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UCRL-8603

UNIVERSITY OF CALIFORNIA
Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

UPTAKE AND DISTRIBUTION OF RADIOACTIVE CARBON FROM LABELLED SUBSTRATES BY VARIOUS CELLULAR COMPONENTS OF SPINACH LEAVES

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January 1959

Printed for the U. S. Atomic Energy Commission

UPTAKE AND DISTRIBUTION OF RADIOACTIVE CARBON FROM LABELLED SUBSTRATES BY VARIOUS CELLULAR COMPONENTS OF SPINACH LEAVES[1]

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INTRODUCTION

Although considerable knowledge concerning the path of carbon in photosynthesis has been obtained by Calvin and co-workers (Bassham and Calvin 1957), little real progress has been made regarding our knowledge of the precise intracellular location of the many reactions of photosynthesis. The chloroplasts, which are responsible at least for the primary photochemical reactions generating the reducing power necessary for the conversion of free carbon dioxide to the level of carbohydrate, have been found to reduce pyridine nucleotides photochemically with a concomitant evolution of oxygen (Vishniac, 1952). It has been suggested by other workers that, in addition to all the enzymes involved in the photosynthetic cycle, enzymes concerned with polysaccharide synthesis (Allen, et. al., 1955) and the respiratory activity of the Krebs cycle (Bassham, et. al., 1956) are also associated with the chloroplasts (i.e., while the chloroplasts contain or are associated with a complement of these enzymes, the enzyme may

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1. The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

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also be present elsewhere in the cells).

The chloroplast may thus be visualized as an entity which is capable not only of fixing and reducing carbon dioxide but also of metabolizing its reduction products via the Krebs cycle to the many organic acids and amino acids which are involved in or originate from Krebs cycle activity. It has been shown (Allen, et. al., 1957) that isolated chloroplasts do fix carbon dioxide, in the light, and that radioactive carbon from labelled carbon dioxide is in fact found in such compounds as phosphoglyceric acid (trace), hexose monophosphates, glucose and various amino acids. These results suggest that the chloroplast actually does contain the carbon dioxide fixation enzymes, together with enzymes converting phosphoglyceric acid (the first product of carbon dioxide fixation) into other substances. In fact, one may obtain a clear supernatent liquid rich in carboxydismutase by simply washing an intact chloroplast preparation with dilute buffer (Fuller, 1955, Lyttleton, 1958, Pon, 1959). This idea is further strengthened by other results (Whatley, 1956) showing that chloroplast fragments which have lost the capacity to fix carbon dioxide can have this ability restored to them by the addition of the clear supernatant liquid obtained after centrifugation of broken chloroplasts.

Bassham, et. al., (1956) using Chlorella, have reported that radioactive carbon from labelled carbon dioxide is not found to any significant degree in glutamic acid (~ 5 min) as long as the cells are in strong light, but is incorporated into this amino acid immediately after the light is switched off. This suggested that a small fraction of the total cellular complement of the respiratory enzymes of the Krebs cycle is associated with the chloroplast, as the incorporation of carbon-14 into glutamic acid upon turning off the light is so rapid that it must occur at the seat of the photochemical reception. Recent

work (Holm-Hansen, in press) has shown that much more carbon from carbon dioxide is incorporated into glutamic acid when the cells were suspended in nutrient solution than when they were in distilled water, and hence there is some question as to the validity of the interpretation of this glutamic acid labelling data. Some attempts (Whatley, 1956) have been made to find direct evidence for the presence of Krebs cycle enzymes in chloroplasts, but without success.

It is known that in plants and animals most of the Krebs cycle enzymes can be found in the mitochondrial fraction of the cells, whereas the enzymes associated with the glycolytic degradation of sugar are soluble in the cell cytoplasm (Neilands, 1955). In view of the evidence demonstrating that some biochemical reactions, at least, are associated with distinct subcellular organelles, it is possible that the chloroplast may represent a body more or less specialized for the fixation and reduction of carbon dioxide, together with some simple transformations of the initial products of this fixation. The conversion of the products of carbon dioxide fixation into the wide variety of metabolites found in the plant may, however, require the collaboration of many enzymatic systems found outside the chloroplast. Assuming that this is the case, the problem arises of the mechanism of transfer of molecules within the cell from one place to another, with the associated time delays, etc.

Although isolated chloroplasts have shown some of the reactions attributed to them in the intact cell, the rate at which carbon dioxide is fixed by these isolated particles is insufficient to account for the activity shown by the intact cell. Allen (1955) reported an activity of isolated chloroplasts corresponding to about 0.004 μmoles CO_2 fixed/min/mg chlorophyll. The rate of carbon dioxide uptake for the intact spinach leaf is not known to us, but the rate for Chlorella is 3-4 μmoles CO_2 fixed/min/mg chlorophyll (Bassham, personal communication) (Hill, 1955). The value given above for the rate shown by the isolated spinach

chloroplasts is thus only about 0.1% that of the maximum rate of intact Chlorella. On the other hand, it has been shown (Arnon, 1956; Thomas, 1957; Holt, 1951) that isolated chloroplasts and their fragments can liberate oxygen in roughly the same amounts as can be obtained from the intact leaf compared on a chlorophyll weight basis. The rate of photosynthetic phosphorylation for the intact leaf has not been determined, but the rates obtained (Jagendorf, 1957; Allen, 1958) with isolated chloroplasts is of the same order or higher than the associated CO_2 uptake by the intact leaf compared on a chlorophyll weight basis. As isolated chloroplasts characteristically show the three groups of reactions typical for photosynthesis (oxygen evolution, photosynthetic phosphorylation and CO_2 reduction), the question arises as to why, when isolated from whole cells, the chloroplasts apparently retain their power to fix CO_2 to only 1% of their former ability, whereas their capacity to liberate oxygen is but slightly impaired.

The observations concerning the loss of activity upon isolating cellular constituents are reflected also in the questions regarding the activity and nature of the initial carboxylation reaction in photosynthesis, namely, the reaction catalyzed by carboxydismutase. This enzyme is capable of carboxylating ribulose diphosphate to form two molecules of phosphoglyceric acid. Racker (1957) has reported that the half maximal velocity of this enzymatic reaction is at a bicarbonate concentration of 2×10^{-2} M. In contrast to this value, intact plants have half saturation CO_2 concentrations (for photosynthesis) in the range of 3.5×10^{-6} M to 2×10^{-3} M (Rabinowitch, 1951). The data of Pon (1959) show that the carboxylating activity of the extracted carboxydismutase system is not greater than 0.5% of the CO_2 uptake rate in the intact leaf. The latter calculation assumes that the enzyme accounts for approximately 10% of the nitrogen of the cell, and is based on the enzymatic CO_2 fixation rate obtained by extrapolation to a total CO_2 concentration of 1.3×10^{-3} M. This concentration corresponds to

0.4% CO_2 in equilibrium with a buffer solution at pH 7.3. From these low turnover rates for carboxydismutase, there arises the problem of its relative inactivity. Several possibilities emerge: (a) the enzyme is not pure in its extracted form, (b) it has been denatured during isolation to an extent that lowers its activity to the observed value, (c) there is some essential cofactor or other enzyme necessary for full activity and which is not provided in the *in vitro* tests, or (d) some form of "active CO_2" is the natural substrate rather than CO_2 or bicarbonate ion.

It is thus apparent that at least for some reactions, the activity of the isolated chloroplasts appears much lower than it must be in the intact cell. The reason for such a decrease in activity could be ascribed either to an environmental change, such as pH (and such other non-specific factors as are mentioned in the preceding paragraph), or to the lack of a necessary collaborator resulting from its physical separation from other cellular constituents. This latter view would regard the many particulate bodies of the cell as being more or less specialized for carrying out reactions which are essential to the overall metabolic balance of the cell. Disruption of the cell and isolation of one component, such as the chloroplast, would eliminate the interactions between cellular components present in the intact cell.

The present investigation was undertaken in an attempt to obtain experimental evidence for such interactions between various parts of a cell. The cellular components of spinach leaves were separated into three fractions (the chloroplasts, the mitochondrial fraction, and the fresh sap), and these fractions were tested, both singly and in combination, for uptake and distribution of radiocarbon and also for assimilation of various carbon-14 labelled substrates. This experiment may be viewed as an attempt to reconstruct the biochemical

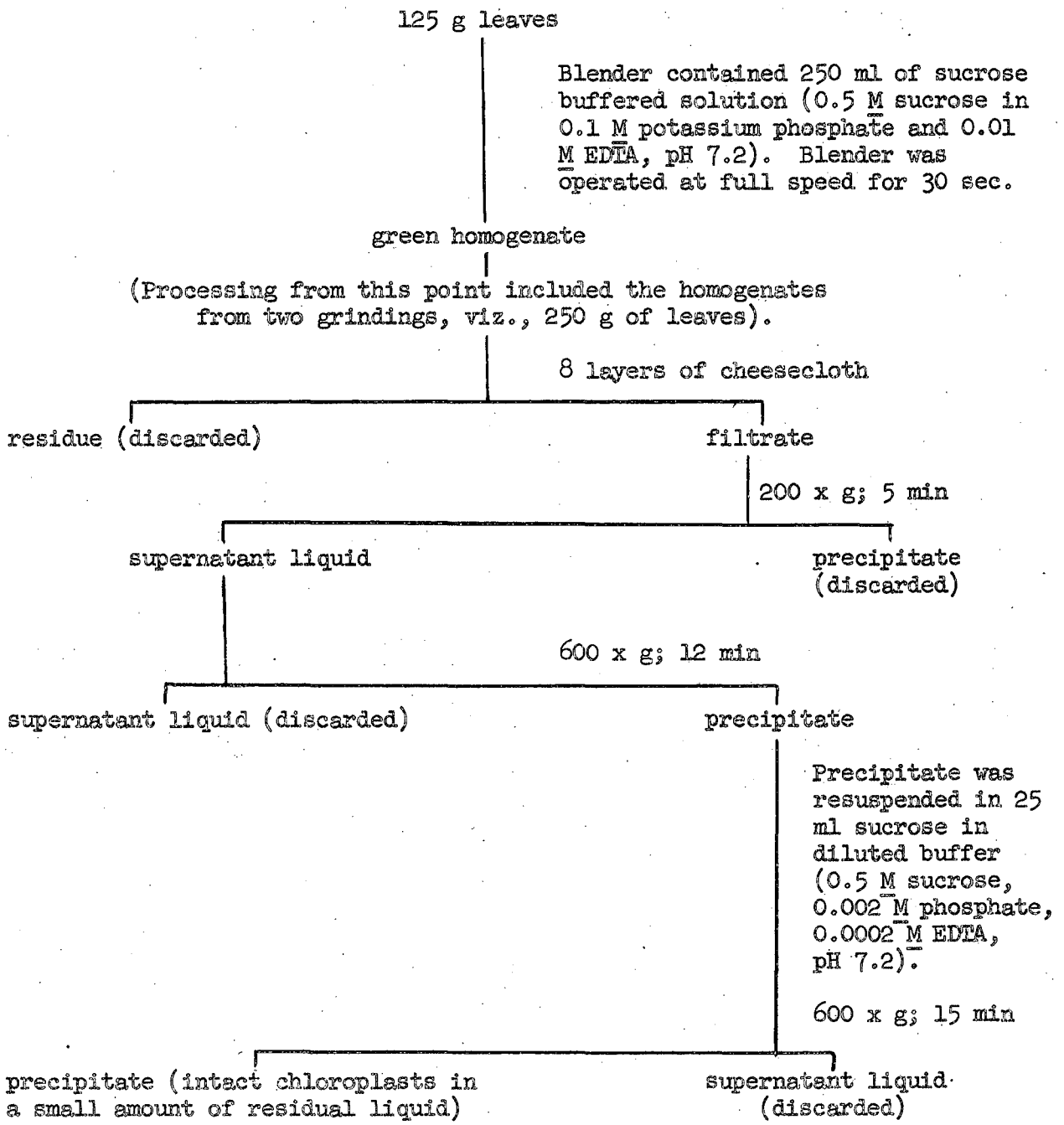
environment of the inside of a cell after various components of that cell have been separated and separately studied.

EXPERIMENTAL PROCEDURE

The following abbreviations are used in this paper: EDTA, ethylenediamine tetra-acetic acid; ADP, adenosine diphosphate; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; TPP, thiamine pyrophosphate; RuDP, ribulose-1,5-diphosphate; PGA, phosphoglyceric acid; FMN, flavin mononucleotide; UDPG, uridine diphosphoglucose; PEP, phosphoenolpyruvate.

Spinach leaves (*Spinacea oleracea*), obtained as fresh as possible from a commercial source, were used to prepare all fractions. For the preparation of whole chloroplasts, mitochondria and sap, the petioles were removed and the leaves were weighed. The leaves were washed thoroughly in cold tap water and drained as dry as possible. For the chloroplast preparation, the leaves were drained dry by "tumbling" in a wire basket, while for the preparation of sap and mitochondria the leaves were further dried by blotting with paper towels. All subsequent operations were carried out at, or near, 0°C. All glassware and centrifugation apparatus were precooled to about 0°C unless otherwise noted.

Preparation of intact chloroplasts: Two hundred and fifty grams of leaves were used for each experiment. Grinding was performed in two batches, the leaves being cut into strips 0.5 cm wide. All centrifugations were performed in an International Portable Refrigerated Centrifuge, Model PR-2. The flow diagram for a typical preparation is given below:



The material was resuspended in the residual liquid with the aid of a Potter-Elvehjem homogenizer equipped with a Teflon* pestle (Suspension C).

