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PHOTOSYNTHESIS STUDIES WITH TRITIATED WATER

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PHOTOSYNTHESIS STUDIES WITH TRITIATED WATER*

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INTRODUCTION

Photosynthesis involves two main processes: the photolytic splitting of water, and the utilization of the "active" hydrogen so produced to reduce the incoming carbon dioxide to carbohydrate, with the concomitant release of molecular oxygen. Detailed studies have been made of the primary quantum conversion process[1] and of the path of carbon[2], and some progress has also been made in the study of the pathway of oxygen evolution[3,4]. The present communication reports experiments designed to follow the uptake of hydrogen from water by Chlorella under conditions of light and dark, and in various media.

In an earlier programme it was proposed to obtain completely deuterated algal cells, in which all the cellular hydrogen was replaced with deuterium (^2H), and then to use ordinary water as a tracer. The incorporated hydrogen was to have been measured with a nuclear magnetic resonance spectrometer[5], which can detect hydrogen (protons) in the presence of deuterons. This programme was abandoned when it was found that such deuterated cells showed distinct pathological characteristics[6], and that the quantities of material required for the subsequent analysis would involve the use of very large amounts of cell material, and would necessitate the isolation of intermediates on a large scale. In addition, deuterium and protium (^1H) are both stable species, and the techniques which had been used so successfully to

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follow the path of carbon by making use of the radioactivity of ^{14}C [2] could not be applied in studies utilizing these hydrogen isotopes.

There remained the possibility of using the radioactive isotope of hydrogen, tritium (^{3}H), to follow the uptake of hydrogen from radioactive water by cells actively carrying out photosynthesis; ultimately all the hydrogen required for photosynthetic and other purposes by these cells must come from water. Many difficulties had to be overcome, not the least of which was the very low energy of the β-particles emitted by tritium, and the large dilution of the radioactive tracer by the relatively enormous amounts of water which are inevitably present in any biological system. The maximum energy of the β-particles emitted by tritium is about 18 KeV, as compared with about 150 KeV for those emitted by ^{14}C. This means that the penetrating power of the particles is very much diminished compared with those from carbon, and their ability to pass through paper and affect photographic film is correspondingly reduced.

Detection of the radioactive materials on paper chromatograms would become considerably more difficult for this reason, and to compensate in part for the weaker radiation, larger amounts of isotope became necessary. In systems in which the cells are supplied with radioactive carbon dioxide, the system can be so arranged that the supply of unlabelled carbon dioxide available to the cells is reduced to a minimum, keeping the specific activity of the added tracer high. This is not possible with water; the best solution of this aspect of the problem was to use cell suspensions much more concentrated than those used for carbon studies, in order to achieve a more favourable substrate-to-cell ratio. This, in turn, presented new difficulties: as the cell concentration in the suspension was increased, the optical density of the suspension rose considerably, and the amount of light passing

through it was correspondingly reduced.

These problems have now been overcome to a considerable extent, and a satisfactory picture of the incorporation patterns of ^3H from ^3H_2O by algae under several experimental conditions has been obtained.

Other photosynthesis studies with water containing heavy isotopes of hydrogen have been concerned mainly with the role of chlorophyll as a hydrogen carrier. Studies by NORRIS, RUBEN AND ALLEN[7], with tritium oxide, and by CALVIN AND ARONOFF[8], using deuterium oxide, failed to demonstrate any excess incorporation of hydrogen from water into chlorophyll during photosynthesis. Recent work by VISHNIAC[9], however, has shown that some four to five times as much tritium is incorporated from water into chlorophyll in the light as in the dark.

METHODS

Cells of Chlorella pyrenoidosa were grown in the continuous culture apparatus described by HOLM-HANSEN, et al.[10] The experimental techniques used in earlier work for the exposure of cells to labelled substrates while carrying on photosynthesis in "flattened test-tubes"[6], or in the conventional "lollipop"[2], were discarded in favour of a new apparatus, designed to achieve a satisfactory degree of illumination of the very dense algal suspension used.

The apparatus provided for the continuous shaking of 6 glass vessels while they were simultaneously illuminated from below. After harvesting of the cells, washing with distilled water, and resuspension in distilled water or other medium, 0.7-0.8 ml of the cell suspension (containing 0.15 ml of wet-packed cells) was pipetted into each of the vessels. These were constructed from glass tubing of about 3.5 cm internal diameter and 7 cm long, to which an optically flat circle of glass was sealed to form a bottom. One ml of liquid in such a vessel forms a layer 1 mm

deep, apart from meniscus effects. A loose-fitting lid was provided with an outlet and an inlet tube; the latter reached nearly to the bottom of the vessel (Fig. 1). During the "pre-adaptation" period before the experiment, in which the cells were allowed to come to a steady metabolic state, the cell suspension was shaken in the light for 30 min, while air containing 1% (v/v) CO_2 was blown over the surface of the cell suspension. The gas mixture was pre-wetted by bubbling through water to minimize evaporation from the cell suspension and to serve as a control measure for the rate of aeration (Fig. 2).

A rectangular glass water bath, fitted with inlet and outlet tubes to permit a constant stream of cooling water to pass through it, was arranged over a bank of eight 6-watt fluorescent lights. At the top of the water bath two rails were fitted along which ran a small carriage consisting essentially of four wheels fixed to a plate having six holes punched in it, in which six of the glass incubation vessels could be placed (Fig. 3). The vessels were suspended partly below the plate so that the bottoms of the vessels reached nearly to the bottom of the water bath. The vessels were prevented from falling through the plate by three glass "ears" on each vessel arranged near the top. A second plate fitted to the carriage about 2 cm below the first, and also containing six holes corresponding with those on the upper plate, prevented too much rattling of the glass vessels; the latter were also taped with collars of adhesive paper to obtain a fairly tight fit. The whole carriage was made to run back and forth along its rails by means of a connecting rod attached to an eccentric on the drive shaft of a stirrer motor. The carriage oscillated about 3 cm at approximately 250 cyc/min. The light intensity at the bottom of the glass vessel was about 2000 ft candles.

As the whole apparatus needed to be as small as possible for use in a sealed box, the fluorescent tubes were of limited length, and while illumination was fairly constant to each vessel, there was some variation between different positions on the

shaker. For this reason the samples were supplied with radioactive substrate one at a time in a serial manner, each flask being moved to one particular position on the shaker for the period of incubation with labelled substrate.

Before the addition of labelled substrates any other additions, such as mineral salts, were made, resulting in a total liquid volume of 0.8 ml. At the start of the experimental period, 0.2 ml of ^3H_2O (specific activity, 5 C/ml, prepared by Whittemore and Lehman by oxidation of tritium gas [11]) was added to the cell suspension, and after a definite incubation period 4 ml of absolute ethanol was added to kill the cells. The shaker was kept in motion while both additions were made to ensure rapid mixing. Unless $NaH^{14}CO_3$ was also used as a substrate, the vessels were kept flushed with the gas mixture during the incubation period.

For experiments on the uptake of labelled water in the dark, the cell suspension was placed in small test tubes bound with black adhesive tape. The tubes were capped with rubber serum stoppers through which passed two hypodermic needles, a long one reaching to the bottom of the tube and a shorter one serving as a gas outlet tube. Once the tubes were capped all additions (substrate, and ethanol for killing) and gas flushing were performed via the longer hypodermic needle.

