Carbon Dioxide Uptake Studies in Algae Grown in Water and Deuterium Oxide

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A procedure is described for studying carbon dioxide uptake in algae using C^{14}-labeled sodium bicarbonate as the source of carbon dioxide. Actively dividing, water grown and deuterium oxide adapted, *Scenedesmus obliquus* and *Chlorella vulgaris* were employed in these studies. Uptake comparisons were made over pH range 6 to 9 using appropriate buffer systems. Uptake was fairly constant in the range pH 6 to 8 for both the aqueous and deuterated algae. Above pH 8 uptake dropped markedly. In general, the deuterated algae showed between 10 and 30% lower uptake than ordinary algae. Greater chlorophyll content is associated with higher carbon dioxide uptake.

RECENT REPORTS (1, 2) indicate that it is possible to culture certain algae in a medium containing better than 99% deuterium oxide. Algae grown under these conditions are a practical source of fully deuterated compounds. A number of monosaccharides and amino acids have been isolated from a hydrolysate of deuterated algae (3). The isolation and characterization of deuterated chlorophylls a and b from chloroplasts of algae and some preliminary experiments on photosynthesis in deuterated algae have also been described (4). The present report deals with further studies on photosynthesis in ordinary and deuterated green algae. Carbon dioxide uptake comparisons are made here between ordinary water and deuterium oxide-grown algae.

The influence of the kinetic isotope effect on photosynthesis is probably among the most significant differences in the culturing of algae in water and deuterium oxide. It is hoped that such comparative studies as are reported here will aid in a better understanding of the complexities involved in the conversion of carbon dioxide to organic compounds by the photosynthetic process.

EXPERIMENTAL

Algae Cultures.—*Scenedesmus obliquus* and *Chlorella vulgaris* were grown autotrophically as stock cultures in 250-ml. culture flasks; there were two such cultures for each organism in both aqueous and deuterated media. The composition of the media has previously been given (2). The volume of the cultures was maintained at 125 ml. The flasks were kept on a mechanical shaker and the algae were continuously illuminated by overhead fluorescent lighting at an intensity of about 700-ft. candles measured at the level of the algae culture. Carbon dioxide was fed as a 5% mixture in nitrogen. The cultures were maintained at room temperature (25–27°).

An algae suspension for an experiment was prepared by centrifuging the harvested cells for 5 minutes at full speed in an International clinical centrifuge. The supernatant liquid was discarded, and the cells were washed once with water or deuterium oxide. The cells were resuspended in fresh solvent, and the packed cell volume was determined by centrifuging for 20 minutes. In all experiments a 1% suspension of algae in buffer solution was prepared by suspending 0.5 ml. of packed cells in 50 ml. of buffer. Only algae from rapidly growing cultures were used in these studies since older cultures were far less active in photosynthesis.

Photosynthesis Flask.—A specially constructed flask was used for the carbon dioxide uptake studies. A ground-glass standard-taper 125-ml. Erlenmeyer flask (male joint) was fitted with a cap (female joint) having a tapered opening extending about 2 cm., over which was fitted a rubber injection-type stopper. Aliquots of the solution within the flask were conveniently removed by hypodermic syringe without loss of carbon dioxide from the system. Experimentation showed that no measurable diffusion of carbon dioxide through the rubber stopper occurred over an 8-hour period. The cap was held tightly to the flask by springs fixed to glass prongs. A circular glass shelf, about 1 cm. in diameter and having a lip 4 mm. high, was fitted to the inner wall of the flask just above the 50-ml. mark. The flask was fitted with a jacket, covering the base and side of the flask above the 50-ml. mark. The algae suspension was maintained at 26° by passing water from a constant temperature water bath through the jacket. The temperature of the algae suspension was determined by inserting a thermometer into the well provided on the side of the flask. Figure 1 shows a diagram of the flask.