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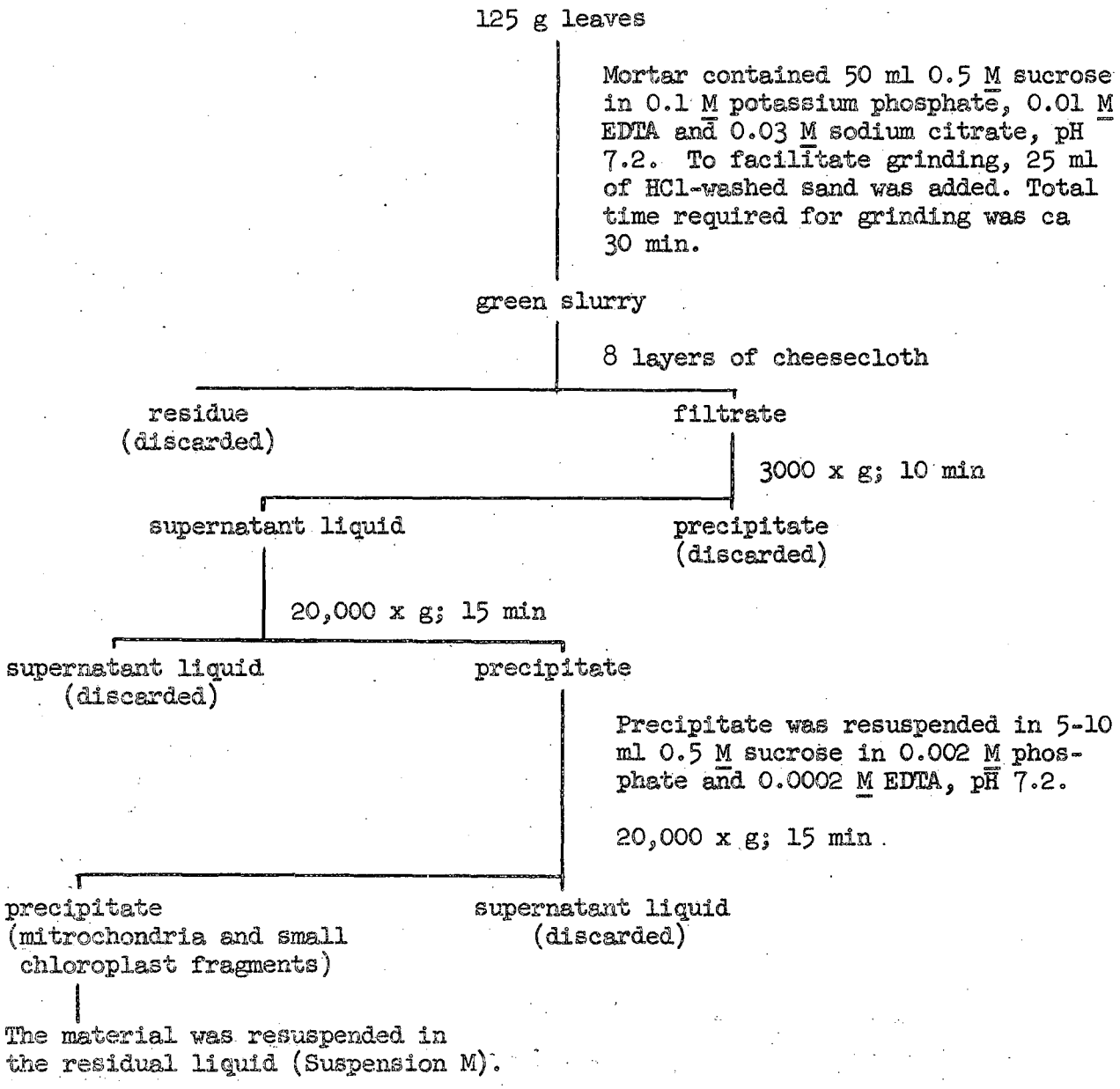
*Teflon is duPont's trademark for its polyfluorohydrocarbon, tetrafluoroethylene.

Preparation of the mitochondrial fraction: The leaves were cut into small pieces (ca 0.5 cm square) prior to homogenization. Low speed and high speed centrifugations were performed, respectively, in an International Portable Refrigerated Centrifuge, Model PR-2 and a Spinco Ultracentrifuge, Model L. The procedure is shown in the following flow diagram:



Preparation of sap: Centrifugations were performed at low and high speeds, respectively, in an International Portable Refrigerated Centrifuge, Model PR-2, and a Spinco Ultracentrifuge, Model L, equipped with a No. 40 rotor. The leaves were cut into small pieces (ca 2 cm square) prior to the following treatment:

150-350 g leaves were placed in the center of five layers of cheesecloth and the cheesecloth was drawn into a bag.

The bag was dipped into liquid nitrogen for 30-60 sec.

frozen leaves

The leaves were crushed with several blows of a hammer while still wrapped in the cheesecloth.

crushed leaves

The leaves were placed in a precooled blender (-15°C). The blender was operated at full speed for 30 sec.

fine powder

The powder was poured into a beaker and stirred constantly until a slurry was obtained. Approximately 30 min was required, and during this time the temperature of the mixture was never allowed to rise above 1-2°C.

green slurry

1,500 x g; 10 min

- precipitate and crust (discarded)
- supernatant liquid

20,000 x g; 15 min

- clear supernatant liquid, greenish yellow. Yield: approximately 10 ml of sap from 50 g leaves.
- precipitate (discarded)

Further treatment of the clear supernatant liquid (sap): A suitable aliquot portion was set aside for the preparation of boiled sap (see below). Another 5 ml portion was adjusted to pH 7.2 at 25°C with 1 N KOH (taking careful note of the volume required). The rest of the supernatant liquid was maintained at 0°C and the pH was adjusted by the addition of a calculated amount of 1 N KOH using the volume determined in the previous step. (This operation was found necessary because the sap pH had a large temperature coefficient; thus, the pH of the sap if adjusted to 7.2 at 0°C would drop to 6.8 when the sap was allowed to warm to room temperature.) The above adjusted solution constituted Solution S.

Preparation of the boiled sap: The sap was heated in a boiling water bath for 5 min. The precipitate was removed by centrifugation and the clear, yellow supernatant liquid was allowed to cool to 25°C before the pH was adjusted to 7.2 (Solution S(b)).

Photosynthesis and dark fixation experiments: The apparatus used for the photosynthesis studies was described by Moses and Calvin (in press). The temperature at which the experiments were carried out was about 18°C; the light intensity was 2,000 f.c. The vessels employed for the dark reaction studies were 10 ml Erlemeyer flasks. Each flask was taped thoroughly with opaque, black adhesive tape and fitted with a rubber stopper. To insure further that no light leaked into the container through the lip of the flask, an aluminum cap was placed over the stopper and the lip. The temperature of the dark fixation vessels was about 25°C.

Loading the reaction vessels: Appropriate volumes of the four mixtures (C, S, M and S (b)) were added, singly and in combination, to each reaction vessel. The total volume in each case amounted to 1.0 ml. The exact volume

of each solution or suspension is listed in the RESULTS section under the various tables. Furthermore, except where otherwise noted, 0.05 ml of a cofactor solution was added to each flask. This solution contained, in a 0.05 ml aliquot portion, the following materials (values are in μmoles): $MgSO_4$, 4; $MnCl_2$, 1.6; ADP, 1; TPN, 0.02; DPN, 0.2; TPP, 1.4; and ascorbic acid, 2.5. Some reaction vessels contained other substrates. These additions were generally limited to 0.1 ml or less and are described in the RESULTS section. The reaction was started by the addition of 0.1 ml of $NaHC^{14}O_3$ (0.0438 M, 1 mc/ml), except in those cases where other radioactive substrates were added. The pH of all solutions (cofactors and substrates) was adjusted to 7.2 just prior to use. Incubation was generally carried out for 30 min. The reaction was stopped by the addition to each flask of 4 ml of absolute alcohol at room temperature; the final concentration of alcohol was thus 80%.

Chromatography and identification of products: The 80% ethanolic suspension was centrifuged and the precipitate was extracted once at 50°C with 3-4 ml of 20% ethanol. The extracts were combined and a suitable aliquot portion (200-500 μl) was subjected to chromatography. Chromatography was carried out on oxalic acid washed Whatman No. 4 paper with phenol-water in the first dimension and butanol-propionic acid-water in the second dimension (Benson, 1955).

The radioactive areas on the chromatograms were located by exposing the paper to duPont X-ray film type 507E. Counting of C^{14} on the paper was accomplished with a Geiger-Mueller tube fitted with a "Mylar" window, and flushed continuously with Q-gas (99.05% He and 0.95% isobutane)(Hayes, 1955). For plate counting, a Nuclear-Chicago automatic counter was employed. Duplicate planchets were prepared, drying the sample together with a drop of 6 N acetic acid. The samples were spread on the aluminum plates using a rotating table and dried with a blower (Calvin, 1949). Preliminary identification of labelled compounds on the paper

was established by their R_f values. Confirmation was achieved by elution of the compound and rechromatography with authentic marker substances. If the compound was in the organic phosphate area of the chromatogram, it was also subjected to dephosphorylation with acid prostatic phosphatase (Schmidt, 1955) and rechromatographed with authentic markers. Exact coincidence between the labelled substance and the marker was taken as the criterion of identity.

Phosphate esters were detected by spraying the paper with the reagent described by Hanes and Isherwood (1949), drying in an oven with a forced air draft at about 60°C and then exposing to sunlight. Amino acids were sprayed with 0.1% (w/v) ninhydrin in 95% (w/v) ethanol and the chromatograms were heated at 100°C for 5 min. Organic acids were sprayed with an 0.04% (w/v) ethanolic solution of bromocresol green, made just alkaline with a little NH_3. Sugars and glyceric acid were detected by dipping the chromatograms into a solution of $AgNO_3$ in acetone, allowing them to dry, and then spraying them with ethanolic NaOH (Trevelyan, 1950). After development of the spots, the excess $AgNO_3$ was washed off with a dilute ammonia solution and the papers were dried. Nucleotides and nucleosides were located by UV absorption on the paper. Those chromatograms of extracts of experiments involving sap generally had enough amino acids and reducing compounds to constitute useful markers for the spray tests.

Chlorophyll determination: Chlorophyll determinations were carried out on the various fractions which contained green material. The method consisted of extracting the material with 80% acetone, clarifying the acetonic solution by centrifugation and reading the supernatant liquid in a spectrophotometer in the wavelength range from 640 mμ to 700 mμ. The details of this procedure are described by Arnon (1949).

For the determination of chlorophyll in the intact leaves, it was found that not all of the green material was extracted by acetone. In this case ethanol was

substituted and heat (steam bath) was applied to hasten the extraction. The concentrated alcoholic solution obtained in this manner was diluted in 80% acetone and treated as indicated above.

Protein determination: The protein content of a sample was determined colorimetrically on the basis of a combination of colors from the biuret reaction and the tyrosine-Folin color reaction. The method is described by Lowry, (1951). The reference protein used to set up the standard curve was obtained by dilute phosphate buffer extraction of the chloroplasts. This extract was subjected to exhaustive dialysis followed by protein determination on a weight basis.

RESULTS

While both the chloroplast and the sap preparations were able to fix carbon dioxide alone, the most striking effect noticed in these experiments on the activity of the chloroplasts was caused by the addition of the cell sap fraction. This is illustrated by the data shown in Table I, from which it can be seen that chloroplasts suspended in sucrose fixed 7.3×10^6 dpm (disintegrations per minute C^{14}), sap fixed 1.5×10^6 dpm, and the combination of chloroplasts and sap fixed 27×10^6 dpm. The addition of the sap thus brought about a fixation of radioactive carbon that was over three times that to be expected from a simple addition of the fixation of each component separately. The distribution of the fixed carbon into the alcohol-soluble compounds is also shown in Table I, and, in addition, representative radioautograms are shown in Figure I.

The distribution of the radiocarbon fixed by the chloroplasts suspended in sucrose was very different from that fixed by the sap fraction alone. The former showed over 70% of the fixed soluble radioactivity in the sugar phosphates and only 15% in the amino acids, while the sap fixed close to 50% of the carbon-14 in the amino acids and only 33% in the sugar phosphates. The percentage of

activity in the sugar phosphates from the sap fixation (33%) was higher than is usually observed in dark fixation experiments by photosynthetic organisms or in CO_2 fixation by nonphotosynthetic organisms (Moses, in press). This may indicate either that labelled aspartic acid was being converted to PGA, giving rise to labelled PGA by a nonphotosynthetic route, or that some of the chlorophyll which was still found in this fraction may have mediated the fixation of some carbon dioxide via a photosynthetic mechanism. The latter possibility is unlikely in view of the results obtained by the addition of the mitochondrial fraction to the sap, as the amount of chlorophyll present in the mitochondrial fraction was greater than that in the sap alone. Neither the total uptake of CO_2 nor the distribution of the radioactivity shown by the sap fraction was altered by the addition of the mitochondrial fraction.

The increase in fixation upon adding the sap fraction to chloroplasts was not caused by an inorganic constituent, as boiling the sap for 5 min destroyed its ability to increase the total fixation of labelled carbon dioxide when added to the chloroplast suspension (Table I). The most noticeable difference between the effects of fresh and boiled sap on the distribution of C^{14} fixed by the chloroplasts, was in the relative amounts found in the sugar phosphates and amino aicds. With chloroplasts and fresh sap some 71% of the total soluble radioactivity was present in the phosphates, whereas chloroplasts and boiled sap fixed 93% of the soluble radioactivity into these compounds, with PGA alone accounting for 42%.

