C^{14} content of a paper chromatogram.

** Uncorrected for loss due to sublimation.

that glycolate-2-C^{14} would label carbon atoms 1, 2, 5 and 6, if the hexoses were formed by the reversal of the Embden-Meyerhof Pathway after the labeling of glycerate. That the present results are in accordance with the expectations is illustrated by experiments 4 and 6 in Table III.

The higher degree of randomization of tracer that was observed when glycolate-1-C^{14} was fed to wheat (Table III, experiment 5) as compared to soybean (experiment 6) was probably caused by a larger portion of the C^{14} which was oxidized to $C^{14}O_2$ plus an unlabeled 1-carbon fragment during the enzymatic conversion of the 2-carbon moiety to a 3-carbon derivative. The radioactive carbon dioxide thus formed would be fixed photosynthetically to yield uniformly labeled hexoses as in experiment 1 of Table III.

Serine-3-C^{14} Feeding Experiment

The results obtained from the feeding of serine-3-C^{14} to wheat leaves are shown in Table VI. Even though the substrate was metabolized slowly during the 10 minute experiment in the light, an appreciable amount of sucrose was formed (about 5 percent of the total activity). Glutamate, alanine and a phosphate ester, which may be phosphoglycolate, could be detected also on the chromatograms although in trace amounts. Degradation of the hexoses from this sucrose showed that they were labeled predominantly in the 1 and 6 positions (Table III, experiment 3). Carbon atom 5 also contained a significant percentage of radioactivity, while

TABLE VI. Products of serine-3-C^{14} metabolism during a period of ten minutes in the light by wheat leaves*.

Compound	c/s	%
Unmetabolized serine	289.5	94.0
Sucrose	16.5	5.0
Glutamate	traces	
Alanine	traces	
Phosphate esters	traces	

* Values are based on the C^{14} content of one paper chromatogram.

atoms 2, 3 and 4 were only slightly active. Since it has been previously demonstrated (60) that glycerate-3-C^{14} is produced from serine-3-C^{14} by corn leaves in 15 minutes, the labeling pattern shown in experiment 3 indicates that glycerate was subsequently incorporated into hexoses by reversal of the Embden-Meyerhof Pathway. This route of hexose formation, however, does not account for the significant randomization of C^{14} which occurred into carbon 5.

Even though the cross-contamination of labeling within the carbon atom pairs 1-6, 1-2 and 2-5 as observed by Busse _et_. _al_. (12) during the fermentation of fructose by a different strain of _Leuconostoc_ _mesenteroides_ were sustained in these experiments, the significance of the data still would not be invalidated. Any cross-contamination that might have occurred between carbon atoms 1 and 2 or 5 and 6 of the hexoses derived from glycolate-2-C^{14} would not change the results since these carbon atoms should have about the same specific activity. The same reasoning applies for cross-contamination between carbon atoms 1 and 6 of hexoses formed from serine-3-C^{14}. Furthermore, the lack of activity in the $C_2 + C_3$ fragment is indicative of the absence of contamination of carbon atoms 2 and 1 and verifies the fact that bacterial fermentation produced little spreading of labeling in the present degradations.

It has been previously shown that a major portion of the photosynthetically fixed $C^{14}O_2$ moves through a pathway involving glycolate (7, 50, 60, 69, 80, 84). Glycolate may function in the translocation of carbon from the chloroplasts to the cytoplasm (36). Since the enzymatic conversion of glycolate to glyoxylate (18) as well as the subsequent reactions leading to the formation of sucrose occur in the cytoplasm, the sucrose labeled from glycolate represents cytoplasmic sucrose in contrast to the reservoir located in the chloroplasts. Due to the fact that glycolate and all of the 3-carbon compounds derived from it during photosynthesis are uniformly labeled even for the shortest experiments (65), the cytoplasmic sucrose formed from glycolate produced during $C^{14}O_2$ photosynthesis should also be uniformly labeled. Since the chloroplastic sucrose produced under similar conditions is unsymmetrically labeled (Table III, experiment 1), it follows that the Glycolate Pathway accelerates the randomization of labeling in the hexoses within the whole cell, and that the contribution of this pathway to the production of uniformly labeled sucrose would not be obvious except as revealed in these studies.

PART II

RESULTS

The Washing Effect

The impairment of the photosynthetic activity of washed algae has been recognized for some time. The origin of such deterioration of the cells has been associated with factors such as damage due to centrifugal force (79), or disturbance of some metabolic system caused by deficiencies of minerals which are induced during preparation of the algae (10, 20, 31, 43, 48). However, in the present report data will be shown which may indicate that washing algae affects also, and perhaps primarily, the utilization of bicarbonate ions.

The effect of 3 washings with distilled water upon the photosynthesis in Chlamydomonas reinhardti is illustrated in Table VII. The capacity to utilize bicarbonate ions was reduced by almost 80 percent. The photosynthetic activity of the washed algae could be restored to about a normal rate by the addition of phosphate or phosphoglycolate ions (pH 7) at a final concentration of 3.3×10^{-3} M. Smaller concentrations of phosphate were less effective, and in fact, 1.7×10^{-3} M was only 70 percent as effective as 3.3×10^{-3} M (Table XIII) and 6×10^{-4} M was much less effective (data not shown). Stimulation of photosynthesis above the rate induced by 3.3×10^{-3} M phosphate was obtained with

TABLE VII. Effect of three washings with distilled water on the capacity of *Chlamydomonas reinhardti* to utilize bicarbonate ions.

Treatment	Total C^{14} Fixed (c/s/ml/10 min. PS*) Unwashed cells**	Washed cells
Control	2,700	546
3.3×10^{-3} M phosphate (pH 7) added prior to PS	2,960	3,100
3.3×10^{-3} M phosphoglycolate (pH 7) added prior to PS	2,560	3,050

* PS stands for photosynthesis.

** Values were corrected for algal dilution, so that they corresponded to the same dilution (1:100) of the washed cells.

1×10^{-2} M, but higher concentrations were inhibitory (Table XIII).