For experiments to study the effect of the radiation from tritium on carbon metabolism, the labelled carbon dioxide was added as 0.1 ml of 0.26 M $NaH^{14}CO_3$(40 μC).

The suspension of cells in 80% ethanol at the end of the incubation period was allowed to stand at room temperature for 2 hrs, and then was centrifuged. The residues were extracted with 2 ml of 20% (v/v) ethanol for 1 hor, and with 2 ml of water overnight. The extracts were pooled and the cell residues discarded. The extracts were evaporated to dryness in vacuo below 40°. The dry residue after evaporation was redissolved in 5 ml of 20% ethanol and again evapprated to dryness; this was repeated twice more, and the residue finally dissolved in less than 1 ml of 20% ethanol, solution being aded by placing one or two small glass beads in the flask

and using these to loosen material from the walls. The purpose of the repeated evaporations was to remove tritium present in compounds in exchangeable positions, leaving the isotope present only in nonexchangeable locations. Were this not done it is likely that almost every compound in the cells would have been labelled(see DISCUSSION).

The water and ethanol released during the evaporations were caught in a trap surrounded by liquid N_2. When each trap became full it was filled with a mixture of vermiculite and cement, stoppered, sealed into a plastic bag, and encased in a lead pipe with a screw cap in preparation for ocean burial.

Owing to the large quantities of radioactivity used, all the operations described above, from the time the vessel containing labelled substrate was first opened until the final evaporation was completed, were performed within a sealed "glove box." A rapid draught of air was maintained through the box by a suction fan which was vented through the roof of the building, and the vacuum pump used for the evaporations, even though outside the box, was exhausted back into the main vent. The whole system inside the box, in addition to the room air, was very carefully monitored to ensure that no radioactive substances escaped from the confines of the box and the venting system.

The final solution of compounds from each sample was chromatographed in toto on a sheet of oxalic acid-washed Whatman No. 4 filter paper. The chromatograms were developed in the first dimension with phenol-water (10 hr) and in the second dimension with n-butanol-propionic acid-water (8 hr)[12]. Radioautograms were obtained by exposing the developed chromatograms to Dupont blue-sensitive single-coated X-ray film 507E for about 2 months[13]. Very faint film images were intensified by blue toning [14]. After location of the spots on the paper the activity in each one was determined by counting with a windowless Scott-type Geiger-Müller

tube placed on the paper[13]. The Scott tube was flushed continuously with a gas mixture consisting of 99.05% (v/v) He and 0.95% (v/v) isobutane. The counter was operated at 1500 V. After being placed on a particular position on the chromatogram, the counting tube was flushed for 5 min before a count (5 min) was taken. Adjacent spots were shielded with a sheet of paper. Compounds containing ^{14}C were counted with similar tubes having a thin "Mylar" window (thickness about 1 mg/cm^2).

Spots on chromatograms were identified by elution, treatment with human seminal acid phosphatase in in 0.02 M-acetate buffer (pH 5) when appropriate, and rechromatographed with authentic markers. Substances containing very little activity were presumptively identified by their chromatographic positions only, comparison being made with chromatograms of cell extracts labelled with ^{14}C run at the same time, the compounds in which had already been identified.

RESULTS

Efficiency of tritium counting.

A spot of tritium-labelled glutamic acid on a chromatogram, of which the counting rate from filter paper with the windowless Scott tube was known, was eluted and aliquots of the eluate dried onto aluminum planchettes. These were counted with the windowless counter. Further aliquots were assayed using a scintillation counter which was known to count 30% of all disintegrations from tritium. The results are shown in Table I. For comparison, ^{14}C compounds counted with a Scott tube having a thin "Mylar" window, about 6% of all disintegrations were counted when the compounds were adsorbed onto filter paper, and about 20% when the compounds were dried as "infinitely thin" films onto aluminum planchettes.

Radiation effects.

A necessary preliminary to further study was to establish that the flux of radiation due to tritium of the specific activity and for the incubation period used (1 C/ml for 3 min) was not liable to damage the cells or cause derangement of their photosynthetic capabilities. The radiation dosage in the conditions used

was 760 reps for the 1 ml sample. Equal aliquots of cells were allowed to carry on photosynthesis in the presence of $NaH^{14}CO_3$ (40 μC) for 3 min in the presence and absence of ^3H_2O (1 C). Chromatograms of the cell extracts were prepared and exposed to X-ray film, a layer of "Mylar" being placed between the paper and the film. The "Mylar" was sufficient to absorb the β-particles from tritium but not those from ^{14}C. A number of compounds were counted for ^{14}C with a thin-window Scott tube and the results are presented in Table II. These compounds normally contain 80-90% of the total soluble ^{14}C fixed by cells supplied with labelled bicarbonate for 3 min in the light. Apart from the changes in glycollic acid, aspartic acid, glutamic acid, and sucrose, no very significant alteration of the photosynthetic incorporation pattern resulted from the presence of tritiated water.

Identity of substances incorporating tritium in the light.

The following substances have been shown by cochromatography with authentic markers to incorporate ^3H from ^3H_2O in the light (phosphates were treated with phosphatase before being chromatographed with markers): sugar monophosphates (containing the monophosphates of glucose, fructose, and possibly other sugars); sugar diphosphates (containing, after treatment with phosphatase, label in ribulose, and a trace in glucose or fructose); phosphoglyceric acid (probably mixed with a little phosphoglycollic acid); uridine-diphosphoglucose (label only in the glucose moiety after 3 min); fumaric acid, malic acid, citric acid, aspartic acid, glutamic acid, alanine, glutamine and a mixed spot of serine and glycine (label mainly in serine). Other substances contained too little activity to permit cochromatography and their identity was inferred from their chromatographic positions, and by comparison with chromatograms of cell extracts labelled with ^{14}C in which these compounds had earlier been identified. These substances were: phosphoenolpyruvic acid, phosphoglycollic acid, succinic acid, glycollic acid (also present after treatment of the sugar diphosphates with phosphatase), and sucrose. The identity of glycollic acid was rendered virtually certain by its high volatility, taken together with its chromatographic

parameters.

Incorporation of tritium from water in the light and dark.

The distribution of tritium incorporated during 3 min incubation periods in the light and dark are shown in Table III. The compounds in which tritium appeared were similar in both conditions of lighting, except for the sugar diphosphates, phosphoenolpyrivate, citrate, glutamine, and one unidentified substance, all of which incorporated no activity in the dark. The total quantity of tritium fixed in the dark, however, was only about one half that fixed in the light. The main differences in distribution were the relatively lesser amounts of tracer incorporated into the sugar phosphates and organic acids, particularly malic acid, in the dark, and the relatively greater quantities incorporated into the amino acids, especially aspartic acid and alanine in the dark. Radioautograms demonstrating these differences are shown in Figs. 4 and 5.

One important difference, difficult to measure, was concerned with glycollic acid. This substance is quite volatile, as evidenced by the fact that even though the film blackening due to this compound was greater than for any other (Fig.4), the activity remaining on the paper when it was counted was only about 4% of that in glutamic acid, the next strongest spot. TOLBERT AND ZILL[15] have reported that 40 ± 10% of the glycollic acid was lost from paper chromatograms during two weeks' exposure to X-ray film, together with another 1-8% lost during development of the chromatograms. A trial experiment in which glycollic acid-^{14}C was added to an extract of Chlorella cells not labelled with ^{14}C, and the mixture evaoprated to dryness four times (see METHODS sections), showed no loss of glycollic acid by volatlization under these conditions, probably due to the relatively high pH of the cell extract (pH 5.7). The chromatograms, on the other hand, having been developed

in the second dimension with an acidic solvent, would lose glycollic acid more readily. In the present work, there was presumably a further loss of glycollic acid in the variable period (1-3 weeks) which elapsed between removing the chromatograms from the X-ray film, and counting the spots on them.