The mitochondrial fraction showed very little fixation of CO_2 (0.4% that of the chloroplast fraction) with the fixed radioactivity being restricted mainly to PGA and aspartic acid (Table I). This fraction did not alter the distribution of radioactivity when added to chloroplasts or sap, but did consistently depress

the total amount of radioactive carbon dioxide fixed by the chloroplast-sap mixture. The reason for the decrease in the rate of carbon dioxide fixed by the chloroplast-sap mixture following the addition of mitochondria is not clear. The effect may represent a physical phenomena of surface adsorption of some required enzyme or reactant on the mitochondria.

The omission of the cofactor supplement resulted in a marked decrease in the total fixation of CO_2 by the chloroplast-sap mixture. The amount of radiocarbon fixed was only 14% of that fixed by the chloroplast and sap mixture when the cofactor supplement had been added (Table I). The effects of the individual components of this cofactor supplement were not separately studied, and it is therefore not possible to state which of the substances in the cofactor mixture was responsible for the large stimulation of fixation.

That the fixation of CO_2 in these experiments does actually represent a photosynthetic uptake of CO_2 and not merely dark reactions is shown by the low total fixation of C^{14} by the combination of chloroplasts, sap and mitochondria when incubated with labeled CO_2 in the dark for 30 minutes (Table I). Over 86% of the fixed soluble radioactivity was incorporated into aspartic acid and malic acid. These two compounds characteristically incorporate radioactive carbon dioxide via well known dark reaction mechanisms (Vishniac, 1957; Meister, 1957). In another experiment, using chloroplast-sap mixture the fixation of carbon dioxide in the light was some 25 times greater than that in the dark.

In order to obtain more information on the effects of sap on the light fixation of carbon dioxide by chloroplasts, the following two experiments were performed: (1) the fixation of CO_2 by the chloroplast and sap mixture was measured at 1, 3, 10 and 30 min after the introduction of the radioactive bicarbonate, and (2) the effect of varying the amount of sap was determined for fixation times of 30 min. The results from the kinetic experiment are shown

in Table II and Figures 2a and 2b together with radioautograms in Figure 3. The fixation of CO_2 by this system was approximately linear with time, and PGA was the dominant radioactive material, especially for the shorter exposure periods to radioactive carbon dioxide. A plot of the radioactivity with time in each compound as a percentage of the total soluble radioactivity shows that PGA and the diphosphate area both had negative slopes, but of these the PGA contained the most activity, having 61% of the soluble activity after one minute of fixation time (Figure 2b). When the amount of sap which was added to the chloroplast suspension was varied, the total fixation was directly proportional to the amount of sap added (Figure 2c).

Except in the 30 minute dark fixation (Table I), no labelled glutamic acid was found in these experiments. It was therefore of interest to see if radioactive carbon would be incorporated into glutamic acid in the dark period following a period of photosynthesis with radioactive CO_2, as has been reported for Chlorella (Bassham, 1956). The results (Table III) show that no radioactive glutamic acid was discernible in the chloroplast and sap mixture after 5 min photosynthesis with radioactive CO_2, followed by a 5 min period of darkness.

The chloroplast-sap mixture did metabolize labelled RuDP. However, since the labelled RuDP was itself contaminated with a variety of other labelled compounds normally found in algae, it was impossible to make a qualitative estimation of the fate of the labelled RuDP. Attempts were made to determine whether RuDP was metabolized by the sap alone or the chloroplasts alone (Table IV). Addition of unlabelled RuDP to a chloroplast suspension in the dark resulted in an increase fixation of labelled carbon dioxide of about 800,000 dpm over that of the dark fixation of a chloroplast suspension in the basence of RuDP. The most pronounced effect on the distribution of fixed carbon-14 following the addition of unlabelled RuDP was that most of the increase in C^{14} fixed was accounted for by an increase

in labelled PGA.

Neither the sap fraction nor the chloroplasts were able to metabolize labelled PGA very rapidly (Table III). The sap fraction did show incorporation of carbon-14 into PEP, glyceric acid, alanine and citric acid. Metabolism of labelled PGA by the chloroplasts was slow, and small amounts of radioactivity only, were found in the hexose monophosphates, maltose, and glycolic acid.

Studies with citric-1,5-C^{14} as a substrate for Krebs cycle activity showed the formation of glutamic, α-ketoglutaric, and other acids by sap, but not by chloroplasts.

In several experiments with the chloroplasts and sap mixture at pH 6.1, there was a marked decrease in the total fixation of CO_2 to about 1% of that obtained at the pH 7.2. The pattern of carbon-14 distribution at pH 6.1 was also very different than that found at pH 7.2 (Figure 4). In this experiment the alcohol-soluble compounds were separated by use of exchange resins into a neutral fraction and an ionized fraction. The neutral fraction, consisting largely of glucose, fructose, sucrose, maltose, and various polysaccharides of glucose, comprised about 50% of the total fixed radioactivity. The ionized fraction, which consisted mostly of the phosphorylated compounds, amino acids and organic acids, contained the remaining 50% of the fixed activity. When the experiment was performed at pH 7.2, the free sugars (corresponding to the neutral fraction in the above experiment contained only 2-3% of the total fixed radioactivity.

Another striking effect caused by lowering the pH of the chloroplast suspension from 7.2 to 5.0 concerned the ability of these particles to carry out transamination. This effect was tested by incubating the sap or chloroplasts fractions with pyruvic acid-3-C^{14} and unlabelled alanine. At pH 7.2, the chloroplasts showed strong transaminase activity as witnessed by the large amount

of radioactivity incorporated into the alanine, whereas at pH 5.0 no radioactivity was evident in alanine. The sap exhibited strong transamination at pH values of 7.2 and 6.1.

DISCUSSION

Although both the chloroplast and sap fractions were very active in assimilating carbon dioxide, especially when mixed together, the mitochondrial fraction had little effect both on the rate of carbon dioxide fixation (a slight depressant effect was noted) and on the distribution of the fixed carbon. This mitochondrial fraction, which was defined as that particulate material which centrifuged down between 3000xg and 20,000xg, most certainly was not a pure preparation of mitochondria, but contained a mixture of particulate matter. This fraction was tested manometrically for oxygen uptake upon addition of a Krebs cycle intermediate (citric acid), and it was found to be active to some degree but not to the extent which would be expected from reported data on mitochondrial activity (Ohmura, 1955). The activity of this fraction was also tested by the addition of labelled citric acid. Its ability to carry on transamination was also tested by addition of labelled pyruvic acid and unlabelled alanine. In both cases there was little or no conversion of the added substrates by the mitochondria.

It is thus apparent that this fraction must have lost activity during the isolation procedure, and the largely negative results obtained upon addition of it to samples of chloroplasts and sap must be interpreted with caution. The tendency of mitochondria to depress the total fixation of CO_2 may represent a physical adsorption of some factor or enzyme by the particulate matter in the mitochondrial fraction. In studies such as those reported in the present communication, it would be of great value to be able to obtain mitochondria

uncontaminated by green chloroplast fragments, and to be able to demonstrate manometrically that the particles possessed strong citric acid cycle activity. Until this is done, it is hard to extrapolate from these experiments to the possible interaction of these particles with the chloroplasts in the intact cell.

The most important observation that emerges from the data is the strong stimulating effect on the total fixation of CO_2 obtained by adding fresh sap to the isolated chloroplasts. This effect is not explicable, based on the knowledge available to us today, in terms of deficiencies of essential cofactors or in terms of osmotic or pH effects, but rather by some catalytic interaction of unknown nature between the two preparations.

The cofactor supplement which was added contained the factors which are commonly accepted as showing a stimulation of chloroplast activity, with the possible exception of a flavin derivative and Vitamin K. It is unlikely that either a flavin or Vitamin K was responsible for the observed effect because they would be expected to resist heating to 100°C for 5 minutes. In fact, it was shown that boiling the sap destroyed its ability to stimulate chloroplast activity. From the knowledge that the rate of carbon dioxide fixation by a chloroplast-sap mixture remained approximately linear for 30 min, and that the stimulation of chloroplast activity by sap was directly proportional to the amount of sap added, it appears unlikely that the sap was merely providing some metabolite or reactant which is necessary for chloroplast activity unless this reactant were of a catalytic nature. It is possible to conceive of the sap providing a heat-labile cofactor or enzyme which can partially overcome a bottleneck, limiting the rate of entry of CO_2 into the photosynthetic cycle.

This could be visualized in one of the following three ways. First, the

sap may be adding a substance which serves as an activator of carbon dioxide, the activated carbon dioxide being the natural substrate for the carboxylation of ribulose diphosphate rather than bicarbonate ion or dissolved carbon dioxide. It is known that the turnover rate of carboxydismutase, when measured after extraction from the cells, is too low to account for the rate at which it must be turning over in the intact cell if the photosynthetically-fixed carbon dioxide is indeed being mediated by this enzyme (see Table V). One of the hypotheses that has been suggested to explain this is that in the in vitro experiments with the enzyme, free CO_2 or bicarbonate has been added as the substrate, and in the intact cell there may be some 'active CO_2' complex which serves as the substrate for the carboxylation of ribulose diphosphate. (Flavin, Castro-Mendoza and Ochoa, 1956; Bachhawat, Woessner and Coon, 1956; Metzner, Metzner and Calvin, 1958).

The second way in which the sap could be stimulating the fixation of CO_2 by the chloroplasts would be if it provided some factor which increased photosynthetic phosphorylation. In the photosynthetic cycle there are two reactions which require high-energy phosphate: the reduction of PGA and the phosphorylation of ribulose monophosphate to RuDP. The present date do not permit a firm decision between these two hypotheses, but the distribution of radioactivity would speak more strongly for the latter alternative. When the diphosphate area was treated with acid phosphatase, most of the radioactivity was found in the fructose and glucose, with about 10-15% in ribulose. If the carbon dioxide substrate were limiting the rate of entry of CO_2 into the cycle, it would be expected that RuDP would accumulate and be the major constituent of the diphosphate area. This is actually found in Chlorella or Scenedesmus under low CO_2 pressure (Wilson and Calvin, 1955). If, on the other hand, the level of ATP were the limiting factor, one would expect the reduction of PGA to triose to proceed

slowly and the amount of labelled RuDP to be low. Both of these circumstances were found in the chloroplast preparations. It should be recalled here that photosynthetic phosphorylation proceeds at a rapid rate in isolated chloroplasts (pH 8; see Table VII) (Allen, Whatley and Arnon, 1958). It is quite possible, however, that photosynthetic phosphorylation was limiting in this case because of pH differences or the lack of some factor. With this possibility in mind, a third alternative must be considered, namely, that the rate of formation of reduced pyridine nucleotide was the limiting factor.

Using the value of 3.0 μmoles CO_2 fixed/min photosynthesis/mg chlorophyll for Chlorella, the spinach chloroplast preparations fixed CO_2 at a maximum rate about 1% that of Chlorella when compared on a mg chlorophyll basis. The actual amount of CO_2 fixed by the spinach chloroplasts was calculated from the specific activity of the bicarbonate introduced and the total uptake of radiocarbon (Table VI).

The question can then be raised as to what is limiting the ability of the chloroplasts to fix and reduce CO_2. It has been seen that the sap, which increased the total fixation markedly, did not significantly alter the pattern of carbon-14 distribution. This may be interpreted to mean that whatever was limiting the ability of the chloroplasts to fix CO_2 before addition of the sap was still operative, even after addition of the sap. If the addition of the sap removed one limitation completely, the new limitation would most likely cause some shift in the incorporation patterns. It is evident that in disrupting the cells and recombining only the chloroplast and sap fractions, not only is the overall organization of the cell destroyed but there is a possibility that the essential cofactors, etc. may have been leached out of the chloroplasts during separation. To obtain more definitive answers as to the nature of the decrease

in photosynthetic ability, it would be helpful to have data from experiments in which the particles were isolated in various ways. It would also be of interest to know the photosynthetic ability of a mash of spinach leaves when the cells were all disrupted but before the actual separation of any components.

Fager (1952) has actually performed experiments with macerates of spinach leaves. Unvortunately, the specific activity of the radioactive carbonate used by him is unknown and therefore it is not possible to calculate the rate of carbon dioxide fixation of the preparation. The experiments, however, brought out two significant facts: (i) the separated chloroplast material containing chloroplasts, grana, etc. did not fix any carbon dioxide, but when this chloroplast material was recombined with the cell sap, the mixture then exhibited the same CO_2 fixing ability as did the complete leaf macerate; (ii) up to 60% of the radioactive tracer was incorporated by this macerate in the PGA fraction.

It should be pointed out that the question of transport of 'active CO_2' through the cytoplasm to the chloroplast remains undecided. Also unsettled is the question as to how much of the products of photosynthesis leaves the chloroplast during optimal conditions for photosynthesis, and the nature of the molecules able to enter and leave the chloroplasts. It is thus possible to conceive of the chloroplast as having limited biosynthetic capabilities, necessitating exchange of substances between the chloroplast and other biosynthetic regions of the cell.

Upon examination of the data showing the distribution of radioactive carbon by chloroplasts with and without the presence of sap, it appears that the sap affected to some degree the conversion of the intermediates involved in the photosynthetic cycle to such other metabolites as amino acids and organic acids. It is possible that this effect of the sap is accomplished by a component of the

sap which can enter the chloroplast, but it is more likely that some component in the chloroplast is moving out of the chloroplast and undergoing further conversion in the sap.