Washing of the algae, however, is not always detrimental, for it may serve to remove undesirable metabolites which may accumulate in the suspending medium, as it is illustrated in Table VIII. Even though 1-day old *Chlamydomonas* were capable of utilizing bicarbonate ions in the presence of the original media, photosynthesis by *Chlorella* of the same age and grown in the low-P medium was very slow. The failure of *Chlorella* to fix bicarbonate when the cells remained in the original medium could not be changed either by lowering the pH of the medium from about 8.2 to 6.9 or by adding phosphate ions to a final concentration of 3.3×10^{-3} M (pH 7) 10 minutes before the photosynthesis experiment was started. Instead, it was necessary that the cells be washed with distilled water and phosphate ions added back before the photosynthetic capacity could be restored. The response of unwashed *Chlorella* may be related to the phosphorus content of the medium during growth, since this same species, when grown in the high-P medium ordinarily used for *Chlamydomonas* (data not shown) was capable of rapid utilization of bicarbonate even if kept in the nutrient solution in which the algae had been cultured for one day. An alternative would be that the behavior of unwashed *Chlorella* may be due to accumulation in the surrounding medium of a substance which is produced by neither of the two *Chlamydomonas* species nor formed by *Chlorella* when grown in the

TABLE VIII. Differential capacity of three species of algae to utilize bicarbonate ions.

One-day Old Cultures	Total C^{14} Fixed* (c/s/ml/10 min. PS)	pH** A	pH** B
Chlamydomonas eugametos (unwashed)	2,284	5.6	6.0
Chlamydomonas reinhardti (unwashed)	2,262	3.3	4.0
Chlorella pyrenoidosa (unwashed)	172	8.2	9.0
Ch. pyrenoidosa (unwashed, pH adjusted to 6.9 with HCl)	256	-	8.5
Ch. pyrenoidosa (unwashed, plus 3.3 x 10^{-3} M phosphate (pH 7) added 10 min. before PS)	344	-	8.5
Ch. pyrenoidosa (washed 3 times plus 3.3 x 10^{-3} M phosphate (pH 7) added at the beginning of PS)	1,712	-	7.8
Ch. pyrenoidosa (washed 3 times without phosphate)	200	-	9.0

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* Values were adjusted for cell dilution on the basis of 1 ml of packed cells per 100 ml of suspension.

** The pH was measured before (A) and after (B) the 10 minute photosynthesis experiment. The pH of the washed algal suspension before PS was about 7.

presence of an excess of phosphate ions. A possible substance could be chlorellin (51, 52, 53, 54) which is known to inhibit photosynthesis, and which seems to be active only when found in the external medium. It is possible that this inhibitory substance may block in some way the mechanism of transport of bicarbonate ions across the cell membrane.

Materials which Stimulated $NaHC^{14}O_2$ Fixation by Washed Algae

The capacity of washed algae to utilize bicarbonate was restored by a number of compounds. Table IX represents a summary of the substances that were tested for stimulation of bicarbonate uptake by all three species of algae. The substances were grouped into three categories according to their relative efficiency at a concentration of 3.3×10^{-3} M and at pH 7, under the standard experimental conditions. In the first group were put those compounds which restored completely the ability of the cells to fix bicarbonate. Since these compounds were not all phosphate esters, the concept was untenable that photosynthesis in the washed cells was limited by the availability of phosphorus for synthesis of the phosphate esters of the photosynthetic carbon cycle. Compounds grouped under the second category restored the photosynthetic fixation of bicarbonate only partially. In the third category were placed those substances which did not show any appreciable effect. This arbitrary classification does not imply that all the substances listed under

TABLE IX. Effect of compounds* on stimulating washed algae to utilize bicarbonate ions.

Degree of Stimulation		
Complete	Some	None
Sodium phosphate	Ammonium chloride	Magnesium chloride
Phosphoglycolate	Tris-hydroxymethyl-aminomethane	Potassium chloride
Phosphoglycerate	Glycine	Sodium chloride
Serine	Glycolate	Sodium sulfate
Aspartate	Ribulose 1,5-di phosphate	Adenosine-tri-phosphate
Asparagine	Glucose-6-phosphate	Minor elements as found in Hoagland's formula (77)
Phosphoserine	Calcium chloride	Sodium arsenate
Glutamate	Sodium bicarbonate	
	Potassium acetate	

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* The compounds were tested at a final concentration of 3.3×10^{-3} M and at about pH 7, except for sodium bicarbonate which was tested at 1×10^{-4} M (pH 8.1), and the minor elements. Three-time washed algae (1:100 cell dilution) were used throughout the experiments.

a given heading had the same degree of activity. To a limited extent, the order in which the compounds were listed represents their decreasing order of effectiveness.

Among the cations which were tested, only ammonium and calcium moderately increased the uptake of bicarbonate. In this connection, Holm-Hansen, et. al. (31) have suggested that the response of the washed algae to NH_4^+ ions may be to relieve a mild nitrogen deficiency. However, this suggestion cannot explain the stimulation from non-nitrogen substances. Addition of phosphate and ammonium ions together stimulated radioactive sodium bicarbonate fixation more than that obtained from either one alone (32). This suggests that there is more than one stimulatory effect. The stimulation by ammonium ions has not been further investigated.

The rather small effect from calcium ions in the standard procedure might have been explained by the fact that the cation was added after washing. Hence, calcium could not have prevented the leakage of cellular phosphorus compounds during the preparation of the algae, as suggested by Schmidt[1]. In order to test this hypothesis, an experiment was performed in which the effect of both washing and resuspending with two calcium chloride solutions was compared against distilled water or phosphate added prior to photosynthesis (Table X). Washing and resuspending Chlamydomonas

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[1] See footnote on page 14.

TABLE X. Effect of washing *Chlamydomonas reinhardti* with two calcium chloride solutions*.

Washing Treatment	Phosphate** Addition During PS	Total C^{14} Fixed (c/s/ml/10 min. PS)	Final pH
Distilled water	0	106	8.5
Distilled water	$3.3x10^{-3}$ M	1,400	7.5
$2x10^{-2}$ M $CaCl_2$	0	360	7.5
$2x10^{-2}$ M $CaCl_2$	$3.3x10^{-3}$ M	954	6.3
$1x10^{-1}$ M $CaCl_2$	0	400	7.0
$1x10^{-1}$ M $CaCl_2$	$3.3x10^{-3}$ M	1,170	6.7

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* The algae were washed 3 times according to the standard procedure.

** The pH of the potassium phosphate solution was 7.

reinhardti in either 2×10^{-2} or 1×10^{-1} M $CaCl_2$ could not prevent the loss of photosynthetic activity. Phosphate in the external medium was required in order to restore activity. That pH could not have been a limiting factor in the experiment is illustrated by the pH values recorded at the end of the 10 minute photosynthesis period. The values fell within the physiological range (pH 4 to 9) determined by Emerson and Green (22) for Chlorella, and by Calvin et. al. (16) for Scenedesmus.