Although it was not possible to measure the amount of activity in glycollic acid with any degree of accuracy, an attempt was made to find the relative amounts of tritium incorporated into this compound in the light and in the dark by densitometric measurements of the amount of film blackening due to this compound. Since both light and dark cell extracts were prepared and chromatographed under identical conditions, such densitometric measurements should have some validity. As a comparison, aspartic acid from each chromatogram was also determined in this manner, and the ratio obtained compared with that derived from direct counting. Table IV shows that on this basis some 6.6 times as much activity was incorporated into glycollic acid in the light as in the dark, although the total activity in all the other compounds was only three times as high as in the light.

Using the comparative film blackening of glycollic acid and aspartic acid, and knowing the activity of the aspartic acid on the chromatograms, it is possible to calculate roughly that the percentages of the total tritium fixed which appeared in glycollic acid were 57% in the light, and 28% in the dark, for 3 min exposure time in both cases.

Effects of ammonia and nitrate on patterns of tritium incorporation.

HOLM-HANSEN *et al*[16], have shown that the pattern of ^{14}C incorporation from $^{14}CO_2$ in the light by *Chlorella* is markedly affected by the presence in the medium of ammonia, and, to a lesser extent, by nitrate. When ammonia was present a much greater fixation of ^{14}C was frequently observed, and the ^{14}C was distributed to a much greater extent among amino acids and organic acids, with a corresponding decrease in the

sugar phosphates and sucrose. Nitrate had a similar, though less marked, effect. Cells supplied with ^3H_2O in the presence of 0.001 M ammonium chloride or 0.001 M potassium nitrate added 2 min before the labelled substrate, showed only small differences from cells incubated in the absence of a nitrogen source, both in total tritium fixed and in its distribution (Table III).

Kinetic studies on the light and dark.

Studies were made to determine the incorporation patterns of tritium in the light and dark after various incubation periods with tracer ranging from 5 sec to 3 min. The results are presented in Tables V and VI. In the light after the shortest incubation period (5 sec), the largest quantities of label by counting appeared in the sugar monophosphates, phosphoglyceric acid, malic acid, aspartic acid, and glutamic acid. Glycollic acid produced the most film blackening even at this short time. The percentages of the total fixed tritium present in the sugar monophosphates, phosphoglyceric acid, and aspartic acid, decreased with time from the shortest to the longest incubation periods, indicating that these substances are early intermediates on the pathways of tritium incorporation (Table V).

In the dark, the earliest substances to become labelled (6.8 sec) were essentially the same as those in the light, though relatively much more activity was present in the amino acids at the earliest periods, and less in the sugar phosphates and organic acids. The sugar monophosphates and aspartic acid were the only substances to show a relative decline in incorporated tritium with increasing incubation periods in the dark (Table VI).

DISCUSSION

Experiments with hydrogen isotopes are bound to present certain problems of interpretation which do not arise with other elements, except possibily oxygen. This results from the ease with which hydrogen in water may exchange with many of the hydrogen atoms in organic molecules, particularly those attached to oxygen and nitrogen atoms.

There are at least six ways in which tritium may be incorporated from water into organic molecules in living tissues. In the present experimental arrangement, in which the cell extracts were repeatedly evaporated to dryness and redissolved in unlabelled water, no tritium would remain fixed in three of these six possible instances. Tritium might enter a molecule by an enzymatic reduction process (perhaps involving a reduced coenzyme), be placed in an exchangeable position (attached to oxygen or nitrogen), and subsequently be relocated to a nonexchangeable position (attached to carbon) and hence remain *in situ* after repeated dilution with unlabelled water. A similar enzymatic incorporation into a nonexchangeable position, perhaps with subsequent relocation into another nonexchangeable position, would have a similar result. On the other hand, after enzymatic incorporation into either an exchangeable or a nonexchangeable position, the tritium may be removed by further enzymatic reactions to an exchangeable position and be lost during evaporation. Further possibilities result from an initial incorporation by nonenzymatic exchange between tritium in water and exchangeable hydrogen in the organic molecules. If such tritium is then removed biochemically to a nonexchangeable position it will be retained during evaporation, but if it is moved to another exchangeable position it will be lost.

In addition to these possibilities involving reduction reactions, water itself may be incorporated as a complete molecule, as in the reaction producing malic acid from fumaric acid. Such a complex series of possibilities is certain to lead to difficulties when interpreting the results, compared, for example, with studies in which labelled carbon dioxide is the added tracer. In the latter case, nonspecific exchange is limited to a small number of possibilities, such as with the carboxyl groups of acids, and such exchange is much slower than that involving hydrogen. Biochemical exchange could also take place in reversible decarboxylation reactions.

It is possible to appreciate a number of places in known sequences of biochemical pathways (carbon reduction cycle, glycolysis, oxidative respiratory pathway, and

tricarboxylic acid cycle) through which tritium could enter biological compounds, either by reduction reactions or by the incorporation of water as a complete molecule. Thus, tritium could rapidly enter glucose-6-phosphate, 3-phosphoglyceraldehyde, glycerol phosphate, glutamic acid, aspartic acid, succinate, alanine, isocitrate, malate, and lactate by reduction reactions, the tritium being sited in the first instance in at least one non-exchangeable position. The formation of 3-phosphoglyceric acid, citrate, isocitrate, and malate by the appropriate reactions involves the uptake of a complete water molecule, with fixation of tritium into at least one non-exchangeable position. Tritium will be incorporated into any molecule which obtains its hydrogen in a reaction involving a transfer from an exchangeable position. The reactions mentioned are but a few of many incorporating hydrogen or water, and there is obviously a wide range of possibilities for the interpretation of results.

In practice the problem is somewhat simplified because of many of the compounds mentioned above are present in such small amounts that they do not incorporate detectable quantities of tracer in short-term experiments. The incorporation patterns of tritium in the light and dark can thus be related to the known patterns of carbon dioxide incorporation under similar conditions. In the light, sugar monophosphates and phosphoglyceric acid are labelled with tritium at an early stage (Table V). The other substances incorporate tritium rapidly (malic, glutamic, and aspartic acids) can be accounted for on the basis of the addition of water to fumaric acid to form malic acid, and the production of glutamic and aspartic acids by reductive amination of their respective keto acids. Although malic and aspartic acids also rapidly incorporate ^{14}C from $^{14}CO_2$ in the light, this is not the case with glutamic acid, and this has led to the suggestion[17] that the passage of carbon from the photosynthetic carbon reduction cycle to the tricarboxylic acid cycle is impaired in some way in the light. The present results with tritium, however, have shown that even though

newly incorporated carbon may not enter glutamic acid, this amino acid is nevertheless being formed in the light, as must be the case when the cells grow under conditions of continuous lighting. This lends support to the conclusion that separate metabolic pools of identical substances which are not in a state of rapid equilibrium with each other, may exist in the algae[18].