It is interesting to note the presence of some labelled phosphogluconic acid in the product of photosynthesis by both chloroplasts alone as well as by the chloroplast-sap combination, (Figures 1a and 1c). This suggests that the pentose cycle for the oxidation of glucose is operating in these preparations and might contribute (Wood, 1955) to the redistribution of the label originally present in carbon atom three into carbon atoms one and two of glucose (Gibbs and Cynkin, 1958).

The distribution of the fixed radiocarbon by the chloroplast-sap mixture was sensitive to pH change. When the pH was changed from 7.2 to 6.1, much more radioactivity was incorporated into the free sugars and oligosaccharides. The same observation has been made by Ouellet (1952) with intact Scenedesmus. His results showed that the lower the pH (down to pH 1.6), the greater was the incorporation rate of radiocarbon into sucrose and polysaccharide material. Since the pH optima of the enzyme systems in plants are not known, no definite correlations can be made between the observed patterns and the pH sensitivity of specific enzymes.

These attemps to 'reconstruct' the biochemical environment of a spinach leaf have been limited by inactivity of the mitochondrial fraction and have so far led to a limiting rate of photosynthesis which is only 1% of that found in the intact leaf. The reason for this reduction in photosynthetic ability upon disrupting the cell and isolating the chloroplasts is not known, but the fact that addition of sap increased the photosynthetic rate more than three times offers some opportunity for further experimentation on this problem.

Addition: Since the completion of the manuscript, Gibbs and Cynkin (1958 a) have reported a CO_2 fixation rate of 0.033 μmole/min/mg chlorophyll by spinach chloroplasts. On this basis, the maximum rate of CO_2 fixed by intact spinach chloroplasts is only about 1% that of the maximum CO_2 fixation rate by intact Chlorella.

Summary

Cellular constituents (chloroplasts, sap and mitochondria), separated from spinach leaves, were allowed to photosynthesize in the presence of radioactive bicarbonate. The radiocarbon fixed by the chloroplasts was distributed mainly in the phosphate esters while the radiocarbon fixed by the sap was located mostly in the amino acids. The mitochondrial fraction fixed very little radioactive carbon dioxide. The rate of carbon dioxide fixation by the recombined mixture of sap and chloroplasts was greater than the sum of the separated components. The distribution pattern of the fixed radiocarbon of the recombined mixture, however, was qualitatively similar to a combination of the radiocarbon distribution of the separated chloroplasts and sap. The results of experiments carried out with these cellular constituents in the dark and in the presence of other substrates, labelled and unlabelled, are discussed.

The authors are indebted to Professor H. A. Barker for his supply of acid prostatic phosphatase.

TABLE I

Total Uptake and Distribution of Carbon-14 from Labelled Carbon Dioxide by Various Combinations of Cellular Constituents of Spinach

Reaction Mixture	C		S		M		C + S	
Reaction Conditions	30 min light		30 min light		30 min light		30 min light	
Disintegrations per minute	7.3×10^6		1.5×10^6		0.05×10^6		27×10^6	
Compound	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%
Diphosphates	17	3.4	--	-	--	-	140	8.4
UDPG	23	4.9	0.4	0.5	--	-	20	1.2
Monophosphates	210	46	2.8	3.1	--	-	420	25
PGA	78	17	21	23	1.0	59	490	29
PEP	--	-	5.2	5.8	--	-	100	5.9
$\sum$ Phosphates	330	72	29	33	1.0	59	1200	69
Aspartic acid	21	4.5	43	48	0.7	42	210	12
Alanine	8.6	1.8	1.1	1.2	--	-	69	4.1
Glutamic acid	--	-	--	-	--	-	--	-
Glycine	36	7.8	--	-	--	-	19	1.1
$\sum$ Amino acids	66	14	44	49	0.7	42	310	18
Fructose	4.0	0.9	--	-	--	-	21	1.2
Glucose	P	P	--	-	--	-	27	1.5
Sucrose	--	-	--	-	--	-	6.8	0.4
Maltose	11	2.3	--	-	--	-	11	0.6
$\sum$ Free sugars	15	3.2	--	-	--	-	58	3.8
Glyceric acid	--	-	2.1	2.3	--	-	59	3.5
Malic acid	19	4.0	13	15	--	-	74	4.4
$\sum$ Organic acids	52	11	16	18	--	-	160	9.3

TABLE I
(Continued)

Reaction mixture	C + M + S		C + S (b)		C + S (no cofactors)		C + M + S	
Reaction conditions	30 min light		30 min light		30 min light		30 min dark	
Disintegrations per minute	16×10^6		7.7×10^6		2.3×10^6		2.5×10^6	
Compound	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%
Diphosphates	15	1.8	48	12	0.8	0.6	--	-
UDPG	6.1	0.7	--	-	--	-	0.3	0.2
Monophosphates	180	22	160	39	44	31	2.3	1.7
PGA	270	33	170	42	36	25	0.8	0.6
PEP	62	7.5	2	0.5	7.7	5.5	1.0	0.8
$\sum$ Phosphates	550	67	380	93	89	62	4.5	3.3
Aspartic acid	110	14	5.2	1.3	6.8	4.8	84	62
Alanine	36	4.3	5.8	1.4	12	8.3	2.2	1.6
Glutamic acid	--	-	--	-	--	-	0.3	0.2
Glycine	35	4.3	7.4	1.8	11	8.2	8.7	6.3
$\sum$ Amino acid	180	22	22	5.2	33	23	95	70
Fructose	--	-	--	-	2.4	1.6	--	-
Glucose	P	P	P	P	--	-	--	-
Sucrose	--	-	--	-	--	-	--	-
Maltose	--	-	--	-	2.6	1.8	--	-
$\sum$ Free sugars	--	-	--	-	4.9	3.4	--	-
Glyceric acid	30	3.6	--	-	11	8.2	3.4	2.5
Malic acid	54	6.6	4.9	1.2	4.5	3.2	34	25
$\sum$ Organic acids	90	11	9.0	2.2	16	11	37	27

Key to Table I:

C: Suspension C, 0.1 ml (0.61 mg chlorophyll and 8.9 mg protein) plus 0.9 ml 0.5 M sucrose buffered at pH 7.2.

S: Solution S, 1.0 ml (0.03 mg chlorophyll and 27.4 mg protein).

M: Suspension M, 0.1 ml (0.44 mg chlorophyll and 5.2 mg protein) plus 0.9 ml 0.5 M sucrose buffered at pH 7.2.

C + S: Suspension C, 0.1 ml plus 0.9 ml Solution S. (This corresponds to between 1.2 and 5.0 times the ratio of sap to chlorophyll which may be in the leaves. The uncertainty lies in the amount of sap in the leaf.)

C + M + S: Suspension C, 0.1 ml plus 0.1 ml suspension M plus 0.8 ml Solution S.

C + S (b): Suspension C, 0.1 ml plus 0.9 ml Solution S (b) (9.9 mg protein).

All other details are described in the Experimental Procedure section.

Disintegrations per minute: This represents the total C^{14} fixed in the alcohol-soluble portion.

cpm: Counts per minute on the paper chromatogram calculated for the entire sample (uncorrected for geometry, self-absorption and coincidence).

%: Percent of the total soluble activity calculated by the summation of all the radioactive spots on the chromatogram.

Phosphates: Sum of the listed phosphates, including those not listed (phosphoglycolic acid and triose phosphate).

Amino acids: Sum of the listed amino acids, including those not listed (serine, citrulline, valine, glutamine and other unidentified ninhydrin-positive spots).

Free sugars: Sum of the listed sugars only.

Organic acids: Sum of the listed acids including citric acid, pyruvic acid, glycolic acid, succinic acid and fumaric acid.

P: Present, but not separated from glycine and therefore counted together with glycine.

TABLE II

Total Uptake and Distribution of Carbon-14 by Chloroplast-Sap Mixture after Varying Times of Exposure to Radioactive Bicarbonate

	Exposure Time to Radioactive Bicarbonate							
	1 min		3 min		10 min		30 min	
Disintegrations per minute	0.37×10^6		1.5×10^6		6.9×10^6		29×10^6	
Compound	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%
Diphosphates	2.3	13	4.4	6.2	12	3.9	43	3.1
UDPG	-	-	-	-	2.4	0.8	17	1.2
Monophosphates	2.0	11	10	14	38	13	280	21
PGA	10	61	41	58	170	57	500	36
PEP	0.3	1.8	2.4	3.4	20	6.7	110	7.9
$\sum$ Phosphates	15	88	58	81	240	81	950	69
Aspartic acid	1.4	7.9	5.2	7.3	18	6.1	180	13
Alanine	-	-	1.1	1.5	3.4	1.1	48	3.5
Glycine	-	-	1.0	1.4	4.2	1.4	45	3.3
$\sum$ Amino acids	1.4	7.9	8.6	12	38	13	310	22
Sucrose	-	-	-	-	-	-	10	0.8
$\sum$ Free sugars	-	-	-	-	-	-	10	0.8
Glyceric acid	-	-	1.5	2.1	5.4	1.8	44	3.2
Malic acid	0.7	4.2	3.0	4.2	13	4.2	42	3.0
$\sum$ Organic acids	0.7	4.2	4.8	6.7	21	6.9	100	7.6

Key to Table II:

Chloroplast-sap mixture: Suspension C, 0.1 ml (0.68 mg chlorophyll and 9.2 mg protein) plus 0.9 ml solution S (0.03 mg chlorophyll and 17 mg protein). The ratio of sap to chlorophyll lies between 4.3 and 1.0 that of the ratio which may be present in leaves.

Disintegrations per minute: Total C^{14} fixed including both the alcohol soluble and insoluble fractions.

Explanations for the rest of the descriptions are given under Table I.

TABLE III

Uptake and Distribution of Radioactive Carbon by Sap and Chloroplasts under Various Conditions

Reaction Mixture	C + S		C + PGA-C^{14}		S + PGA-C^{14}	
Conditions	5 min light, then 5 min dark		30 min light		30 min light	
Disintegrations per minute	4.0×10^6					
Compound	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%
Diphosphates	0.8	0.4	-	-	-	-
UDPG	-	-	-	-	-	-
Monophosphates	15	8.3	20	13	-	-
PGA	89	50	130	83	190	76
PEP	14	8.1	-	-	28	11
$\sum$ Phosphates	120	67	150	98	220	87
Aspartic acid	22	12	-	-	-	-
Alanine	9.6	5.0	-	-	9.1	3.6
Glycine	3.3	1.9	-	-	-	-
$\sum$ Amino acids	42	23	-	-	9.1	3.6
Maltose			2.6	1.6	-	-
$\sum$ Free sugars	-	-	2.6	1.6	-	-
Glyceric acid	3.6	2.0	-	-	24	9.6
Malic acid	11	6.4	-	-	-	-
Organic acids	17	9.8	1.0	0.7	25	10

Key to Table III:

C + S: This chloroplast-sap mixture is identical to that in Table II.

C + PGA-C^{14}: Suspension C, 0.1 ml (0.68 mg chlorophyll and 9.2 mg protein) plus carboxyl labelled PGA prepared by reacting RuDP with $NaHC^{14}O_3$ in the presence of carboxydismutase (Mayaudon, 1957). The concentration of the PGA used in this experiment was unknown. Unlabelled bicarbonate was added in place of radioactive bicarbonate.

S + PGA-C^{14}: Solution S, 1.0 ml (0.03 mg chlorophyll and 19 mg protein) + PGA-C^{14}.

Disintegrations per minute: Total C^{14} fixed in the alcohol soluble fraction only.

Explanations for the rest of the descriptions are given in Table I. Other details of the experiment are described in the Experimental Procedures section.

TABLE IV

The Effect of Added RuDP on the Fixation of $C^{14}O_2$ by Chloroplasts in the Dark

Reaction Mixture	C		C + RuDP	
Conditions	30 min dark		30 min dark	
Disintegrations per minute	0.43×10^6		1.2×10^6	
Compound	cpm $\times 10^{-3}$	%	cpm $\times 10^{-3}$	%
Diphosphates	-	-	-	-
UDPG	-	-	-	-
Monophosphates	-	-	-	-
PGA	19	55	47	81
PEP	-	-	-	-
$\sum$ Phosphates	20	58	49	85
Aspartic acid	6.0	17	3.5	6.1
Alanine	-	-	1.8	3.0
Glycine	-	-	0.2	0.4
$\sum$ Amino acids	10	30	5.5	9.5
Maltose	-	-	-	-
$\sum$ Free sugars	-	-	-	-
Glyceric acid	-	-	-	-
Malic acid	2.8	8.0	3.4	5.9
$\sum$ Organic acids	4.0	12	3.4	5.9

Key to Table IV:

C: Suspension C, 0.1 ml (0.68 mg chlorophyll and 9.2 mg protein) plus 0.9 ml 0.5 M sucrose buffered at pH 7.2.

C + RuDP: Same as above except that 0.01 ml of RuDP solution containing about 0.1 μmole of RuDP was added. The RuDP was prepared according to the method described by Horecker, et.al. (1956).

Disintegrations per minute: Total C^{14} fixed in the alcohol soluble fraction only.