A slight stimulation of C^{14} fixation was observed by the addition of excess carrier bicarbonate during an experiment with completely washed Chlorella pyrenoidosa (Table XI). The total amount of bicarbonate added per treatment was increased stepwise but the specific activity of C^{14} was held constant. Since both C^{12} and C^{14} were used, a correction for isotopic dilution was made according to the formula given below, in order to calculate total fixation rates.

$$\text{Correction factor} = \frac{(\mu\text{moles of } C^{12} + \mu\text{moles of } C^{14})}{\mu\text{moles of } C^{14}}$$

Since increasing bicarbonate concentrations had some stimulatory effect upon photosynthesis, the influence of the presence of this anion during washing of the algae was investigated (Table XII). Chlorella pyrenoidosa cells were both washed three times and resuspended with 1×10^{-3} M (Suspension A) or 1×10^{-1} M $NaHCO_3$ (Suspension B). The pH of these solutions was 7.9 and 8.1, respectively. Also,

TABLE XI. Effect of increasing amounts of bicarbonate ions on photosynthesis of washed *Chlorella pyrenoidosa*.

Treatment	Total C^{14} Fixed* (c/s/ml/10 min. Ps)	Final pH
Control (6.7 x 10^{-4} M $NaHC^{14}O_3$)	168	7.4
3.3 x 10^{-5} M $NaHCO_3$ + 3.3 x 10^{-5} M $NaHC^{14}O_3$	230	6.5
3.3 x 10^{-4} M $NaHCO_3$ + 3.3 x 10^{-4} M $NaHC^{14}O_3$	268	7.1
3.3 x 10^{-3} M $NaHCO_3$ + 3.3 x 10^{-3} M $NaHC^{14}O_3$	600	9.0

* Values were corrected for isotopic dilution according to the formula given in the text. The correction factor was 2.

TABLE XII. Photosynthesis by algae washed with bicarbonate.

Treatment	Total C^{14} Fixed* (c/s/ml/10 min. Ps)	Final pH
Suspension A + 6.7×10^{-4} M $NaHC^{14}O_3$	189	7.4
Suspension A + 3.3×10^{-3} M phosphate (pH 7) + 6.7×10^{-4} M $NaHC^{14}O_3$	1,390	8.1
Suspension B + 6.7×10^{-4} M $NaHC^{14}O_3$	3,620	8.6
Suspension B + 3.3×10^{-3} M phosphate (pH 7) + 6.7×10^{-4} M $NaHC^{14}O_3$	4,220	8.7

Suspension A: *Chlorella pyrenoidosa* both washed (3 times) and resuspended with 1×10^{-3} M $NaHCO_3$.

Suspension B: Same as suspension A except that 1×10^{-1} M $NaHCO_3$ was used.

* Values were corrected for isotopic dilution. The correction factors used were 2.5 and 151, respectively for suspensions A and B.

the effect of the addition of the standard amount of phosphate ions was tested during photosynthesis with the bicarbonate treated cells. Preparing the algae with 1×10^{-3} M bicarbonate solution did not prevent the loss of photosynthetic ability, but activity could still be restored by 3.3×10^{-3} M phosphate (pH 7). Preparation with 1×10^{-1} M bicarbonate, on the other hand, was effective for controlling the deleterious action of washing. This amount of bicarbonate was much larger than the amount of phosphate necessary for restoring photosynthetic activity. The stimulation of C^{14} fixation by 0.1 M bicarbonate (alone or in combination with phosphate) occurred at the high pH values, 8.6 to 8.7. Speculation why this occurred will be discussed in more detail later.

The combination of phosphate and bicarbonate was further explored. Accordingly, increasing amounts of phosphate (pH 7) were added to Chlorella cells which had been both washed (3 times) and resuspended with either distilled water or 1×10^{-3} M $NaHCO_3$ (Table XIII). The presence of 1×10^{-3} M bicarbonate in the medium resulted in a small increase of C^{14} fixation which cannot be ascribed to pH. In the presence of phosphate and bicarbonate, the rate of C^{14} fixation increased until the phosphate concentration reached a level of 6.6×10^{-3} M and the pH had dropped to about 7.5. When phosphate was added to cells resuspended in water, the highest rate of fixation was attained with a concentration of 1×10^{-2} M. In the absence of bicarbonate,

TABLE XIII. Effect of increasing phosphate concentrations on the photosynthetic activity of algae washed either with distilled water or a bicarbonate solution.*

Washing Treatment	Phosphate** Addition During PS	Total C^{14} Fixed (c/s/ml/10 min. PS)	Final pH
Distilled water	0	47	8.4
Sodium bicarbonate	0	210	8.4
Distilled water	1.7×10^{-3} M	817	7.4
Sodium bicarbonate	1.7×10^{-3} M	1,010	7.8
Distilled water	3.3×10^{-3} M	1,210	7.6
Sodium bicarbonate	3.3×10^{-3} M	1,890	7.6
Distilled water	6.6×10^{-3} M	1,730	7.3
Sodium bicarbonate	6.6×10^{-3} M	3,280	7.5
Distilled water	1×10^{-2} M	2,000	7.3
Sodium bicarbonate	1×10^{-2} M	2,630	7.4
Distilled water	1.3×10^{-2} M	1,310	7.2
Sodium bicarbonate	1.3×10^{-2} M	3,180	7.3

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* Sodium bicarbonate -C^{12} at 1×10^{-3} M was used both for washing and resuspending the alga *Chlorella pyrenoidosa*, according to the standard procedure.

** The pH of the potassium phosphate solution was 7.

a slight decline in effectiveness of the phosphate ions occurred at the highest concentration, which may reflect some unbalanced condition of the medium.

The Effect of Inhibitors

Inhibitors were used to provide data which might help to explain the phenomenon of stimulation or recovery of radioactive bicarbonate utilization by thoroughly washed algae. Uranyl acetate and sodium arsenate were investigated. According to Few et. al. (23), uranyl ions cause a major reduction in the charge density of purely phosphate-type colloids such as protoplast membrane lipid. With respect to carboxyl-type colloids, on the other hand, uranyl ions are not as effective. Frenkel (24) reported that photosynthesis in Chlorella was not inhibited by uranyl concentrations as high as 1×10^{-2} M. This is remarkable because of the generally high sensitivity of photosynthesis to heavy metals (58). Although Frenkel indicated that endogenous respiration in Chlorella was only slightly affected by uranyl chloride, respiration attributed to added glucose was 80 percent inhibited by a 1×10^{-3} M concentration of this salt. The latter observation suggests that transport of glucose across the cell membrane was more drastically inhibited by the uranyl ions in the external medium than subsequent metabolism inside the cell.