In the dark, the early incorporation of tritium is mainly into substances associated with the tricarboxylic acid cycle and with pyruvate (alanine, malic, acid, aspartic acid, and glutamic acid) as has also been found with the dark fixation of carbon dioxide[19], and less appears in sugar phosphates (Table VI). Less total tritium is incorporated in the dark, and different incorporation patterns are seen, demonstrating that some, at least, of the tritium taken up in the light must be specifically associated with new synthesis.

The large amounts of activity observed in glycollic acid, particularly in the light, may also be interpreted in terms of comparative biochemistry. The fact that so much tracer was present in this compound suggests that it arose by reduction , possibly of glyoxylic acid, and that glycollic acid is not merely an excess product originating in transketolase reactions as proposed by WILSON AND CALVIN[20]. Further, these authors found that the incorporation of ^{14}C into glycollic acid increased when the cells were placed in a low carbon dioxide environment, and it is possible that in spite of flushing the cell suspension with 1% carbon dioxide during the experiment, the cells were relatively short of it owing to the dense cell suspensions (15%) used. Nevertheless, the relative amounts of glycollic acid in these studies were far greater than those observed when the carbon dioxide concentrationwas lowered to 0.003%[20], and the very heavy labelling with tritium indicates that glycollic acid plays a more significant role in hydrogen metabolism than that resulting from carbon dioxide deficiency. In addition, the rapid appearance of label in glycollic acid (5 sec) suggests that its role in photosynthesis is an early one. Evidence

has been obtained that photosynthetic reductions take place through phosphopyridine nucleotides[21]. It thus seems likely that glycollic and glyoxylic acids may play a role in the transport of hydrogen from photochemically reduced pyridine nucleotides possibly to phosphoglyceric acid, as earlier proposed by ZELITCH[22]. The initial supply of glycollic acid could originate from the transketolase reaction[20], by reduction of glyoxylic acid formed in the glyoxylic cycle [23], or be produced by deamination of glycine[24]. Thelatter is known to appear quite early when algae are incubated in the light with $^{14}CO_2$ [25].

Labelled glyoxylic acid has not been observed. However, if such a hydrogen transport system between glycollic and glyoxylic acids were to functbn,it is reasónable to suppose that the (labelled) hydrogen atom which would be added to glyoxylic acid to form glycollic acid would also be the same one as was later passed on to other substances. Hence, label need never have appeared in glyoxylate, even though its reduction product was highly active. The relative pool size of glyoxylic acid is almost certainly much less than that of glycollic acid, since the remaining unknown spots in $^{14}CO_2$ incorporation chromatograms are all very weak, and glyoxylic acid has not been identified in such experiments.

TOLBERT[26] has recently made the interesting observation that when chloroplasts were supplied with $^{14}CO_2$, glycollic acid was the only labelled compound to be secreted into the medium; no phosphorylated compounds appeared. It seems reasonable that if no phosphorylated sugars were able to pass through the chloroplast membrance, pyridine nucleotides would be similarly restricted, and Tolbert has suggested that glycollic acid may act as a hydrogen carrier between chloroplasts, in which photosynthetic reduction takes place, and other parts of the cell.

VISHNIAC'S observation[9] of a light-induced appearance of tritium from water in chlorophyll is not incompatible with the results here reported. He describes his incorporated isotope as "relatively labile" to exchange with water and suggests that it is the enolizable hydrogen on the isocyclic ring. Such label would have been lost by back exchange in the present procedure. An examination of the chromatograms in the lipid area revealed a level of tritium activity lower than anything observed in the phosphates and amino acids reported. This suggests that the compounds in this area (chlorophyll, carotenoids, and lipids) do not rapidly acquire tritium in nonexchangeable positions either by a cycling process or by new synthesis.

In spite of the many difficulties of manipulation and interpretation, it seems likely that the use of tritium will eventually enable us to learn something of the path of hydrogen, though not with the ease or completeness which studies with ^{14}C have permitted investigation of the path of carbon in photosynthesis.

SUMMARY

A study has been made of the incorporation patterns of tritium from tritium oxide by Chlorella cells under conditions of light and dark. A new apparatus has been designed for use in photosynthesis experiments which necessitate the employment of dense cell suspensions and substrates of high specific activity (1 C/ml). The incorporation patterns of $^{14}CO_2$ in the presence and absence of tritiated water showed little evidence of physiological damage due to radiation.

The substances incorporating tritium were essentially the same as those incorporating ^{14}C from $^{14}CO_2$. However, the percentage distribution of the tracer among the labelled compounds showed considerable differences from the carbon pattern. At the shortest incubation periods in the light, tritium appeared mainly in the sugar monophosphates, phosphoglyceric acid, aspartic acid, glutamic acid, and malic acid. These substances also incorporated label most rapidly in the dark, though in

this case a greater percentage of the activity fixed appeared in the amino acids. About three times as much tracer was fixed in the light as in the dark. The total activity fixed, and its distribution, was affected to some extent by the presence of ammonium or nitrate ions in the medium.

Glycollic acid was labelled very early, and was the most active compound present. A scheme for hydrogen transport in photosynthesis involving an alternate oxidation and reduction of glycollic and glyoxylic acids is proposed. The difficulties of interpreting the results of biochemical studies with hydrogen isotopes due to non-specific exchange reactions are discussed.

TABLE I

EFFICIENCY OF TRITIUM COUNTING

A sample of tritium-labelled glutamic acid of known counting rate was eluted from a chromatogram and aliquots of the eluate were counted with a windowless Scott tube after being dried onto aluminum planchettes. Further aliquots were counted in a scintillation counter of known efficiency (30% for tritium).

Means of counting	counts/min	efficiency (%)	relative counting rate
On paper, before elution, with windowless Scott tube	7,900	0.26	1.0
On aluminum planchette, after elution, with windowless Scott tube	104,000	3.48	13.2
Scintillation counter, after elution (reference point)	896,000	(30.0)	113.4
Absolute disintegration rate	2,987,000	100.0	378.1

TABLE II

EFFECT OF HIGH SPECIFIC ACTIVITY TRITIATED WATER ON THE INCORPORATION OF LABELLED CARBON DIOXIDE INTO SEVERAL SUBSTANCES

Cells were exposed in the light for 3 min to $NaH^{14}CO_3$ (40 μC/ml) with or without ^3H_2O (1 C/ml). Values are given as counts/min for the substances in a standard aliquot of the cell extracts (containing 83 μl of wet-packed cells).

Substrates	$^{14}CO_2$	$^{14}CO_2$ plus ^3H_2O
Dose of radiation received in 3 min (reps)	0.48	760.48
Sugar diphosphates	31,400	34,200
Sugar monophosphates	189,000	181,000
Phosphoglyceric acid	105,000	84,600
Uridinediphosphoglucose	28,100	21,400
Aspartic acid	13,500	7,700
Glutamic acid	3,230	2,100
Alanine	19,800	14,900
Malic acid	25,700	21,200
Fumaric acid	3,980	3,570
Glycollic acid	24,900	38,000
Sucrose	9,750	4.050
Total	453,000	412,000

TABLE III

INCORPORATION OF TRITIUM FROM TRITIATED WATER IN THE LIGHT AND DARK; EFFECT OF THE PRESENCE OF AMMONIUM CHLORIDE AND POTASSIUM NITRATE ON THE UPTAKE OF TRITIUM FROM TRITIATED WATER IN THE LIGHT

Cells suspended in 0.002 M KH_2PO_4 buffer, pH 7.05. Concentrations of NH_4Cl and KNO_3, 0.001 M. Cells incubated with ^3H_2O (1 C/ml) in the presence of unlabelled carbon dioxide for 3 min in the light or dark. The values given are the percentages of the total tritium fixed which appeared in each compound.