TABLE V

Comparison of Carbon Dioxide Fixation Rates *in vivo* and *in vitro* Systems

Plant or Preparation	Investigator	Rate of CO_2 Fixation at 1.3×10^{-3} M, Total CO_2 Species.[a] μmole/min/mg protein	% of *in vivo* CO_2 Fixation Rate
Intact *Chlorella pyrenoidosa*	Bassham (personal communication)	0.4[b]	100
Enzyme preparation from *Spinacea oleracea*	Weissbach (1956)	0.27[c]	68
Enzyme preparation from *Spinacea oleracea*	Racker (1957)	0.44[c]	11
Enzyme preparation from *Spinacea oleracea*	Jakoby, et.al. (1956)	0.019[d]	4.8
Enzyme preparation from *Tetragonia expansa*	Pon (1959)	0.02[d]	5.0

a. This concentration is equivalent to 0.4% in equilibrium with a pH 7.3 buffer.

b. The CO_2 fixation rate for the *in vivo* system assumes that 20% of the wet weight of algae is equal to the dry weight, 50% of the latter being equal to the weight of the protein in the cells. It should be emphasized that these comparisons also assume that all of the protein within the cell constitutes the carboxylation enzyme.

c. These fixation rates were calculated by substituting the values for the Michaelis constant for bicarbonate and the maximum valocity of the reaction (quoted in the respective references) into the Michaelis-Menten equation.

d. These values were obtained by assuming that the rate of fixation is directly proportional to the bicarbonate concentration.

TABLE VI

Carbon Dioxide Fixation by Various Chlorophyll-containing Materials

Materials	Investigator	Max. Fixation Rate μmole CO_2/min/mg chlorophyll	Final Conc. Total Species of CO_2, i.e., $(CO_2)+(HCO_3^-)$	Remarks
Intact chloroplasts in sap	This paper	2.9×10^{-2}	3.8×10^{-3} M	pH 7.2
Intact chloroplasts in NaCl	Allen, et.al. (1955)	4.2×10^{-3}	5.0×10^{-4} M	pH 7.2
Intact *Chlorella pyrenoidosa*	Hill and Whittingham (1955)	3.1	Unknown	
Intact *Chlorella pyrenoidosa*	Bassham (personal communication)	4.0	(0.4%) = 1.3×10^{-3} M	Assumed A.Q. =1.0; pH 7.3
Sambucus nigra green leaves	Willstatter (1951)	2.5	(5% CO_2)	
Sambucus nigra yellow leaves	Willstatter (1951)	44	(5% CO_2)	
Helianthus annuss	Willstatter (1951)	5.3	(5% CO_2)	
Intact *Spirogyra*	Thomas (1957)	1.5	(5% CO_2)	A.Q. = 1.0
Spirogyra chloroplast fragment	Thomas (1957)	8.9	(5% CO_2)	A.Q. = 1.0

* A.Q. = assimilatory quotient (the ratio of CO_2 to O_2)

TABLE VII

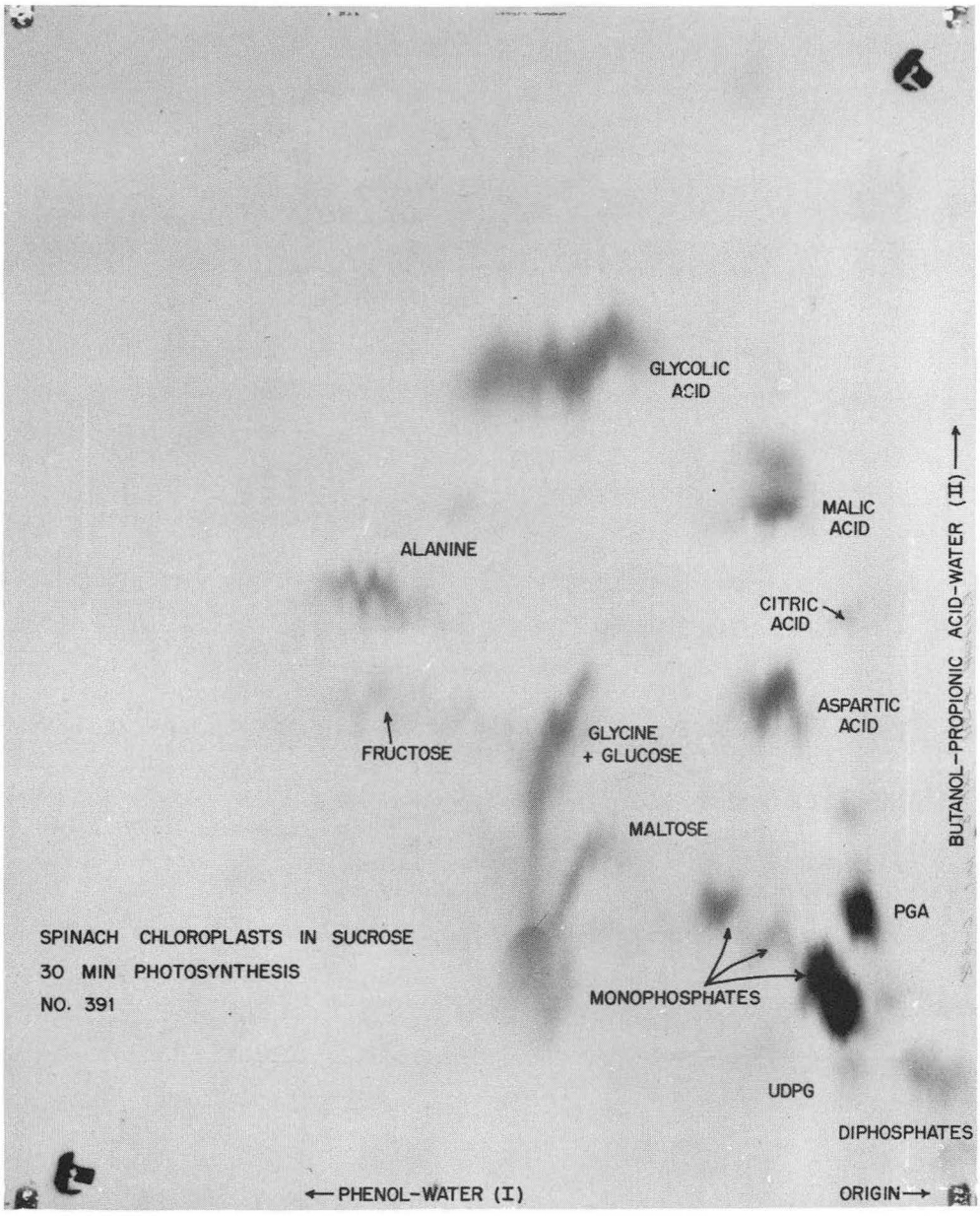
Maximum Initial Rate of Hill Reaction and Rate of Photolysis

Preparation	Investigator	Oxidant	Temp., °C	Max. initial rate of Hill reaction μmole/ O_2/min/mg chlorophyll
Intact spinach chloroplasts	Arnon, et.al. (1956)	p-benzoquinone	15	0.75
Intact *Chlorella pyrenoidosa*	Hill and Whittingham (1955)	(CO_2)		3.1
Spirogyra, chloroplast fragment	Thomas (1957)	(CO_2)	18	3.1
Spirogyra, intact	Thomas (1957)	(CO_2)	18	1.5
Chloroplast suspension from *Phytolacca americana*	Holt, et.al. (1951)	2,6-dichlorobenzene indophenol		1.6

Rate of photosynthetic phosphorylation with dilute NaCl-washed chloroplast fragments (Allen, et. al., 1958): 8.5 μmole inorg. phosphate esterified/min/mg chlorophyll.

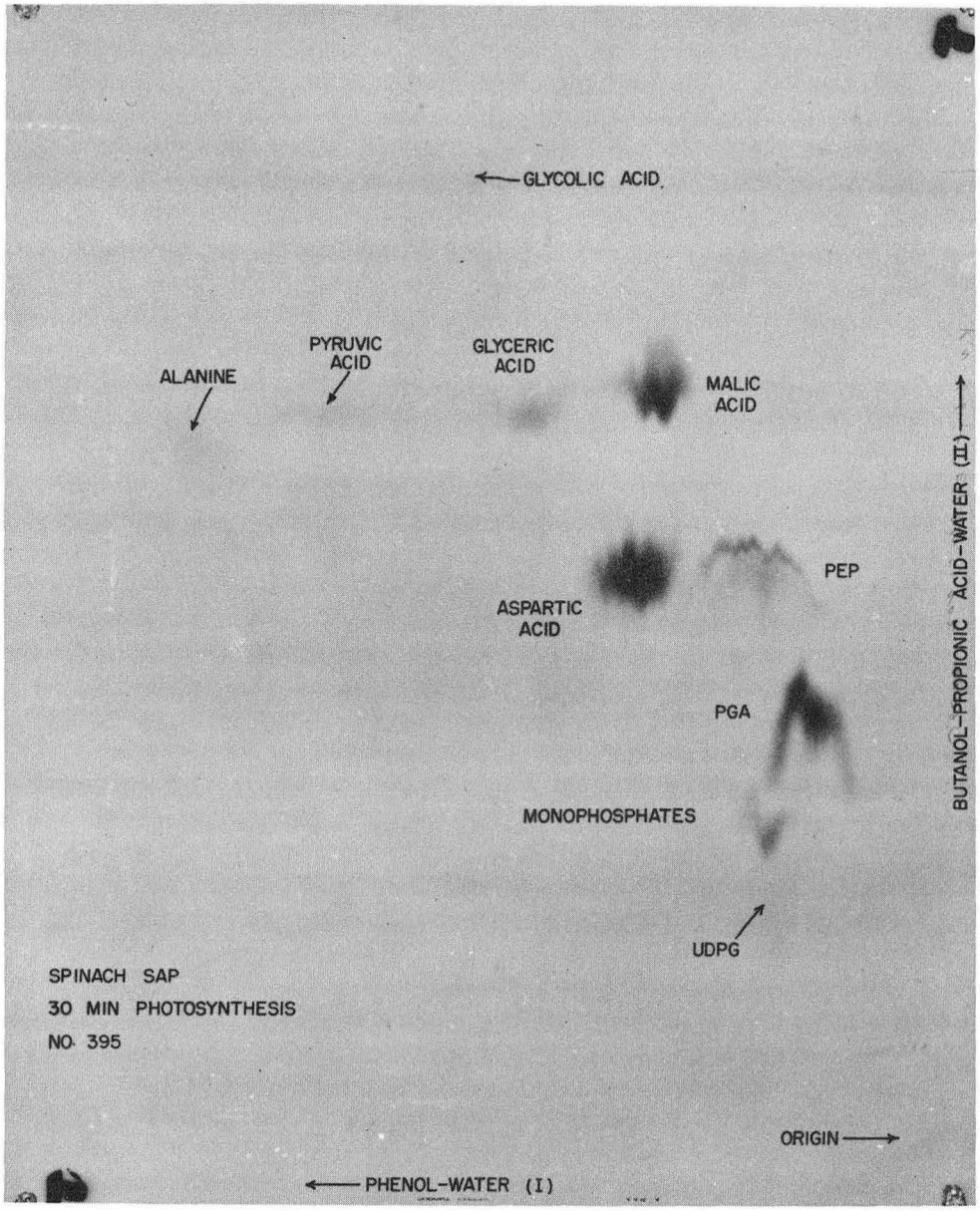
Figure 1. Radioautograms showing distribution of radioactive carbon by various cellular constituents of spinach. (a) chloroplasts in sucrose; (b) sap; (c) chloroplasts plus sap; (d) chloroplasts plus boiled sap; (e) chloroplasts plus mitochondrial fraction plus sap in the dark for 30 min.

For experimental details see Table I.