Arsenate, on the other hand, is known to interfere in the metabolism of phosphorus, and specifically with the formation of high energy phosphate compounds (58). Presumably, arsenate might also act as a competitive inhibitor of phosphate during uptake of bicarbonate.

Uranyl Inhibition

The effect of uranyl ions on the utilization of bicarbonate by 3-time washed algae was studied first by adding increasing amounts of uranyl acetate[1] to *Chlamydomonas reinhardti*, simultaneously with the standard concentration of either phosphate, phosphoglycolate or serine (Table XIV). Uranyl, at a concentration of 6.6×10^{-4} M, inhibited the rate of $NaHC^{14}O_3$ fixation over a 10 minute period of illumination in the presence of 3.3×10^{-3} M phosphate, phosphoglycolate or serine, respectively by 32, 27 and 48 percent. Increasing the uranyl concentration to 1.7×10^{-3} M caused no appreciable change in the degree of inhibition in the presence of phosphoglycolate. However, this same uranyl concentration reduced the photosynthetic activity 75 percent in the presence of phosphate, and almost 95 percent in the presence of serine. At 3.3×10^{-3} M, uranyl proved to be equally toxic in the presence of any of the three substances, although phosphoglycolate still showed some activity. The extreme sensitivity of the serine stimulation to uranyl ions cannot be explained by a comparison of the differential

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[1] The pH of the solution of this salt could be brought only to 4.1 with KOH, for beyond this point it began to form a precipitate.

TABLE XIV. Effect of uranyl acetate on the stimulatory action from addition of phosphate, phosphoglycolate and serine* to washed *Chlamydomonas reinhardti*.

Treatment	Total C^{14} Fixed (c/s/ml) 5 minutes	10 minutes	Final pH
Control	155	475	7.4
$3.3x10^{-3}$ M phosphate	1,064	2,240	7.0
$6.6x10^{-4}$ M uranyl + phosphate	806	1,520	6.9
$1.7x10^{-3}$ M uranyl + phosphate	292	570	5.8
$3.3x10^{-3}$ M uranyl + phosphate	100	0	4.7
$3.3x10^{-3}$ M phosphoglycolate	810	1,740	7.0
$6.6x10^{-4}$ M uranyl + phosphoglycolate	852	1,274	6.0
$1.7x10^{-3}$ M uranyl + phosphoglycolate	688	1,236	5.9
$3.3x10^{-3}$ M uranyl + phosphoglycolate	180	200	5.2
$3.3x10^{-3}$ M serine	--	1,480	7.0
$6.6x10^{-4}$ M uranyl + serine	--	770	--
$1.7x10^{-3}$ M uranyl + serine	--	100	4.7
$3.3x10^{-3}$ M uranyl + serine	--	0	--

* The pH of the phosphate, phosphoglycolate and serine solutions was adjusted to 7. The algae were washed 3 times and resuspended with distilled water.

ability of uranyl to reverse the charge density of the anionic groups, as discussed by Few *et. al.* (23).

The concentration of uranyl ions which inhibited the utilization of bicarbonate in the presence of all three stimulatory compounds was 3.3×10^{-3} M, or three times less than the concentration which Frenkel reported as harmless to photosynthesis in *Chlorella*. If it is assumed that Frenkel was correct and that *Chlamydomonas* and *Chlorella* respond in a similar manner towards uranyl ions, it follows that the activity of the stimulatory compounds was not concerned with the photosynthetic fixation of $C^{14}O_2$ but with some other related process.

In a second experiment (Table XV), uranyl acetate was used at a constant concentration of 1.7×10^{-3} M, in conjunction with 3.3×10^{-3} M alanine or ammonium chloride, as well as phosphate, phosphoglycolate or serine. The alanine stimulation was inhibited by uranyl ions exactly the same as serine, while the ammonium stimulation was not affected. In fact, it seemed as if the ammonium and the uranyl ions had acted in a synergistic fashion. The higher resistance of photosynthesis to uranyl inhibition in the presence of phosphoglycolate may be explained by the assumption that phosphoglycolate, which has two anionic groups, could bind more uranyl than either phosphate, serine or alanine. Inhibition by binding of uranyl ions to some anionic group on the cell surface would occur only when an excess of free uranyl ions were available. Presumably, the

TABLE XV. Differential response of enhanced photosynthesis in presence of a constant concentration of uranyl acetate.*

Treatment	Total C^{14} Fixed (c/s/ml/10 min. PS)	Final pH
Control	324	7.0
$3.3x10^{-3}$ M phosphate + uranyl	100	5.2
$3.3x10^{-3}$ M p-glycolate + uranyl	1,360	5.7
$3.3x10^{-3}$ M serine + uranyl	100	4.7
$3.3x10^{-3}$ M alanine	1,680	7.6
$3.3x10^{-3}$ M alanine + uranyl	176	5.0
$3.3x10^{-3}$ M ammonium chloride	1,000	6.7
$3.3x10^{-3}$ M ammonium chloride + uranyl	1,540	6.9

* Uranyl acetate was added at a final concentration of 1.7 x 10^{-3} M. Chlamydomonas reinhardti, prepared as usual, was utilized in these experiments.

site affected by the uranyl ion is active in the mechanism of bicarbonate transport across the cell membrane or some other process related to photosynthesis.

The stimulation of photosynthesis by ammonium ions is probably a different phenomenon than the stimulation by phosphate, as already suggested (page 41). In the first place, one is a cation and the other an anion. Secondly, phosphate stimulation was inhibited by uranyl ions, while ammonium stimulation was not sensitive to this cation. In fact, a higher rate of stimulation of photosynthesis was obtained in the presence of both uranyl and ammonium ions.

Arsenate Inhibition

The influence of arsenate on the utilization of radioactive bicarbonate by algae was studied first with *Chlamydomonas eugametos*. The cells were centrifuged once and the supernatant was discarded. The algal pellet was then resuspended in distilled water. After adaptation in the photosynthetic apparatus sodium arsenate was added to the algal samples (3-ml aliquots) at a final concentration of 6.6×10^{-4}, 1.7×10^{-3} M or 3.3×10^{-3} M. Immediately afterwards, 20 µl of 0.1 M $NaHC^{14}O_3$ was added and the algae were allowed to photosynthesize for 5 or 10 minutes (Table XVI). The addition of arsenate up to 3.3×10^{-3} M concentration had no inhibiting effect on the photosynthetic activity of partially washed algae.