Light conditions	Light	Dark	Light	Light
Additions of salts	none	none	NH_4Cl	KNO_3
Sugar diphosphates	3.3	0.0	2.4	1.6
Sugar monophosphates	16.1	11.1	12.6	12.3
Phosphoglyceric acid plus phosphoglycollic acid	5.4	8.4	5.1	5.3
Phosphoenolpyruvic acid*	0.5	0.0	0.4	0.4
Uridinediphosphoglucose	2.6	2.9	1.2	3.7
Fumaric acid	1.6	**	1.9	0.9
Malic acid	18.4	3.6	22.2	7.8
Citric acid	0.7	0.0	1.5	1.1
Succinic acid*	1.9	1.3	1.2	1.5
(Glycollic acid*)	(1.4)	(**)	(0.3)	(0.3)
Aspartic acid	8.1	26.5	9.4	14.8
Glutamic acid	32.5	33.2	29.8	31.4
Serine plus glycine***	3.8	1.1	2.6	2.1
Alanine	4.1	12.0	7.1	12.1
Glutamine	0.8	0.0	1.3	3.2
Unidentified substance 1	0.0	0.0	1.3	1.1
Unidentified substance 2	0.2	0.0	0.0	0.0
Unidentified substance 3	0.0	0.0	0.0	0.6
Total in sugar phosphates	27.9	22.4	21.7	23.3
Total in organic acids	22.6	4.9	26.8	11.3
Total in amino acids	49.3	72.8	50.2	63.6
Total in unidentified substances	0.2	0.0	1.3	1.7
Total activity fixed (μC/ml of wet-packed cells)****	32.4	17.1	33.8	34.2

* Identity not confirmed by cochromatography with authentic marker

** Trace of blackening on film.

*** Activity mainly in serine.

**** Excluding glycollic acid; determined by summation of the activities of all the spots on the chromatograms.

TABLE IV

ACTIVITY INCORPORATED INTO GLYCOLLIC ACID IN THE LIGHT AND DARK

The relative densities of film blackening due to glycollic acid in the light and dark as determined by densitometric measurements are given. The blackening due to aspartic acid is given for comparison, together with the actual counting rate for aspartic acid from the two chromatograms. All film densities were referred to the density of aspartic acid in the light. For experimental details, see Table III.

Sample	Film Densities		Counting rate
	Glycollic acid	Aspartic acid	Aspartic acid (counts/min)
Incubated in the light	16.5	(1.0) (reference point)	1951
Incubated in the dark	2.5	1.5	3347
Ratio: $\frac{\text{light sample}}{\text{dark sample}}$	6.6	0.67	0.58

TABLE V

KINETIC STUDY OF THE INCORPORATION OF TRITIUM FROM TRITIATED WATER IN THE LIGHT

The cells, suspended in distilled water, were incubated in the light with ^3H_2O (1 C/ml) in the presence of unlabelled carbon dioxide for the periods shown below. The values given are the percentages of the total tritium fixed which appeared in each compound.

Incubation period (sec)	5	10	30	60	180
Sugar diphosphates		8.1	12.4	9.4	4.7
Sugar monophosphates	31.4	24.7	16.2	16.4	12.6
Phosphoglyceric acid plus phosphoglycollic acid	10.3	8.3	5.2	5.2	3.1
Uridinediphosphoglucose					2.1
Fumaric acid					1.0
Citric acid					0.7
Malic acid	15.5	13.6	26.3	19.0	15.2
(Glycollic acid*)	(**)	(**)	(1.1)	(1.4)	(3.1)
Aspartic acid	15.9	13.1	11.5	9.1	6.5
Glutamic acid	26.8	19.7	22.8	23.6	32.1
Alanine		5.5	5.5	7.4	6.7
Serine plus glycine***		5.7		6.9	8.1
Sucrose*		1.3		3.0	7.2
Total in sugar phosphates	41.7	41.1	33.8	31.0	22.5
Total in organic acids	15.5	13.6	26.3	19.0	16.9
Total in amino acids	42.7	44.0	39.8	47.0	53.4
Total in sugars	0.0	1.3	0.0	3.0	7.2
Total activity fixed (μC/ml wet-packed cells)****	1.3	2.5	5.5	8.4	24.1

* Identity not confirmed by cochromatography with authentic marker

** Blackening on film but no appreciable count.

*** Activity mainly in serine.

**** Excluding glycollic acid; determined by summation of the activities of all the spots on the chromatograms.

TABLE VI

KINETIC STUDY OF THE INCORPORATION OF TRITIUM FROM TRITIATED WATER IN THE DARK

The cells, suspended in distilled water, were incubated in the dark with $^{3}H_2O$ (1 C/ml) in the presence of unlabelled carbon dioxide for the periods shown below. The values given are the percentages of the total tritium fixed which appeared in each compound.

Incubation period (sec)	6.8	30	60	180
Sugar diphosphates	1.9			0.6
Sugar monophosphates	14.4	11.5	9.4	7.7
Phosphoglyceric acid	7.9	10.6	4.7	8.1
Phosphoenolpyruvic acid*		0.6	0.1	0.1
Phosphoglycollic acid*		2.1	4.9	0.3
Uridinediphosphoglucose			2.5	2.4
Malic acid	4.1	4.0	6.0	2.5
Citric acid	1.2	1.5	1.6	0.3
(Glycollic acid*)	(0.4)	(0.4)	(0.1)	(0.1)
Aspartic acid	48.3	41.7	31.5	30.9
Glutamic acid	16.5	19.9	25.9	32.4
Alanine	5.7	7.9	13.1	13.8
Glutamine				0.6
Unidentified substance 1		0.4	0.3	0.1
Unidentified substance 2				0.4
Total in sugar phosphates	24.2	24.8	21.6	19.2
Total in organic acids	5.3	5.5	7.6	2.8
Total in amino acids	70.5	69.5	70.5	77.7
Total in unidentified substances		0.4	0.3	0.5
Total activity fixed (μC/ml wet-packed cells)**	1.4	4.3	7.1	13.2

* Identity not confirmed by cochromatography with authentic marker.