$NaHC^{14}O_3$ Stock Solution.—A stock solution of C^{14}-labeled sodium bicarbonate was prepared by dissolving 2 mc. of isotope in 4 ml. of pH 9 phosphate buffer solution. The solution was assayed by diluting a 25-λ aliquot to the mark of a 50-ml. volumetric flask. A 100-λ aliquot of this solution was added to 15 ml. of liquid scintillator in a 5-dr. vial and the system was counted in a Tri-Carb

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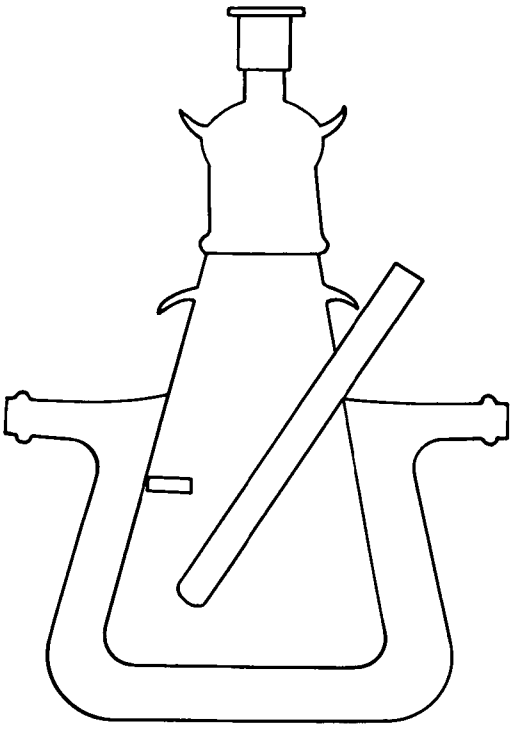

Fig. 1.—Schematic diagram of photosynthesis flask.

liquid scintillation spectrometer (model 314). A count rate of about 30,000 counts/min./100 λ was observed. This was compared to a standard.

General Procedure.—In all photosynthetic experiments 50 mg. of sodium bicarbonate and 25 λ of $NaHC^{14}O_3$ solution (12.5 μc.) were placed on the shelf of the photosynthesis flask. The algae suspension, prepared as described above, was added to the flask, below the shelf, with the aid of a long stem funnel. The flask was capped and, at zero time, was shaken vigorously to distribute the sodium bicarbonate and isotope throughout the algae suspension. The flask was placed on the shaker where the algae were permitted to photosynthesize. At 90-minute intervals for a total of 6 hours a small aliquot was removed from the flask by hypodermic syringe, centrifuged for 3 minutes, and 100 λ of the supernatant solution was counted.

Calibration Curves.—*Aqueous System.*—At pH 7.0 and below, a substantial portion of the sodium bicarbonate is present as carbon dioxide, part of which is in solution and part in the gas phase. The equilibria involved may be illustrated as follows

$[CO_2]$ gas phase

$\Downarrow\Uparrow$

$[CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-]$ aqueous phase

$\Downarrow\Uparrow$

[CO_2 taken up by algae[1]]

A calibration curve was necessary in order to determine the amount of carbon dioxide in the gas phase and the carbon dioxide plus other species in the liquid phase from a measurement of the C^{14} content of an aliquot of the liquid phase. Theoretically, this information could be obtained by calculation, but some of the necessary constants are not available for the deuterium oxide system. An empirical calibration curve supplied a more certain answer.

To the shelf of each of a series of 5 flasks was added respectively: 50 mg., 40 mg., 30 mg., 20 mg., and 10 mg. of sodium bicarbonate. This was followed by the addition of 25 λ of $NaHC^{14}O_3$ solution to the shelf of each flask. Fifty milliliters of buffer solution was added to the flasks below the shelf. The flasks were capped and shaken mechanically for 90 minutes. An aliquot of 100 λ was counted. Since the specific activity (counts/min./mg.) of the sodium bicarbonate was known, the amount of sodium bicarbonate in the aqueous phase was readily determined. The amount of carbon dioxide, as sodium bicarbonate equivalent, in the gas phase was then known. As an illustration, when 50 mg. of sodium bicarbonate and 25 λ of $NaHC^{14}O_3$ solution were used in pH 6.0 buffer, the specific activity was 300,000 counts/min./mg. of sodium bicarbonate. After equilibration, the activity in the liquid phase was observed to be 8,500 counts/min./100 λ or 4,250,000 counts/min./50 ml. This represented 14.2 mg. of sodium bicarbonate in the aqueous phase and 35.8 mg. of sodium bicarbonate equivalent in the gas phase as carbon dioxide. Corresponding values were obtained for the other weights of sodium bicarbonate which were employed. A calibration curve was constructed by plotting mg. of sodium bicarbonate equivalent in the gas phase *vs.* mg. of sodium bicarbonate in the aqueous phase. A straight line was obtained which passed through the origin.