In another experiment (Table XVII), arsenate was

TABLE XVI. Effect of sodium arsenate* on the photosynthetic activity of partially washed algae.

Treatment	Total C^{14} Fixed (c/s/ml/10 min. Ps)	Final pH
Control	1,808	6.9
6.6×10^{-4} M arsenate	2,140	8.2
1.7×10^{-3} M arsenate	1,776	---
3.3×10^{-3} M arsenate	1,562	---

* The pH was 8.2. Arsenate was added to *Chlamydomonas eugametos* cells which were centrifuged once and then resuspended with distilled water.

TABLE XVII. Effect of sodium arsenate of the photosynthetic activity of completely washed algae*.

Treatment	Total C Fixed (c/s/ml/10 min. PS)	Final pH
Control	215	6.9
6.6×10^{-4} M arsenate	340	8.2
1.7×10^{-3} M arsenate	350	8.2
3.3×10^{-3} M arsenate (pH 7.1)	140	7.2

* The alga *Chlamydomonas reinhardti* was used in this experiment. The preparation of the cells was as usual. Except in the last treatment, the pH of the arsenate solution was 8.2.

added as above to 3-time washed Chlamydomonas reinhardti. Arsenate had no inhibiting or stimulating effect on completely washed cells. The results from these experiments suggest that arsenate ions were not taken up during the duration of the experimental conditions. If arsenate had been absorbed to any appreciable extent, it should have inhibited photosynthesis in Chlamydomonas, since at least two steps in the photosynthetic carbon cycle are known to be susceptible to arsenate poisoning. One is the reduction of 3-phosphoglycerate to 3-phosphoglyceraldehyde, which is catalyzed by triosephosphate dehydrogenase and requires both ATP and TPNH. The other step is the phosphorylation of ribulose-5-phosphate to ribulose-1, 5-diphosphate, which is catalyzed by phosphopentokinase (70).

Arsenate inhibition was also studied with 3-time washed Chlamydomonas reinhardti, in the presence of phosphate or phosphoglycolate (Table XVIII). Incubating the algae for 10 minutes in 3.3×10^{-3} M arsenate prevented phosphate or phosphoglycolate from stimulating the rate of photosynthesis when added later. However, when the algae were first incubated for 10 minutes in a phosphate or phosphoglycolate solution, the subsequent inhibition by arsenate did not occur. The simultaneous addition of phosphate or phosphoglycolate and arsenate just prior to the addition of $NaHC^{14}O_3$ was nearly as effective as incubating the algae with phosphate and then adding arsenate. These results may be indicative of a type of competitive inhibition between the

TABLE XVIII. Effect of incubation* with arsenate or some stimulatory substance on the capacity of washed *Chlamydomonas reinhardti* to utilize bicarbonate ions.

Treatment	Total C^{14} Fixed (c/s/ml/10 min. PS)
Control	262
3.3×10^{-3} M arsenate (pH 8.2)	190
3.3×10^{-3} M phosphate (pH 7)	2,360
10 min. incubation with arsenate followed by phosphate	470
10 min. incubation with phosphate followed by arsenate	1,575
Simultaneous addition of arsenate and phosphate	1,395
3.3×10^{-3} M phosphoglycolate (pH 7)	1,347
10 min. incubation with arsenate followed by p-glycolate	124
10 min. incubation with p-glycolate followed by arsenate	1,357
Simultaneous addition of arsenate and p-glycolate	950

* Incubation consisted of adding the substance under study during the 10 minute period of light and temperature adaptation prior to the photosynthesis experiment. In all cases, arsenate, phosphate or phosphoglycolate were added in the proportion of 10 umoles per 3 ml of algal suspension, or 3.3×10^{-3} M.

two anions for the same site, although more detailed research needs to be done before this idea can be developed.

Influence of pH on the Utilization of Bicarbonate Ions

It was found in previous experiments that the stimulation of bicarbonate utilization by completely washed algae occurred at pH values near neutrality. Hence, it appeared logical to examine the influence that the pH of the medium may have on the effectiveness of the so-called stimulatory compounds. Several factors determined the final pH of the algal suspension after a 10 minute photosynthetic experiment. One factor was a slow and small increase in pH during photosynthesis as if dissolved CO_2 as carbonic acid were being removed from the medium. Another factor was the 20 µl of 0.1 M $NaHC^{14}O_3$ solution which was added to the algal culture. A 0.1 M $NaHCO_3$ solution has a pH of about 8.2. Generally the C^{14}-bicarbonate mixture was prepared in a small excess of free base in order to insure against loss of $C^{14}O_2$. The solution was therefore a mixture of $NaHCO_3$ and $NaCO_3$ and as a result even a small amount of the bicarbonate solution when added to the algal suspension raised the pH to above 8. To counteract the alkalinity of the bicarbonate potassium hydrogenphosphate and trishydroxymethylaminomethane (tris) buffers were chosen. The pH of 0.1 M buffer solutions was adjusted with HCl to predetermined values and 10 µmoles of each were added to 3 ml of completely washed <u>Chlorella pyrenoidosa</u> (Table XIX). Both phosphate and tris buffers were effective

TABLE XIX. Influence of pH of the medium on the stimulation of photosynthesis by two buffer solutions*.

Treatment		Total C^{14} Fixed (c/s/ml/10 min. PS)	Final pH
Control		203	8.3
Potassium phosphate,	pH 7.0	1,953	7.4
	7.5	938	7.9
	8.0	375	8.3
	8.8	300	8.5
Tris,	pH 7.7	816	7.8
	8.0	524	7.9
	8.5	210	8.6
	9.0	96	9.1

* The buffers were adjusted to the desired pH with 0.1 N HCl. Ten umoles of each buffer were added to 3-ml aliquots of *Chlorella pyrenoidosa* cells which were prepared as usual.

stimulators of CO_2 fixation when the final pH was maintained around 7 to 7.8. Above pH 8 there was no stimulation of photosynthesis by these buffers. The lack of stimulation at higher pH values cannot be explained solely on the basis of an adverse pH effect on normal photosynthesis by unwashed or partially washed algae since these pH values fell within the accepted physiological range of 4 to 9 (16, 22).

When the effectiveness of the phosphate buffer was investigated at pH values less than 7 (Table XX), it was found that the rate of photosynthesis was equally stimulated between the range 4 to 6. In this lower pH range the predominant ionic form of the buffer was $H_2PO_4^-$ (11), which was necessary to neutralize the alkalinity of the bicarbonate.