** Excluding glycollic acid; determined by summation of the activities of all the spots on the chromatograms.

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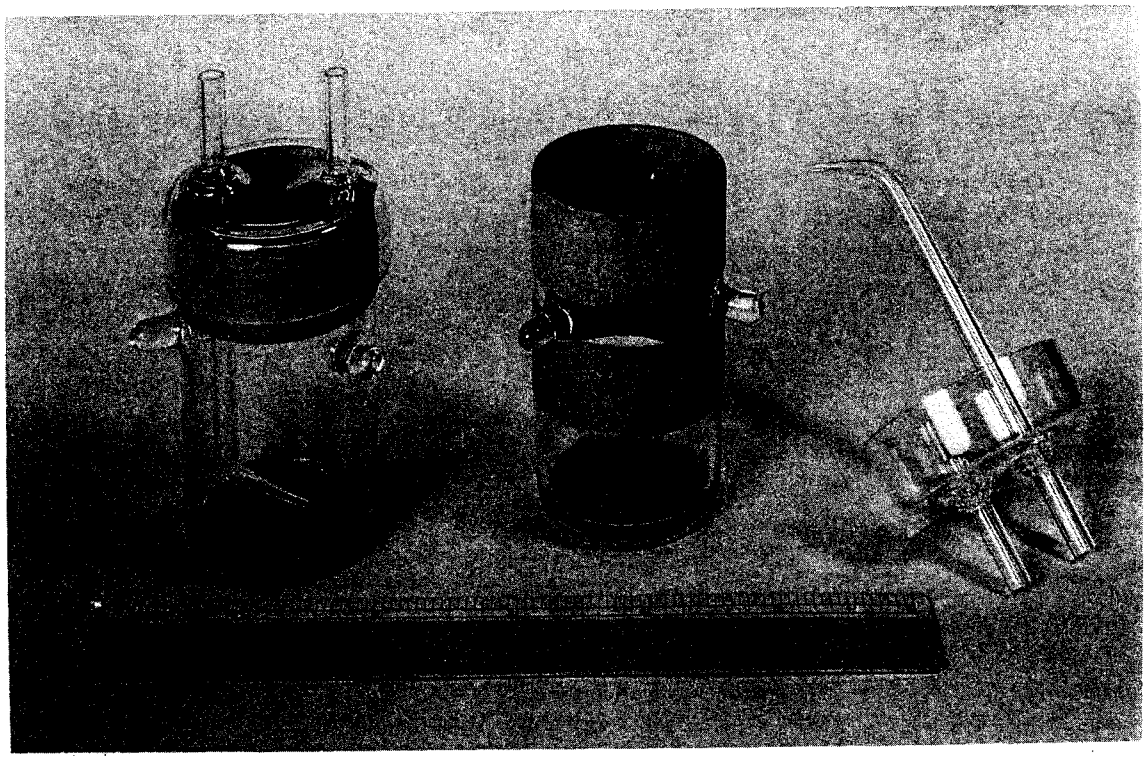
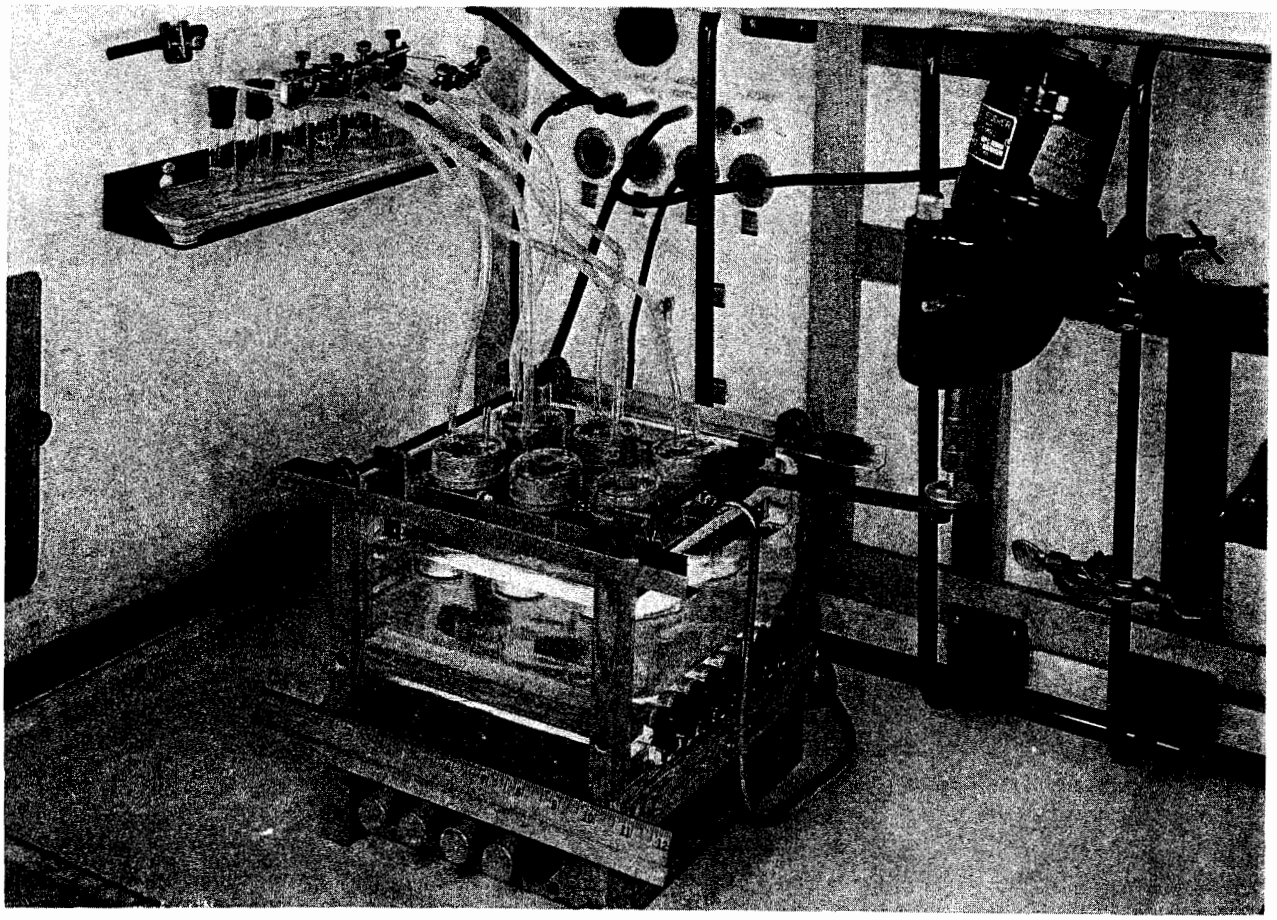