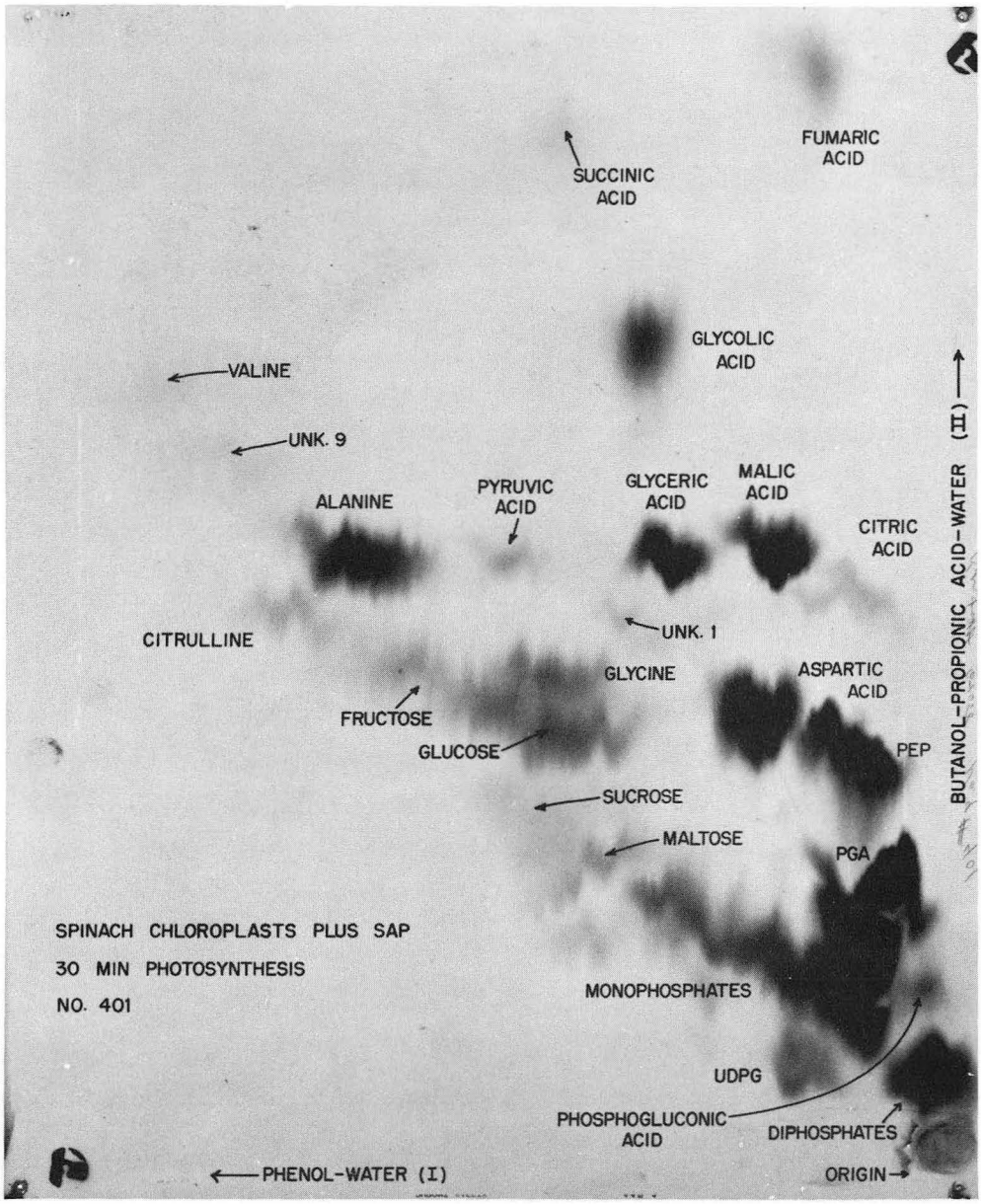
ZN-2161

Fig. 1a



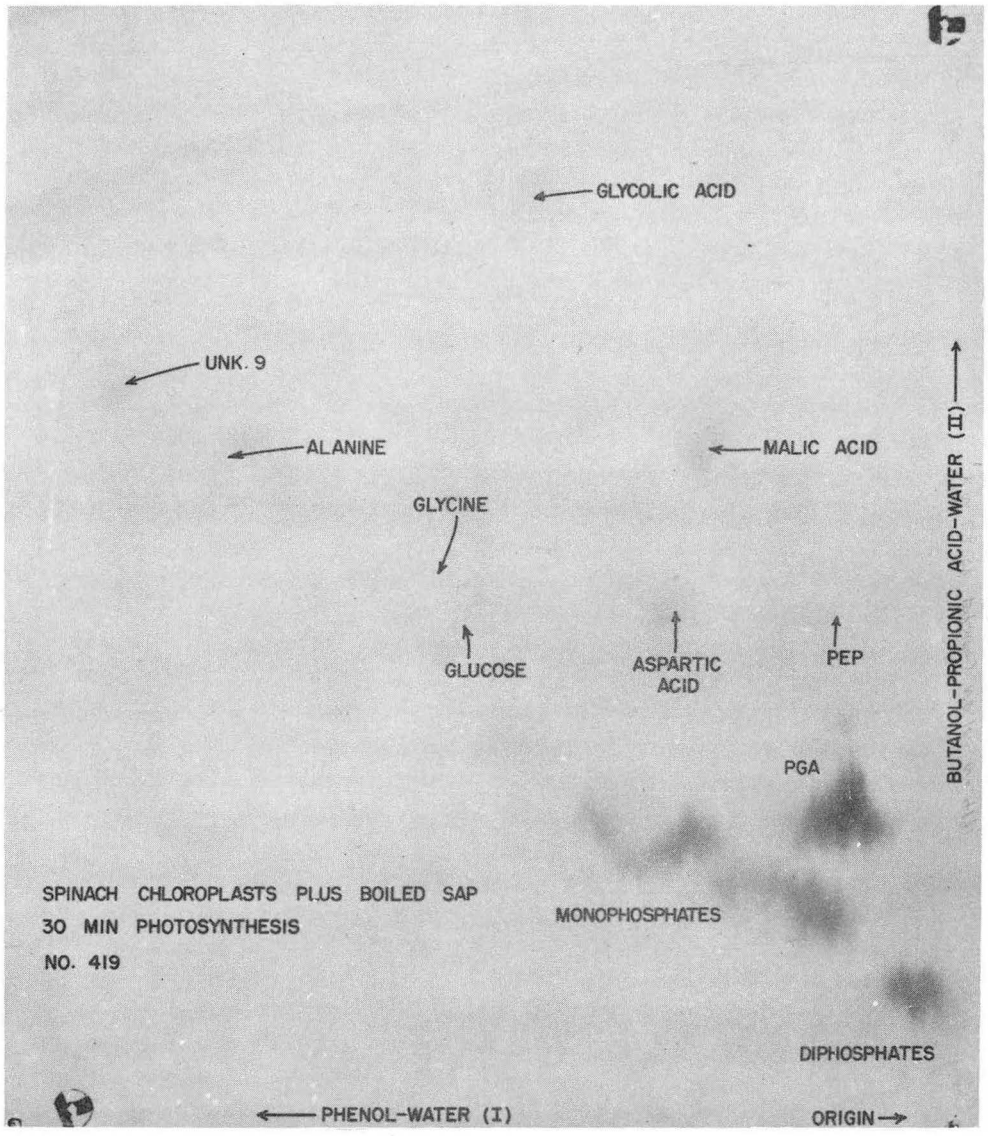
ZN-2159

Fig. 1b



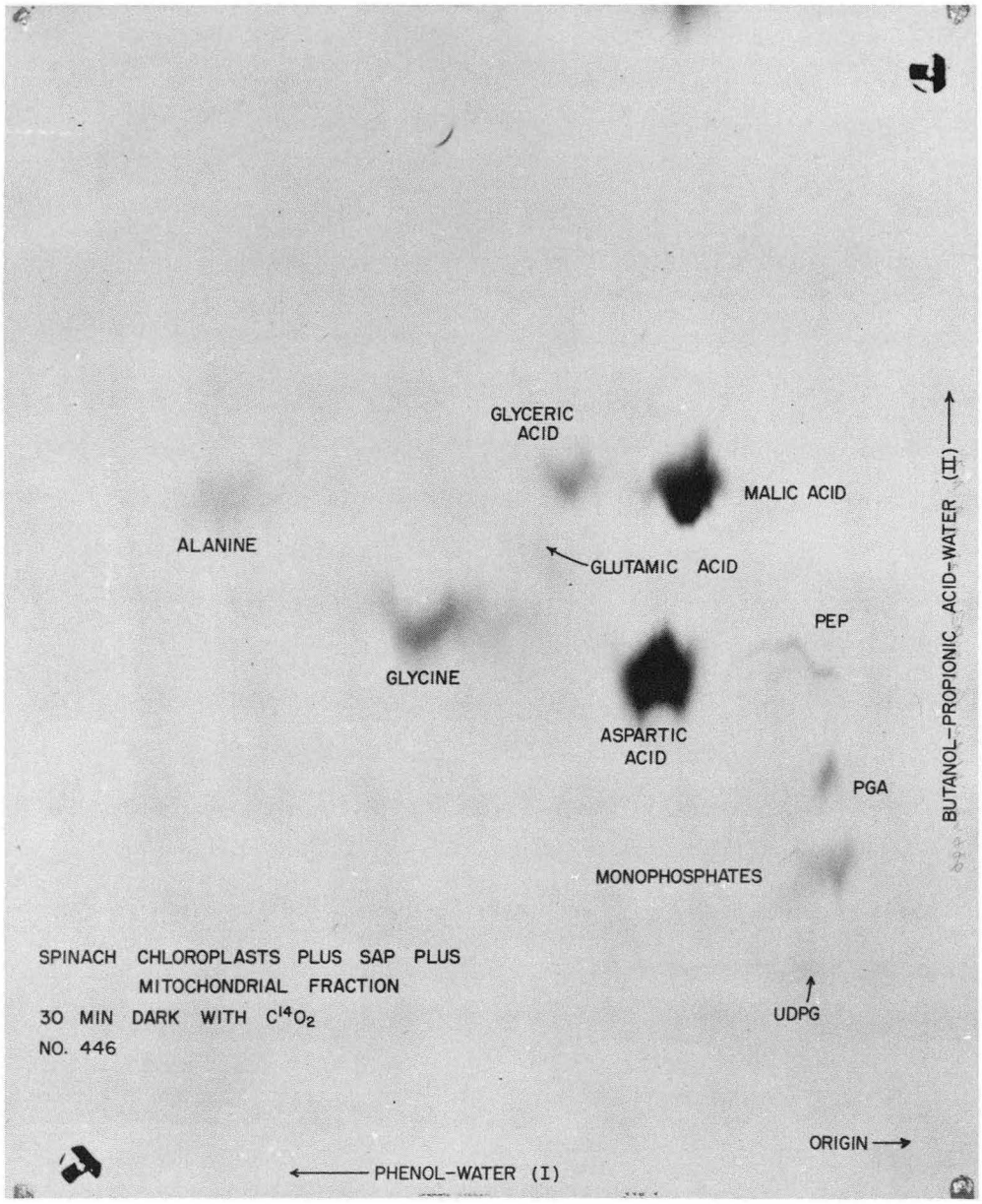
ZN-2156

Fig. 1c



ZN-2158

Fig. 1d



ZN-2160

Fig. 1e

Figure 2. (a) Uptake of radioactive carbon dioxide by chloroplasts plus sap after varying lengths of time with labelled carbon (the filled circle is from the 5 min light, then 5 min dark experiment).

(b) Effect of varying amounts of sap on total fixation of carbon dioxide by spinach chloroplasts.

(c) Amount of radioactivity in PGA and diphosphates as percent of the total soluble radioactivity.

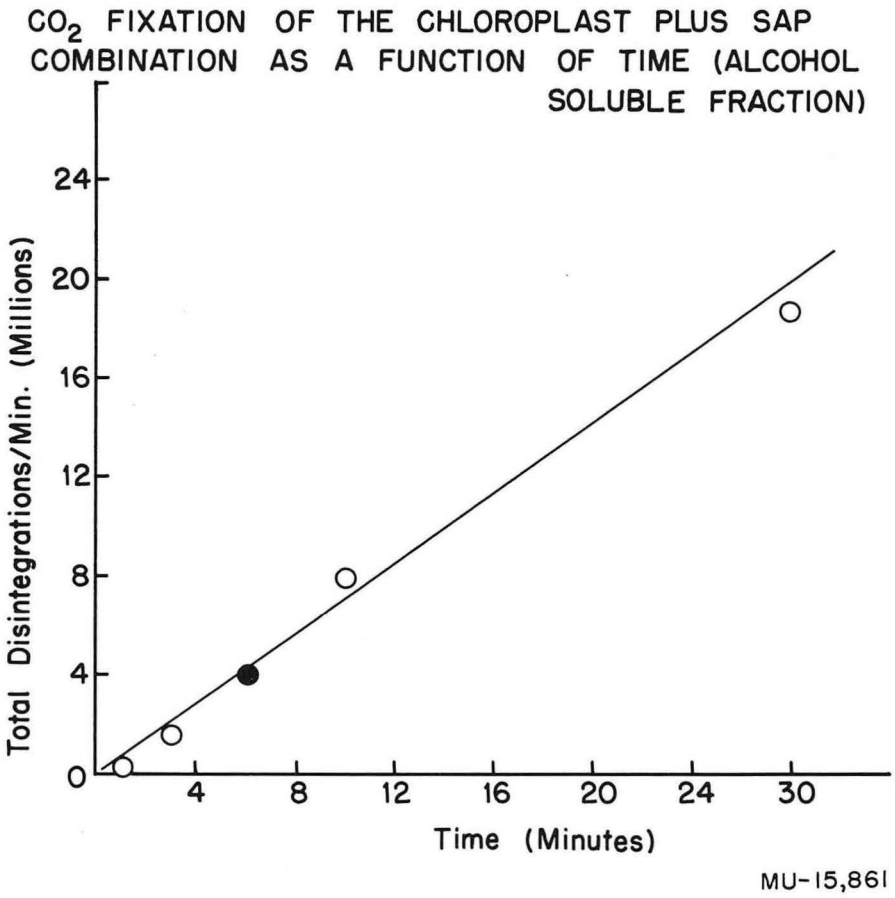

CO_2 FIXATION OF THE CHLOROPLAST PLUS SAP COMBINATION AS A FUNCTION OF TIME (ALCOHOL SOLUBLE FRACTION)
Total Disintegrations/Min. (Millions)
0
4
8
12
16
20
24
4
8
12
16
20
24
30
Time (Minutes)
MU-15,861