The above results indicate that a pH effect could partially explain the stimulatory action from the buffer. To investigate this possibility without buffer, it was necessary to add experimental amounts of 0.01 N HCl to a washed algal suspension (<u>Chlorella pyrenoidosa</u>), so that when the $NaHC^{14}O_3$ was added, the final pH was in the range of 4 to 7 (Table XX). The results of this experiment indicated that there was a stimulation of C^{14} fixation when the final pH was 7 or less, even though no phosphate buffer was added. It appears, therefore, that the $H_2PO_4^-$ ions acted as a weak acid which in the presence of $HC^{14}O_3^-$ ions would release $C^{14}O_2$ according to the equation

$$H_2PO_4^- + HC^{14}O_3^- \rightleftharpoons HPO_4^= + H_2O + C^{14}O_2$$

TABLE XX. Effect of pH on the utilization of bicarbonate ions by washed algae.

pH Adjustment*		Total C^{14} Fixed (c/s/ml/10 min. PS)	Final pH
Control		570	7.5
Potassium phosphate,	pH 4	2,565	6.0
	5	2,540	6.2
	6	2,620	6.6
	7	2,120	7.6
1×10^{-2} N HCl,	pH 3	2,020	3.7
	3.8	1,330	7.0
	4.8	540	8.2

* A 0.1 M solution of KH_2PO_4 was adjusted to the desired pH values with KOH. Then each buffer was added to completely washed *Chlorella pyrenoidosa* cell, prepared as customary, at a final concentration of 3.3×10^{-3} M. When HCl was used, the pH of about 10-ml aliquots of the same algal suspension was adjusted before the period of adaptation.

Thus, at pH values below or near neutrality, the stimulatory activity of phosphate, phosphoglycolate and the amino acids can be best explained in terms of availability of $C^{14}O_2$. The data suggest that at the low CO_2 concentrations normally encountered in photosynthesis, the CO_2 is the active species which enters the cell. If bicarbonate ion enters the cell it does so at a slower rate. This concept, however, does not account for the arsenate inhibition during bicarbonate utilization (Table XVIII). The stimulation due to excess carrier bicarbonate (Table XII) would have to be accounted for by mass diffusion or active transport of bicarbonate ions. Additional data are given in Table XXI concerning photosynthesis rates in the presence of higher concentrations of carbonate buffers which were added just prior to the 10 minute test period. The rate of C^{14} fixation was highest at about pH 8.5, when both phosphate and carrier bicarbonate were added at a final concentration of 3.3×10^{-3} M. These results seem to be in disagreement with the idea that only CO_2 which can exist below pH 8 was effective in photosynthesis. It is necessary to assume that bicarbonate ions can also diffuse or be transported into the cell. The decline of the rate of C^{14} fixation which was observed at pH values above 8.5 might be due to the adverse effect of the higher pH values.

Feeding Radioactive Compounds which Stimulate Photosynthesis

In order to determine whether phosphate or serine

TABLE XXI. Influence of high pH values on the stimulatory effect of phosphate.

Treatment*	Total C^{14} Fixed (c/s/ml/10 min. PS)	Final pH
6.7 x 10^{-4} M $NaHC^{14}O_3$ and $NaC^{14}O_3$	140	8.6
3.3 x 10^{-3} M phosphate (pH 7)	1,760	7.9
3.3 x 10^{-4} M $NaHCO_3$ + phosphate	1,815	8.2
1.7 x 10^{-3} M $NaHCO_3$ + phosphate	2,770	8.4
3.3 x 10^{-3} M $NaHCO_3$ and $NaCO_3$ + phosphate	3,470	8.5
3.3 x 10^{-3} M $NaHCO_3$ and $NaCO_3$ + phosphate	1,740	8.7
3.3 x 10^{-3} M $NaHCO_3$ and $NaCO_3$ + phosphate	995	9.0
3.3 x 10^{-3} M $NaHCO_3$ and $NaCO_3$ + phosphate	432	9.6
3.3 x 10^{-3} M $NaCO_3$ + phosphate	100	10.3

* The same amount of 6.7 x 10^{-4} M $NaHC^{14}O_3$ was present in all treatments. The values were corrected for isotopic dilution as needed. The same amount of 3.3 x 10^{-3} M phosphate buffer was present in all treatments except the first one.

were absorbed by the washed algae during the 10 minute photosynthesis experiments, P^{32}-labeled phosphate (1 mc/ml) and serine-3-C^{14} (100 µc/ml) were added to washed Chlorella pyrenoidosa cells. The experiments were run in the presence of the corresponding carriers, so that the final concentrations were 3.3×10^{-3} M. The P^{32} experiments were done with algae grown either in the standard low-P medium, or in the high-P medium used for Chlamydomonas. Both media were used to investigate whether the phosphorus content of the original culture solution could affect to any appreciable extent the uptake of phosphorus by washed algae (Table XXII). Even though the photosynthetic activity of the washed algae was markedly stimulated by adding either phosphate or serine to the medium, only a very small amount of P^{32} or serine-C^{14} was found with the cell fraction (extract plus residue) after the short experimental period. In the case of P^{32}, similar results were obtained whether the algae were grown in a low-P or a high-P medium, thus suggesting that in both kinds of Chlorella cells, the internal reservoirs of phosphorus did not influence the amount of phosphorus uptake during the experiment. The amount of P^{32} found in the supernatant and the washing fractions together, represented about 97 percent of the total added radioactivity. Less than 1 percent was detected in the cell extract, and the remaining 2 percent was associated with the algal residue and the millipore filter. This fractionation procedure was described in the Methods section. To what extent the amount

TABLE XXII. Absorption of labeled phosphate or serine by washed *Chlorella pyrenoidosa* during stimulation of bicarbonate uptake.