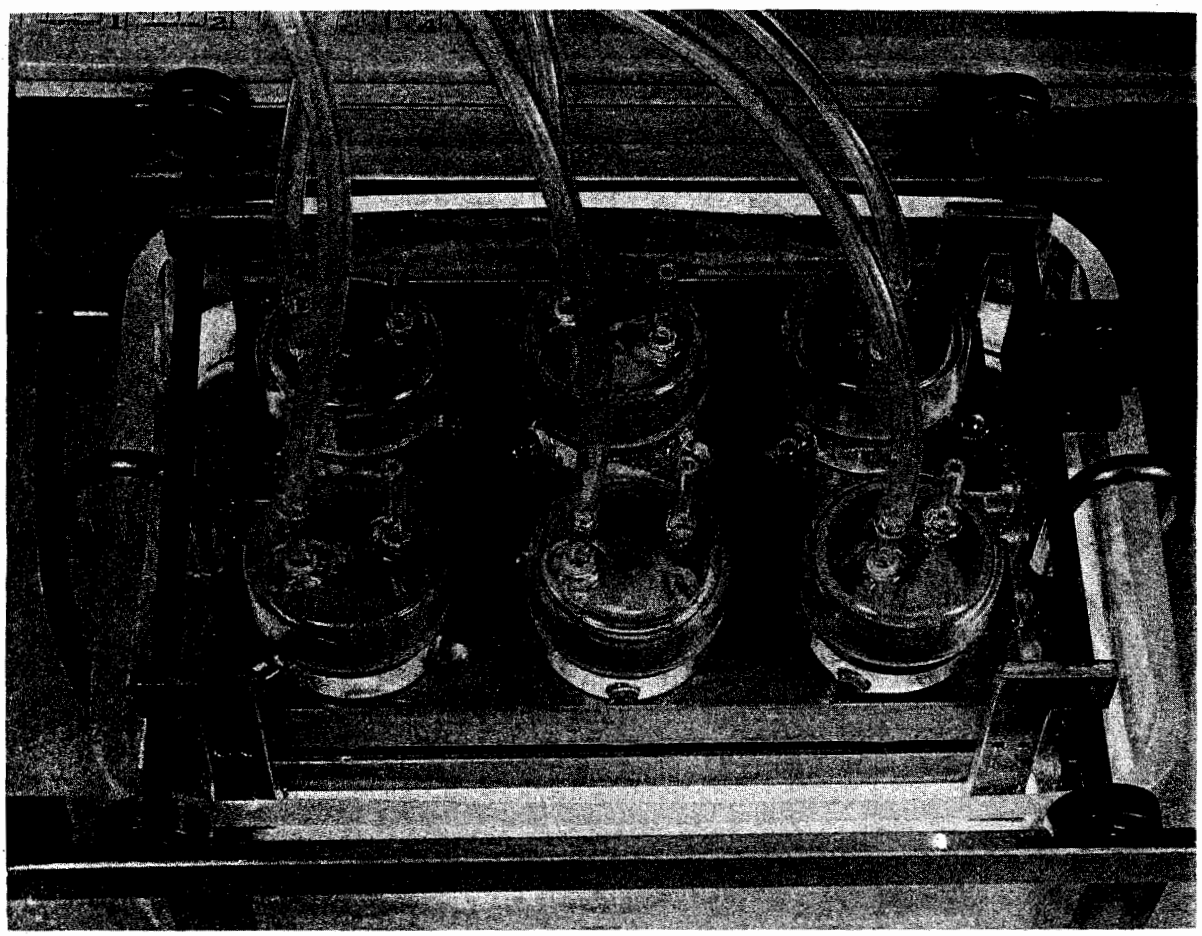
ZN-1867

Fig. 1. Close-up of glass vessels for incubation of algae, showing details of lids.



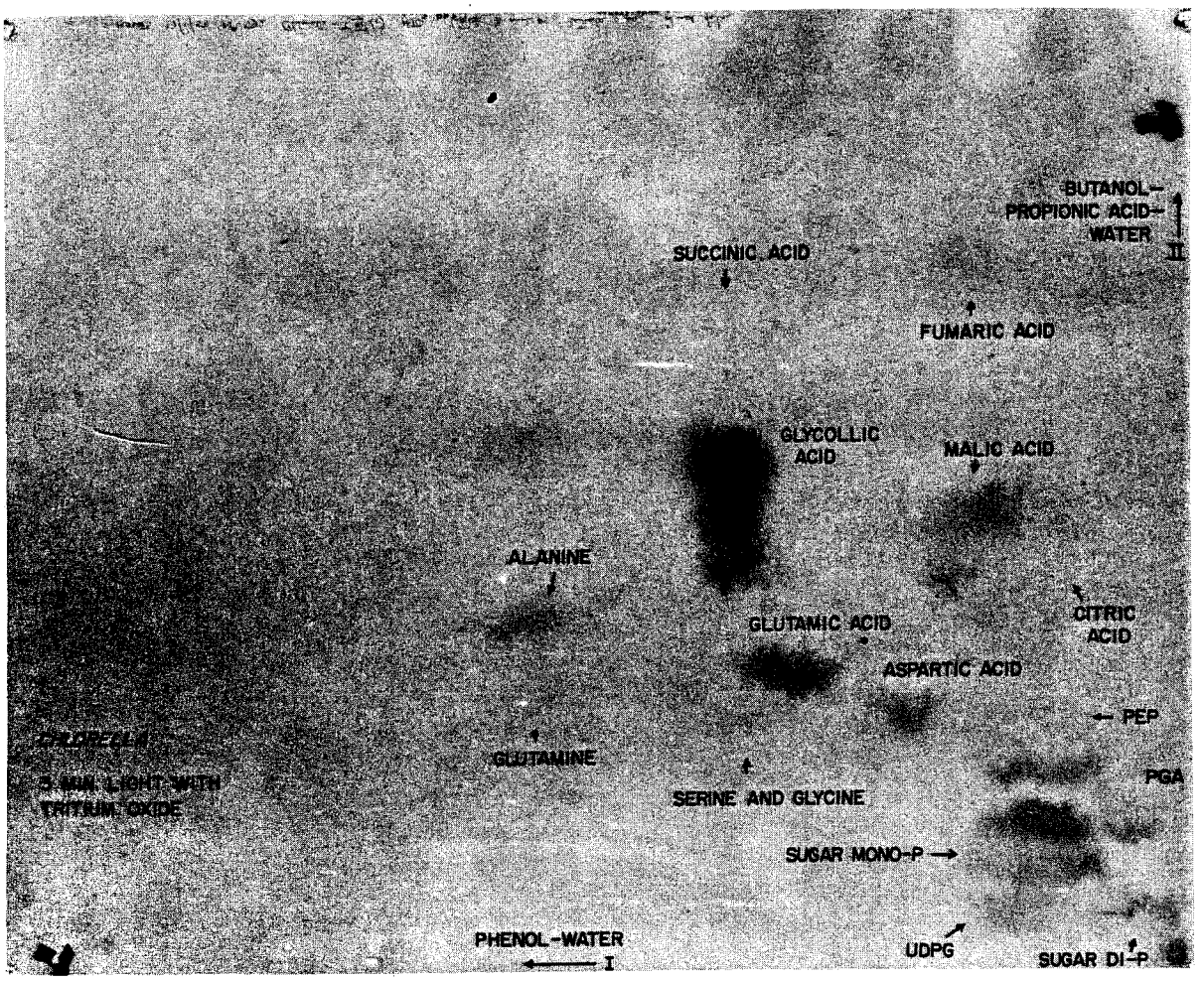
ZN-1868

Fig. 2. General view of apparatus, showing shaker, motor and bubbling assembly.