For pH 6.0 a phosphate buffer system (KH_2PO_4 + NaOH) was used. For pH 7.0 a tris(hydroxymethyl)aminomethane-hydrochloric acid buffer system was used. The calibration curve for pH 7.0 with this buffer was not linear. More carbon dioxide was bound in the liquid phase than expected, due probably to the formation of carbamate. Since the calibration curves apply only to the vessels and conditions used in this study, the curves are not given here.

The "tris" buffer system was used for those studies at pH 8.0, 8.5, and 9.0. Since the carbon dioxide in the gas phase at pH values of 8.0 and above is negligible, a calibration curve was not necessary here. The observed activity in the aqueous phase of blank runs did not deviate from the theoretical value, and the direct count of the liquid phase was all that was required at these pH values.

Deuterium Oxide System.—The equilibria involved in the carbon dioxide uptake studies with deuterium oxide-grown algae are essentially the same as those involved in the aqueous system except that deuterium replaces hydrogen. Calibration curves were prepared as described for the aqueous system. The buffer solution corresponding to pH 6.0 was prepared from NaOD and KD_2PO_4. The apparent pH as observed with a Beckman pH-meter (model G) was 5.9. The buffer solutions in deuterium oxide (D_2O) corresponding to pH values of 7.0, 8.0, 8.5, and 9.0 were prepared from tris(hydroxymethyl)-aminomethane (in which all of the exchangeable hydrogen was replaced by deuterium) and DCl. All buffers in D_2O were prepared using the same stoichiometry as for the H_2O buffers. The apparent pH was measured with the pH-meter and an ap-

[1] Species other than carbon dioxide may be involved.

propriate correction (5) to the reading was made. The pD values of the buffer solutions in D_2O corresponding to the aqueous buffer values were 6.3, 7.3, 8.3, 8.8, and 9.3.

Effect of pH on Carbon Dioxide Uptake.—Carbon dioxide (or bicarbonate) uptake was studied over a 6-hour period. The algae were agitated continuously at 26° at a light intensity of 700-ft. candles. Comparison was made between algae grown in water and deuterium oxide over a pH range of 6.0 to 9.0. At pH values (and corresponding pD values) of 8.0, 8.5, and 9.0% uptake was determined directly from the counts/min./100-λ aliquot of the aqueous phase at zero time and the counts/min./100-λ aliquot after an interval of photosynthesis. It was assumed that at these pH values there was a negligible amount of carbon dioxide in the gas phase. For pH (and pD) values of 6.0 and 7.0 the carbon dioxide in the gas phase must be taken into account. The total count rate in the liquid phase was determined from the count rate in a 100-λ aliquot of the solution. The amount of bicarbonate in the liquid phase was readily determined, and the amount of sodium bicarbonate equivalent in the gas phase was ascertained from the calibration curve. The sum in the aqueous phase and the gaseous phase was then subtracted from the original amount. This represented the amount taken up by the algae. Per cent uptake was then calculated. The data for these experiments are shown in Figs. 2–5.

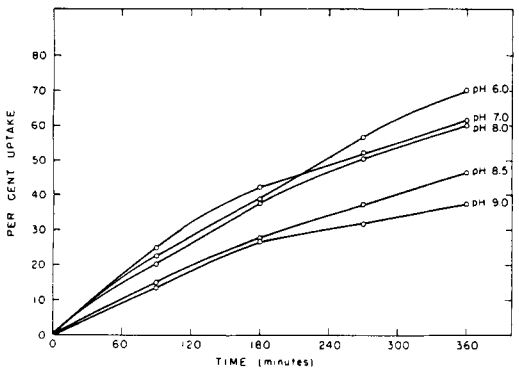

Fig. 2.—Uptake of $NaHC^{14}O_3$ by ordinary *C. vulgaris* in water as a function of pH and time.