Treatment	Total C^{14} Fixed (c/s/ml/10 min. PS)
Controls	
Distilled water	188
Low-P cells + 3.3 x 10^{-3} M phosphate (pH 7)	2,486
High-P cells + phosphate	2,444
Low-P cells + 3.3 x 10^{-3} M serine (pH 7)	897

Labeled Compounds	c/s/ml/10 min.				Activity/ml of whole sample
	Supernat.	Washing	Extract	Residue	
Low-P cells + 10 uc P^{32} + 3.3x10^{-3} M carrier phosphate	975	98	9	24	1,027
High-P cells + 10 uc P^{32} + carrier phosphate	967	91	8	37	1,045
Low-P cells + 10 uc of serine-3-C^{14} + 3.3x10^{-3} M carrier serine	345	36	7	28	--

The treatments receiving P^{32} or serine-3-C^{14} also received 10 umoles of C^{12} sodium bicarbonate in order to stimulate photosynthesis.

of radioactivity left on the filter represents actual metabolism of the added P^{32}-phosphate was not determined. But even if all this residual radioactivity was attributed to the cells, the total amount of P^{32} that was taken up was very small compared to the strong stimulation of photosynthesis caused by phosphate. In considering this finding, one must remember that rather large concentrations of phosphate ions were used to restore photosynthesis to the washed algae, and that just catalytic amounts were not sufficient.

When serine-3-C^{14} was used, the pattern of distribution of radioactivity among the various cell fractions was essentially the same as for the P^{32}-labeled phosphate experiments. There was, however, a slightly higher percentage of C^{14} associated with the cell extract and the residue left on the filter.

The very low absorption of phosphate by washed Chlorella may reflect the fact that the algae were mostly mature cells, since the youngest ones were probably lost during the process of washing. Another limiting factor for phosphorus uptake may have been the absence of any nitrogenous compound in the medium. Corroborating data for these two assumptions has been reported by Schmidt (66), who studied the uptake of phosphate by synchronized cultures of Chlorella pyrenoidosa. He observed that the time and amount of phosphate absorbed by the cells were related to the nitrogen source and age of the culture. Thus, ammonium ions were more effective than nitrate ions, and the rate of absorption decreased during the last stages of cellular growth (nuclear and cell division). Therefore, it is not surprising that washed Chlorella cells did not absorb appreciable amounts of phosphate during the 10 minute experiments. The important fact is that a significant absorption of phosphate or serine apparently was not an obligatory step in the process of bicarbonate utilization by the washed algae.

Products of Photosynthesis

The alcoholic extracts of washed Chlamydomonas eugametos and Ch. reinhardti were obtained from experiments in which the algae were treated with phosphate or phosphoglycolate, both at pH 7 and then exposed to $NaHC^{14}O_3$. The insoluble cell residues were removed by centrifugation at full speed for about 5 minutes in a clinical centrifuge. The supernatant fractions were then evaporated and subjected to the standard procedures of chromatography and autoradiography (6). The C^{14}-labeled products of 10 minute photosynthesis were identified according to their relative R_f values from inspection of the chromatograms and autoradiograms. In addition, glutamate, aspartate and serine were also identified by cochromatography. Poor separation of glycine and serine made impossible the separate determination of radioactivity in these two compounds (Table XXIII).

In general, both species of Chlamydomonas produced about the same compounds regardless of treatment. Perhaps

TABLE XXIII. Percentage distribution of C^{14} among products of photosynthesis as affected by different stimulatory compounds*.

	Ch. eugametos			Ch. reinhardti		
Compound	Water	Phosphate	P-glycolate	Water	Phosphate	P-glycolate
Phosphate esters	25.3	21.7	18.5	17.3	29.4	16.0
Glycolate	17.3	16.8	2.2	2.2	0.5	1.4
Glutamate	3.9	3.2	14.9	4.7	2.5	21.2
Glycerate	3.1	1.8	10.9	20.9	14.5	7.5
Aspartate area	6.8	3.9	15.4	7.2	4.7	13.0
Serine + glycine	1.8	4.5	5.4	0	4.0	0
Lipids	11.6	17.2	2.9	13.0	18.0	8.5
Others	23.4	12.9	17.6	15.9	10.2	20.4
Origin	6.8	18.0	12.2	18.8	16.2	12.0

* Phosphate and phosphoglycolate were added to 3-washed algae at a final concentration of 3.3×10^{-3} M. The duration of the photosynthesis experiments was 10 minutes.

when the algae were stimulated with phosphoglycolate, they had a tendency to produce different amounts of glycolate, glutamate, glycerate and aspartate, but the significance of this observation is doubtfull on account of the small number of repetitions. Incorporation of C^{14} into phosphate esters was not stimulated by addition of orthophosphate or phosphoglycolate. If the water washed algae had had a phosphorus deficiency, one would expect to find a much less percentage of the C^{14} in the phosphate esters and an increase in the percent of radioactivity incorporated into this fraction upon addition of phosphate ions. These changes did not occur, suggesting that the washing procedure had not caused any phosphorus deficiency which may have reduced the reservoirs of the sugar phosphates encountered in the path of carbon in photosynthesis.

DISCUSSION

Much of the photosynthesis research in recent years has been done with washed algae cultures to which was added a tracer amount of $NaHC^{14}O_3$. The cultures were generally washed one or more times by centrifugation in order to remove most of the nutrient salts which otherwise would overload subsequent paper chromatograms. In addition, unwashed cultures often gave poor photosynthetic rates which may be explained by such toxic substances as Chlorellin (51, 54), which the algae excrete into the medium. During subsequent photosynthetic tests some investigators have at times added back to the washed algae a small amount of phosphate buffer, generally around pH 7. The effect of the washing treatment on photosynthesis has not been thoroughly investigated. I have found that algae washed three times with water lost 80 to 90 percent of their photosynthetic ability as measured by the fixation of $NaHC^{14}O_3$ at pH 8.2. At first this phenomenon was not attributed to a pH effect, because of numerous reports that photosynthesis by algae was unaltered between pH 4 to 9. However, examination of those compounds which were most effective in restoring photosynthesis to the washed algae indicated that they all were potential buffers below pH 8. Phosphate, phosphoglycolate, serine and tris buffer were used the most often to restore photosynthesis for the washed algae. The addition of such compounds at pH 7 to the algal cultures did indeed lower the final pH of the suspension after addition of the $NaHC^{14}O_3$ to values between 7 and 8. In this pH range part of the bicarbonate would be converted to CO_2. Consequently, the data can be explained best by the working hypothesis that $C^{14}O_2$ enters the algae much faster than $HC^{14}O_3$ ions. From the action of the buffers a constant amount of $C^{14}O_2$ would be released and disolved in the water. As the algae absorbed the CO_2 more would be released from the bicarbonate without a significant change in pH because of the action of the buffer. The results emphasize the need for buffer and pH control during tracer photosynthesis experiments with algae. A pH range between 5 and 7 but not over 7.5 is recommended. Too low a pH would cause the loss of much of the $C^{14}O_2$ as gas to the atmosphere, or would necessitate working in closed and aerated apparatus. Of course, one could gas the algae cultures with $C^{14}O_2$, but this procedure would involve a much more elaborate apparatus.