Fig. 2a

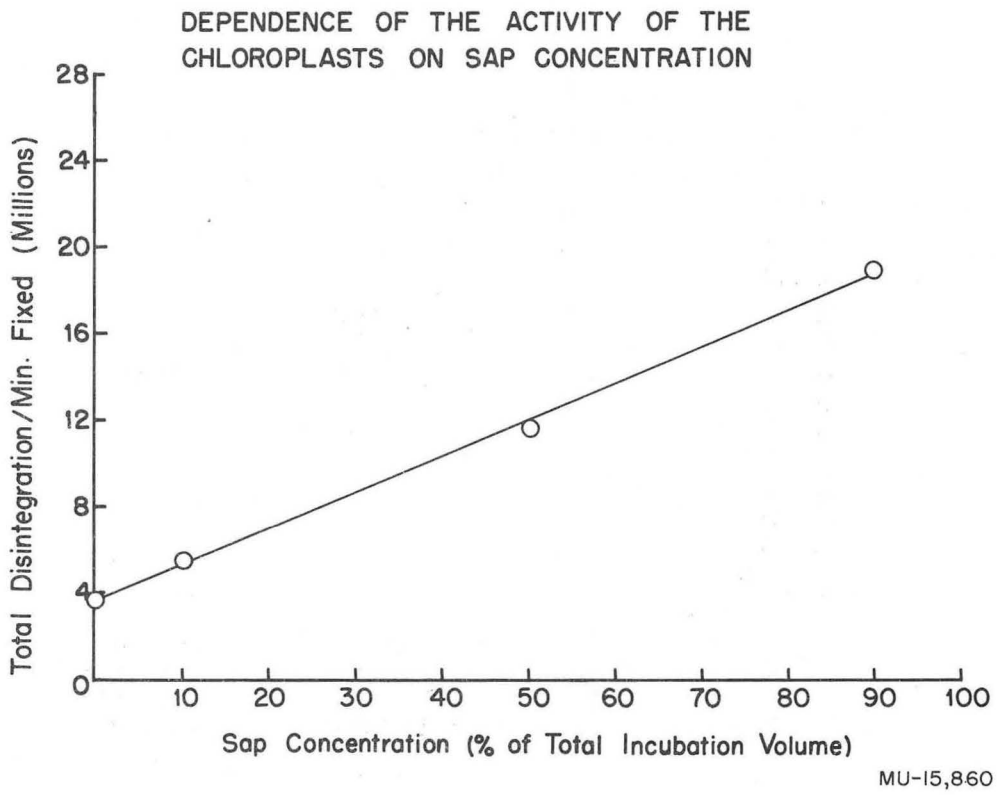

DEPENDENCE OF THE ACTIVITY OF THE CHLOROPLASTS ON SAP CONCENTRATION
Total Disintegration/Min. Fixed (Millions)
0
4
8
12
16
20
24
28
10
20
30
40
50
60
70
80
90
100
Sap Concentration (% of Total Incubation Volume)
MU-15,860

Fig. 2b

RATE OF DISAPPEARANCE OF PGA AND DIPHOSPHATES

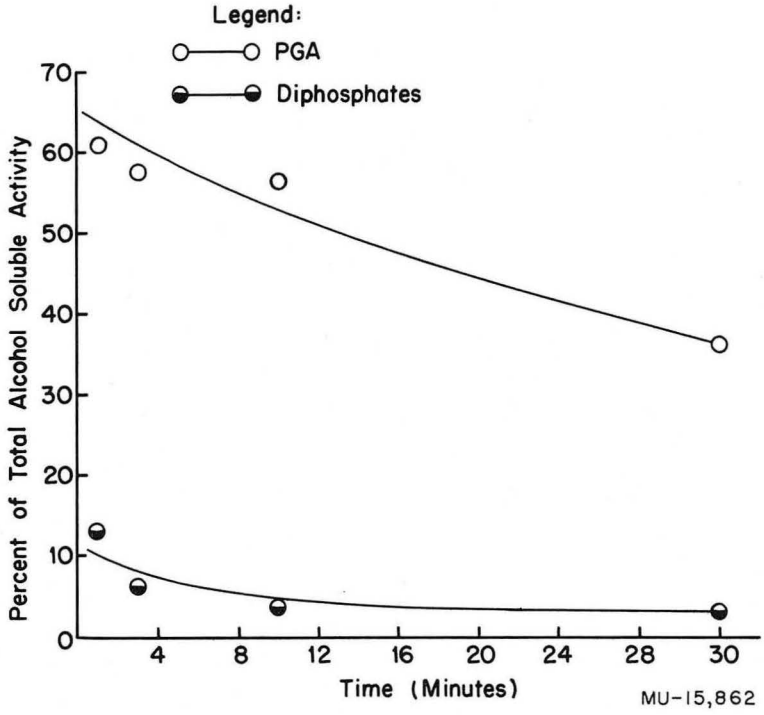
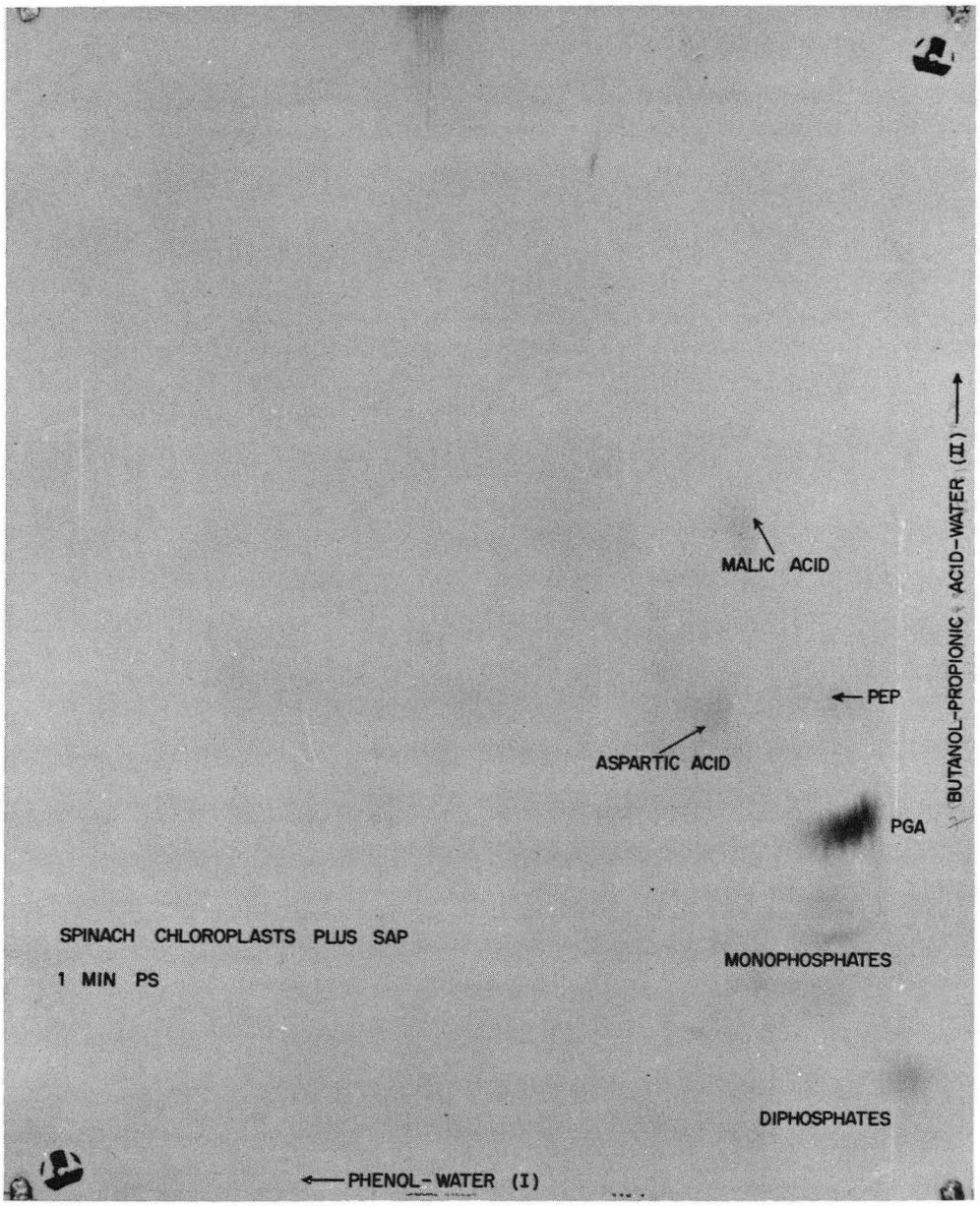


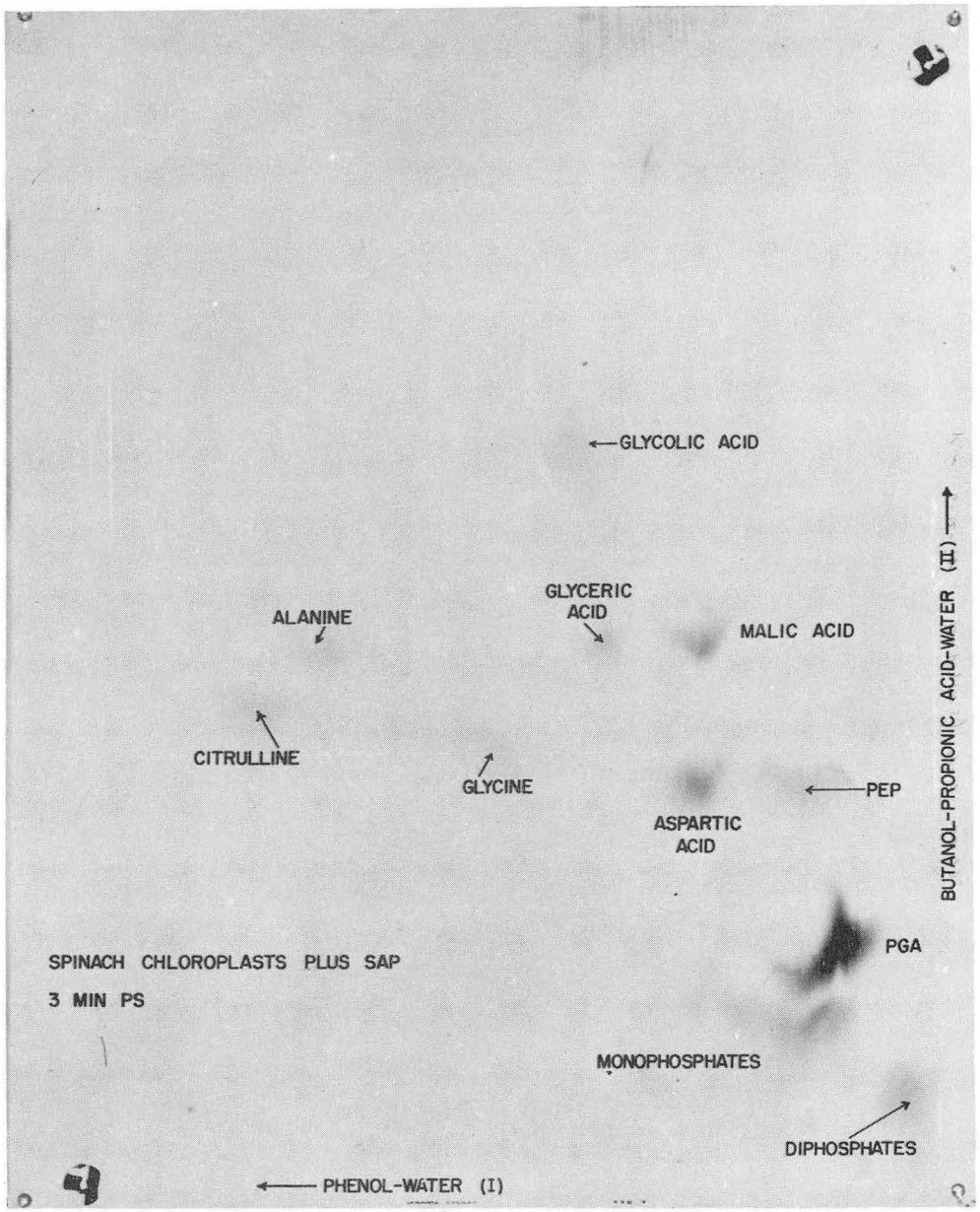
Fig. 2c

Figure 3. Radioautograms showing pattern of carbon-14 distribution by chloroplast-sap mixture after varying lengths of time of exposure to labelled carbon dioxide (see Table II). (a) 1 min; (b) 3 min; (c) 10 min; (d) 30 min.