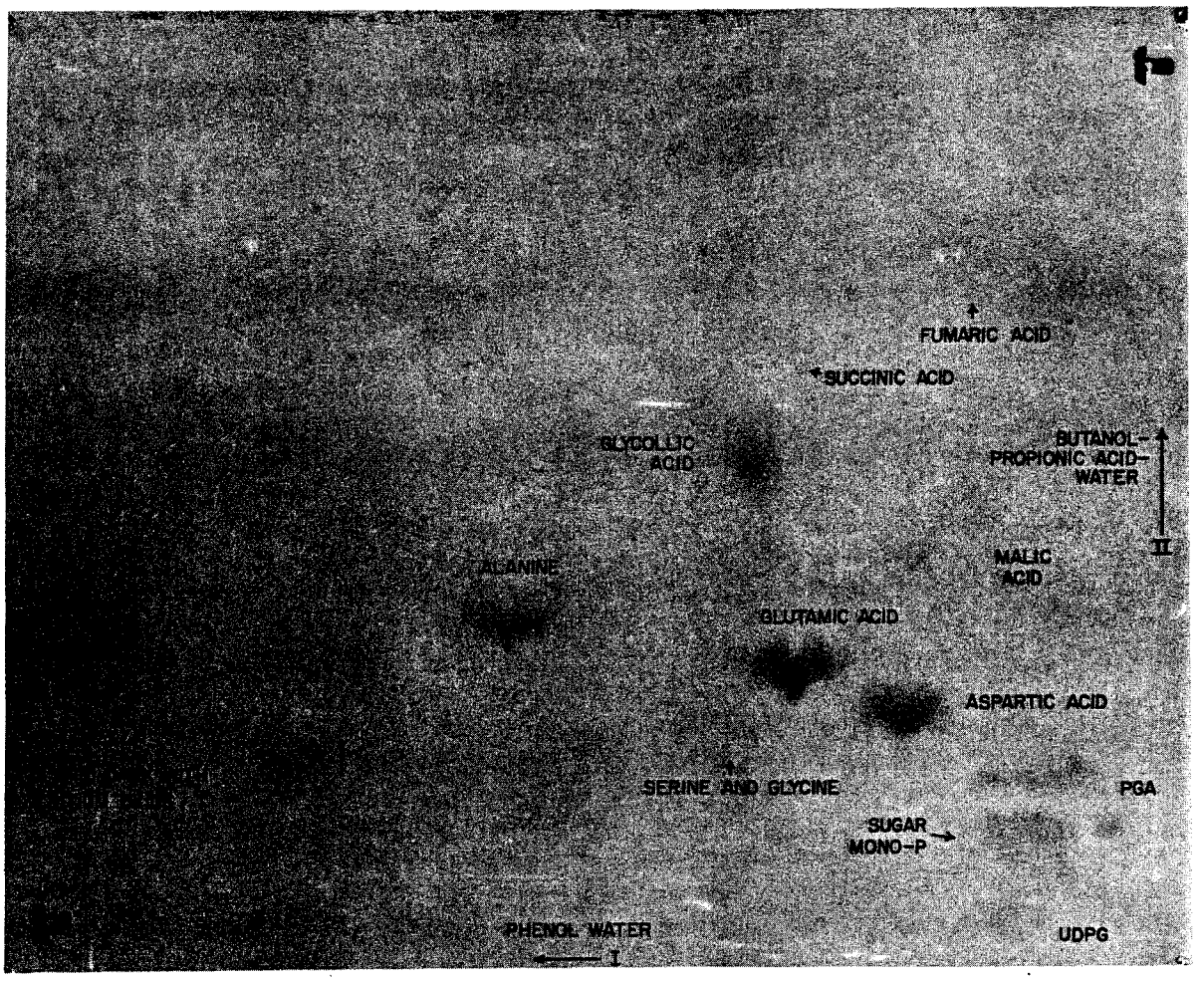
ZN-1869

Fig. 3. Close-up of shaker and glass incubation vessels from the top.



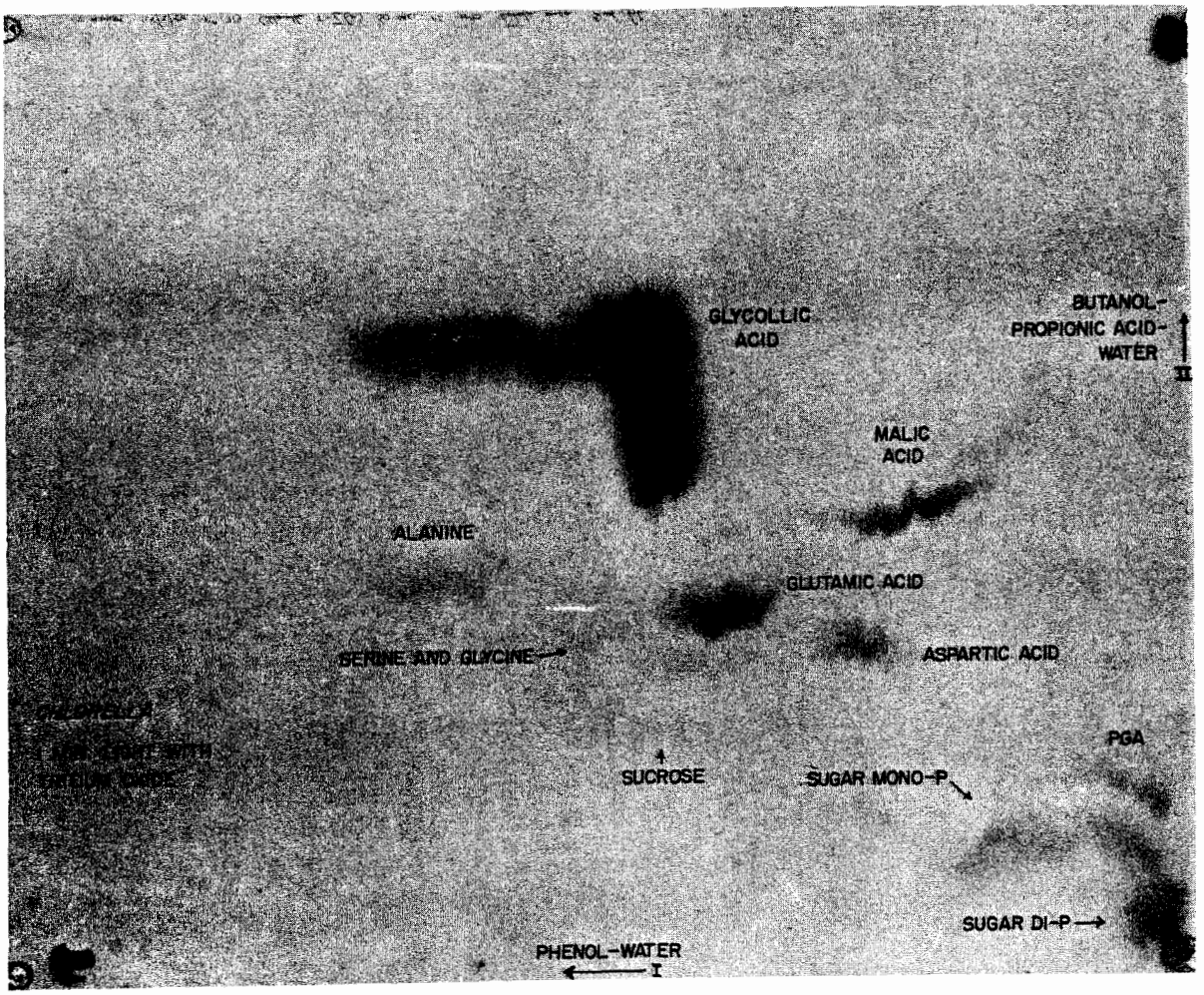
ZN-2008

Fig. 4. Radioautogram of extract from Chlorella exposed to tritiated water for 3 min in the light. Key to abbreviations: Sugar mono-P, sugar monophosphates; PEP, phosphoenolpyruvic acid; PGA, phosphoglyceric acid; UDPG, uridinediphosphoglucose; Sugar di-P, sugar diphosphates.



ZN-2006

Fig. 5. Radioautogram of extract from Chlorella exposed to tritiated water for 3 min in the dark. Key to abbreviations: see caption to Fig. 4.



ZN-2007

Fig. 6. Radioautogram of extract from Chlorella exposed to tritiated water for 1 min in the light. Key to abbreviations: see caption to Fig. 4.