The above hypothesis was substantiated by several additional types of experiments. (a) Buffers were not necessary for restoring activity to the washed algal cultures providing the final pH was lowered to about 7 by addition of HCl. (b) Radioactive labeled buffers, phosphate and serine were not abosorbed by the cells in significant amounts during the course of the experiments. Thus, there was not evidence that these buffers entered the cell or participated in the photosynthetic process. (c) Even though the rate of photosynthesis was reduced 90 percent at pH 8.2 and above, the C^{14} labeled products of photosynthesis did not seem to be significantly altered. This suggests that availability of $C^{14}O_2$ was the limiting factor rather than alteration of the fixation products.

In the course of this work several deviations arose from the postulate. (a) At high bicarbonate concentrations, around 10^{-2} and 10^{-3} M, normal rates of photosynthesis occurred even at pH 8.2 or above. This may be explained by mass diffusion of bicarbonate into the cells which occurs too slow at 10^{-4} and 10^{-5} M bicarbonate concentrations which are encountered with the tracer research method. (b) Uranyl (UO_2^{++}) at concentrations equal to that of the buffers completely negated the stimulatory effect from the buffers even though the pH remained far below 8. Uranyl ions have been reported to be without effect upon photosynthesis by partially washed algae. (c) Arsenate ions if added to the algae 10 minutes before addition of the phosphate or phosphoglycolate buffers also blocked photosynthesis. This inhibition by arsenate did not occur if the buffers were added first. As mentioned in the result section, the arsenate was probably not absorbed by the cells. There is no ready explanation for these latter two observations.

The three algae used in this work were selected because of their ability to synthesize and secrete large amounts of glycolate. Tolbert and Zill (76) showed that _Chlorella pyrenoidosa_ converted 3 to 12 percent of the total C^{14} fixed during 10 minutes of photosynthesis into glycolate and excreted it into the supernatant fluid. The two Chlamydomonas cultures synthesized 50 percent of the C^{14} fixed during 1 to 10 minutes of photosynthesis into glycolate and excreted most of it[1]. One hypothesis about the glycolate excretion was that it involved an active glycolate-bicarbonate shift or transport mechanism across the cell membrane (76).

A major difference between the photosynthesis experiments on glycolate excretion mentioned in the preceeding paragraph and all the experiments in Part II of this dissertation was in the preparation of the algae for experimentation. The glycolate excretion studies were done with either unwashed or once washed algae from which treatments no reduction in photosynthesis rate was imposed. All of my experiments were done with algae which had been washed three times and which had a severely inhibited photosynthetic rate at pH 8.2 when bicarbonate was added. The washed algae, however, would photosynthesize normally at pH values (7 or below) where CO_2 could exist in the medium. These considerations generate speculation that the unwashed or once washed cells could produce and excrete their own acid or buffer for releasing CO_2 from bicarbonate. This acid would be the excreted glycolic acid. This release of glycolic acid would occur near the cell surface and be titrated by the $NaHCO_3$ to give sodium glycolate and CO_2.

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[1] P. C. Kearney, E. Jimenez and N. E. Tolbert, unpublished.

This speculation is supported by the fact that the glycolate excretion phenomenon occurred only above pH 5.0 to 5.5 and in the presence of low CO_2 and bicarbonate concentrations (76). The present hypothesis about glycolate excretion differs from that proposed by Tolbert and Zill, in that it does not involve a glycolate-bicarbonate anionic shift, but rather the release of acid by the cells and the generation of CO_2 in the medium which diffuses into the cells.

SUMMARY AND CONCLUSIONS

PART I

A Glycolate Pathway has been proposed from the metabolic conversion of glycolate to sugars. This scheme first involves the synthesis of serine from two glycolate molecules through the intermediates glyoxylate and glycine. The serine was further converted to glycerate, which in turn was incorporated into hexoses by reversal of the Embden-Meyerhof Pathway. This route was substantiated by feeding leaves for short periods of time C^{14}-labeled glycolate and serine-3-C^{14}, and then isolating and degrading the C^{14}-labeled sucrose which was formed. Glycolate-2-C^{14} produced hexoses labeled in carbon atoms 1, 2, 5 and 6. Serine-3-C^{14} was converted into 3, 4-labeled hexoses. These data corroborate other investigations which purport to show that glycolate is a major photosynthetic product which enters the cytoplasm from the cloroplast. The enzymes of the Glycolate Pathway are cytoplasmic. Since the glycolate produced by $C^{14}O_2$ photosynthesis is uniformly labeled, its subsequent metabolism would produce uniformly labeled hexoses in the cytoplasm. This is in contrast to the predominately 3, 4-labeled hexoses of the photosynthetic carbon cycle inside the chloroplasts. The rate of randomization of label in the hexoses of the whole cell is therefore accelerated by the contribution of the Glycolate Pathway.

PART II

The effect of washing algae with water before subsequent photosynthesis experiments with $NaC^{14}O_3$ was investigated. An explanation was sought for the reason why better photosynthetic rates were obtained when the washed algae were resuspended in phosphate buffer. Upon washing algae three times with distilled water, the photosynthetic rate of fixation of $NaHC^{14}O_3$ at pH 8.2 or above was inhibited 80 to 90 percent. However, the rate of photosynthesis could be restored to normal when the pH was lowered to 7 or below by buffers such as phosphate, phosphoglycolate, or amino acids. This phenomenon was best explained by the assumption that $C^{14}O_2$ could enter the cells many times faster than the bicarbonate ions. Thus the buffers served the purpose of holding the pH in a range where the $NaHC^{14}O_3$ was converted to $C^{14}O_2$. The buffers when labeled with tracers were shown not to enter the cells. The products of C^{14} fixation by the washed algae were not altered by the presence or absence of the buffers. At bicarbonate concentrations 100 fold higher than generally used with C^{14} tracer research, a normal rate of photosynthesis occurred even at pH values of 8.2 or higher. Presumably bicarbonate ions could enter the algae at a slow rate which was compensated for by higher concentrations. The restoration of active photosynthetic rates by phosphate or serine buffers was prevented by uranyl ions or arsenate even though the pH was lowered well below 7.

If the algae which were washed three times produced less glycolate during photosynthesis than algae washed only once, these results could then be interpreted to indicate that normal production and excretion of glycolic acid by algae may be a mechanism for converting the bicarbonate of the medium to CO_2, which in turn can be absorbed by the cells.

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