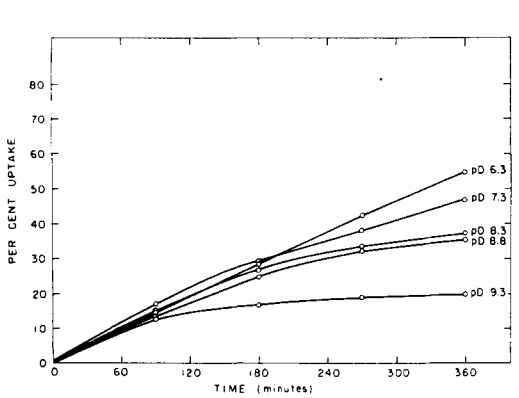

Fig. 3.—Uptake of $NaHC^{14}O_3$ by deuterated *C. vulgaris* in deuterium oxide as a function of pD and time.

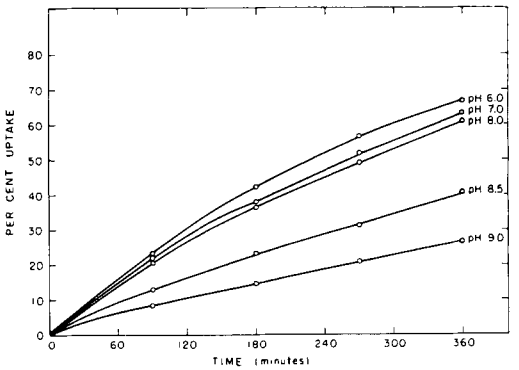

Fig. 4.—Uptake of $NaHC^{14}O_3$ by ordinary *S. obliquus* in water as a function of pH and time.

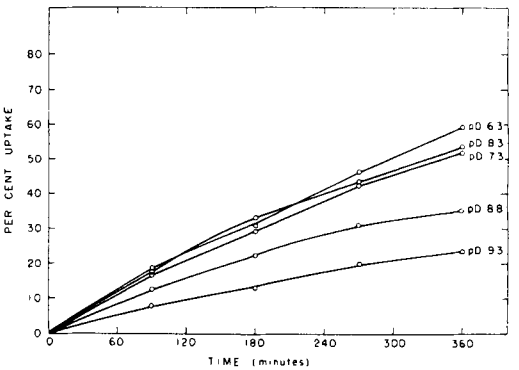

Fig. 5.—Uptake of $NaHC^{14}O_3$ by deuterated *S. obliquus* in deuterium oxide as a function of pD and time.

Radioactive Excretion Products.—Tolbert and Zill (6) have reported that at pH values greater than 4.5 glycolic acid is an excretion product of *Chlorella*. Experiments were therefore conducted to determine whether significant amounts of glycolic acid or other radioactive organic compounds were excreted into the medium in these studies. Samples of algae suspensions were centrifuged and the supernatant liquid transferred to a second tube. Concentrated hydrochloric acid was added (50 λ) and the solution was flushed with nitrogen. A 250-λ aliquot was then counted. In no instance was the count rate sufficiently above background to indicate the presence of significant amounts of radioactive metabolic products. This was further confirmed by applying the color reaction with 2,7-dihydroxy naphthalene as described by Calkin (7) and used by Tolbert and Zill in their work.