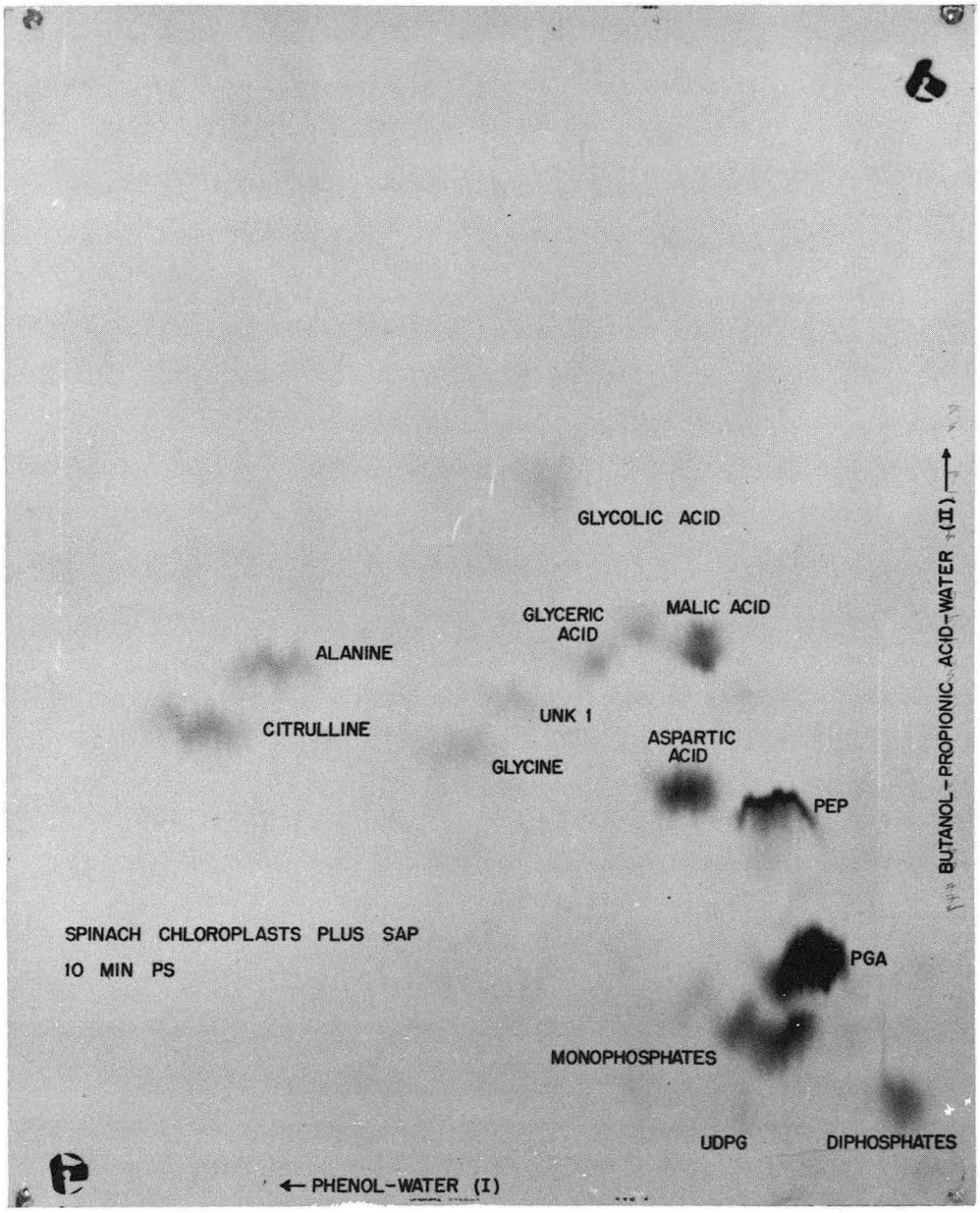
ZN-2162

Fig. 3a



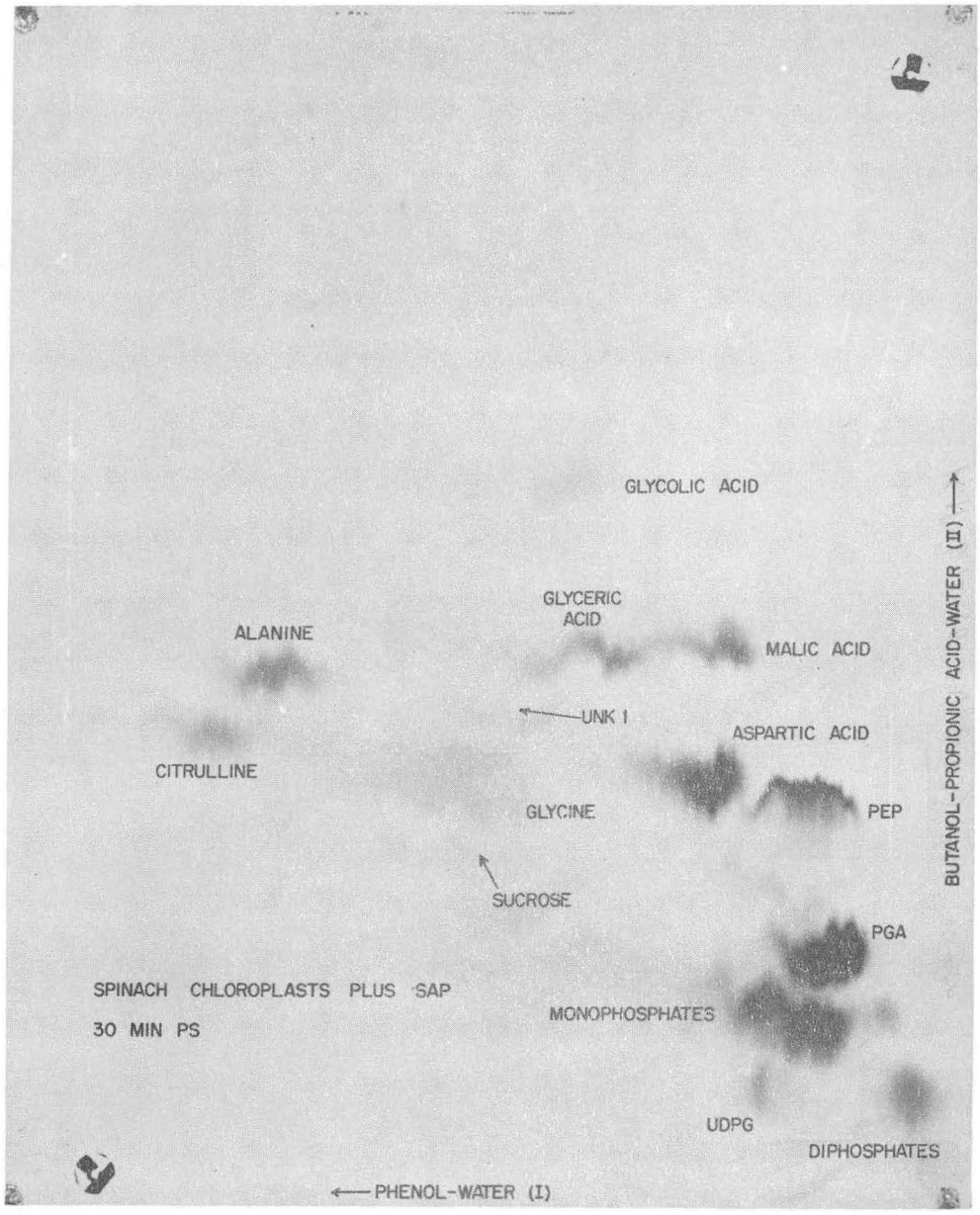
ZN-2157

Fig. 3b



ZN-2164

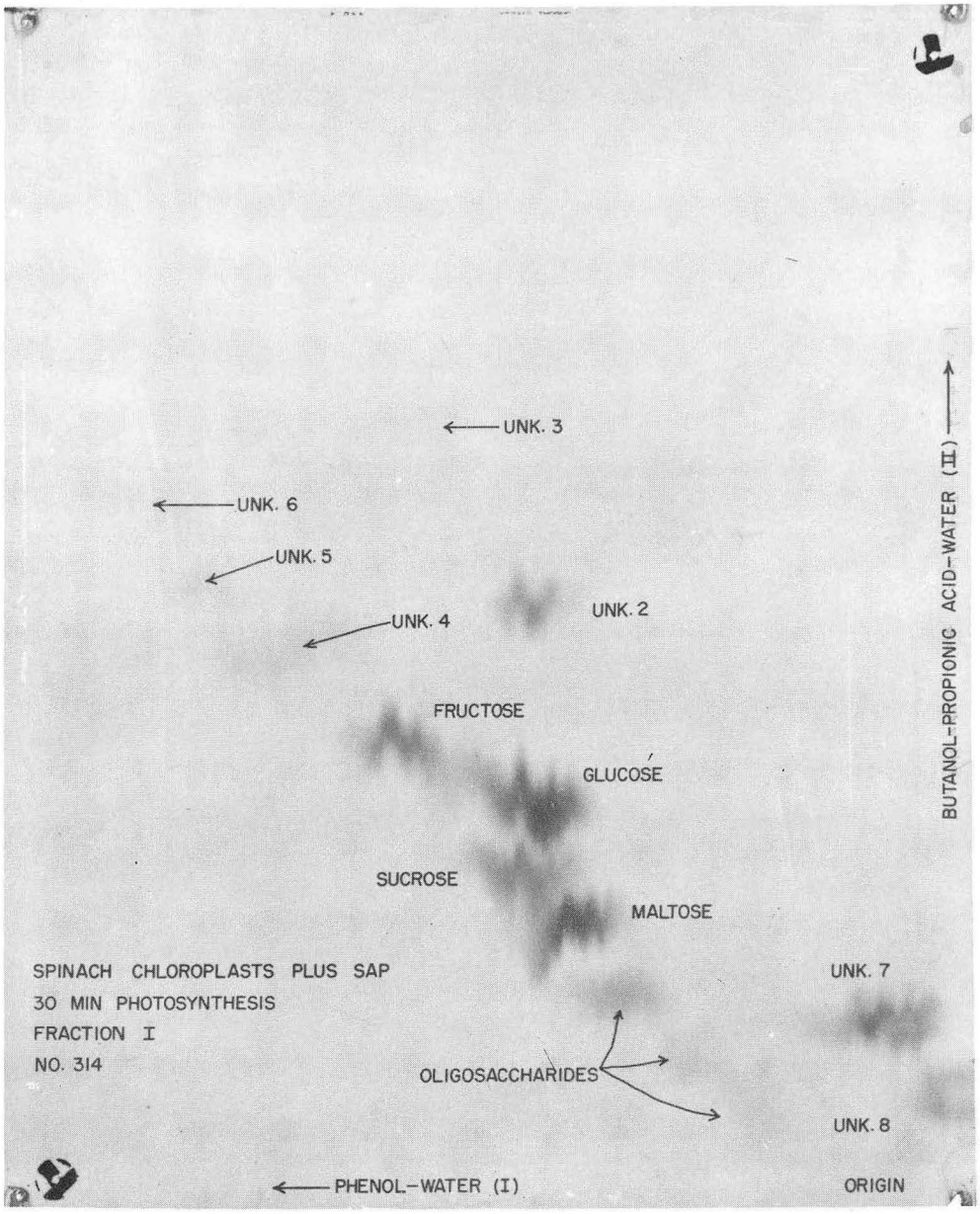
Fig. 3c



ZN-2163

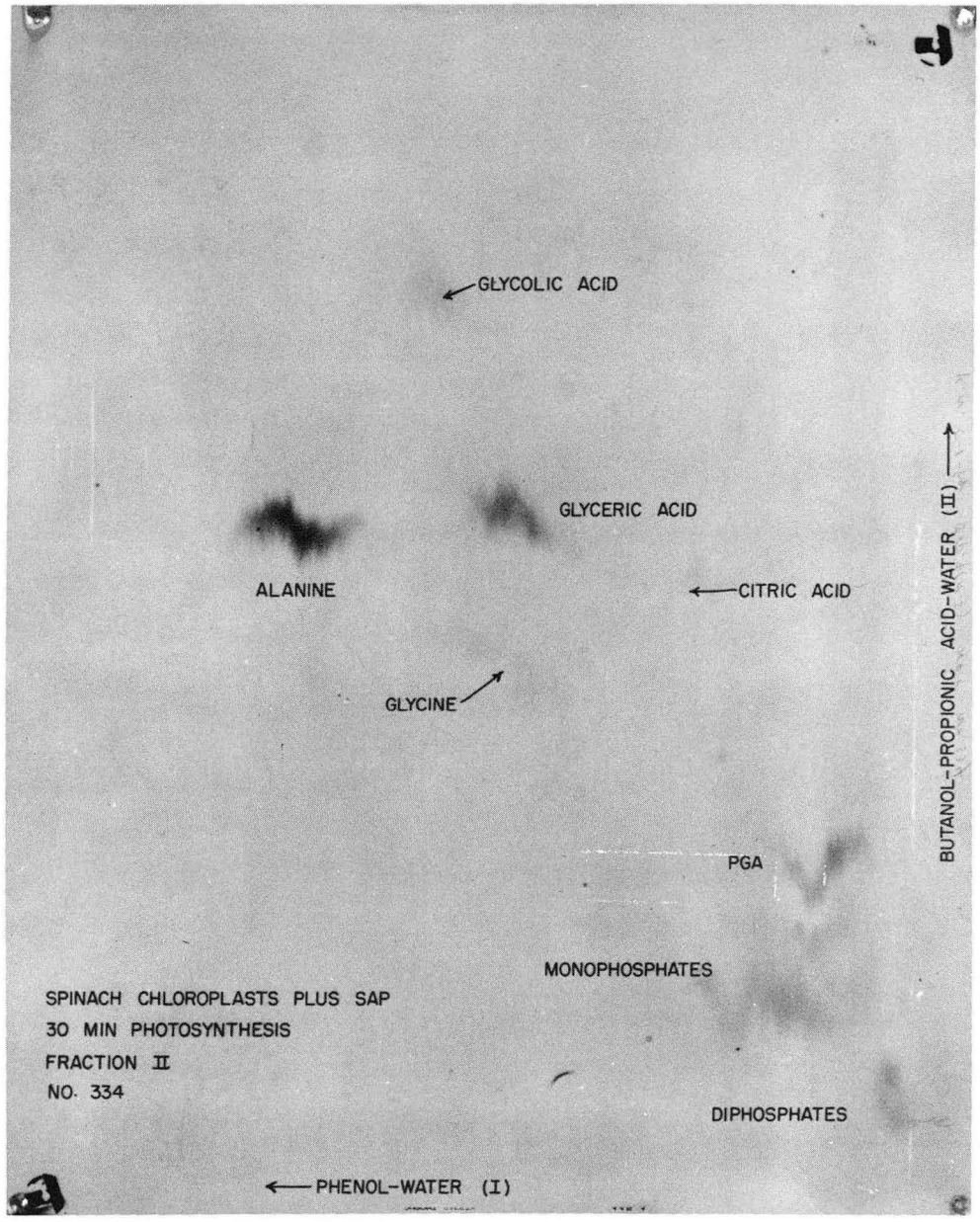
Fig. 3d

Figure 4. Radioautograms showing pattern of distribution of radioactive carbon by chloroplast plus sap mixture at pH 6.1. (a) The neutral fraction (Fraction I); (b) the ionized fraction (Fraction II).



ZN-2166

Fig. 4a



ZN-2165

Fig. 4b

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