Chlorophyll Content.—The chlorophyll content of the algae used in this study was determined. Exactly 0.5 ml. of wet packed cells (representing 0.1 Gm. of dried cells) was transferred to a 15-ml. centrifuge tube. About 10 mg. of sodium bicarbonate was added, followed by the addition of 10 ml. of actively boiling water. The tube was then placed into boiling water for 1 minute, cooled under tap water, and centrifuged. The supernatant liquid was discarded. Ten milliliters of a mixture of ethyl ether (2 volumes) and methanol (1 volume) was added, and the tube was shaken vigorously until its contents were

thoroughly mixed. The tube was centrifuged and the supernatant liquid was transferred to a separator. This extraction procedure was repeated twice. An additional 30 ml. of ether-methanol solvent mixture was added to the separator and the solution was extracted several times with saturated salt solution, followed by extraction with distilled water. The chlorophyll layer was transferred to a 100-ml. volumetric flask and was diluted to the mark with ether. Absorption was determined with a Cary model 14 recording spectrophotometer at 660.0 and 642.5 mμ, and both total chlorophyll and chlorophyll a were calculated by the method of Comar (8). The data are recorded in Table I.

TABLE I.—DETERMINATION OF TOTAL CHLOROPHYLL AND CHLOROPHYLL a IN DEUTERIUM OXIDE AND WATER-GROWN ALGAE

Algae	Total Chlorophyll,[a] %	Chlorophyll a,[a] %	Total Chlorophyll D_2O/H_2O	Chlorophyll a, D_2O/H_2O
C. vulgaris				
H_2O	3.44	2.57	0.779	0.747
D_2O	2.68	1.92	...	...
S. obliquus				
H_2O	2.45	1.84	0.955	0.880
D_2O	2.34	1.62	...	...

[a] Grams per 100 Gm. of dried algae.

DISCUSSION

The availability of fully deuterated algae has made possible a comparative study of photosynthesis in both deuterium oxide-adapted and water-grown algae. The replacement of hydrogen by deuterium probably affects the relative rates of reactions involved in photosynthesis, and does not interfere with the pathways by which carbon dioxide is converted to organic compounds. This is substantiated by the fact that the same metabolites, but which contain deuterium where hydrogen is normally found, have been isolated (3). Identical, but deuterated, chloroplast pigments have also been extracted (4). In other studies (9) essentially similar radioautograms have been obtained using the techniques described by Bassham and Calvin (10). Although similar compounds were identified, it is quite possible that the relative abundances may differ. The course of photosynthesis, at least in its earlier phases, appears to be substantially the same in both varieties of algae.

Figures 2–5 show carbon dioxide uptake by deuterated and water-grown algae as a function of time and pH (or pD). It is apparent that the water-grown algae take up carbon dioxide to a greater extent than the deuterated algae over a 6-hour study period. Greater differences are to be noted between the *Chlorella* cultures than between the *Scenedesmus* organisms. Although there is a greater disparity in uptake between the aqueous and deuterated *Chlorella*, it is interesting to note that in an earlier report (2) it was shown that adaptation in deuterium oxide occurs more readily with *Chlorella* than with *Scenedesmus*.

In general, under the conditions of the experiments described here, deuterated algae showed between 10 and 30% lower uptake than ordinary algae. The influence of variation in pH on bicarbonate uptake by ordinary and deuterated algae was studied. Since the electrochemistry of D_2O and H_2O is different, it is essential to distinguish between kinetic isotope effects and changes in uptake rate due to pH sensitivity. Solutions in H_2O and D_2O made up stoichiometrically identical will have different ion concentrations and activities. Suitable corrections were made for the observed values in the deuterium oxide systems. Uptake was fairly constant in the range pH 6 to 8 for both ordinary and deuterated algae. The uptake is thus insensitive to pH changes in this range. Therefore, differences in uptake are attributable to metabolic effects involving deuterium rather than pH sensitivity. Above pH 8, uptake dropped markedly and at pH 9, the algae showed signs of damage in a short period of time.

The question of whether carbon dioxide, bicarbonate ion, or both are utilized by algae as a source of carbon has been considered by numerous workers. Evidence in the literature is very conflicting. This matter is discussed by Krauss (11). Emerson and Green (12) claim that sufficient carbon dioxide is released in carbonate-bicarbonate buffer systems for photosynthesis even at high pH values. The equilibrium curve shows that at pH values above 8, only traces of carbon dioxide are present and, therefore, it appears improbable that sufficient carbon dioxide is released to satisfy the requirements of algae cultures. Osterlind (13) has found that *Scenedesmus quadricauda* assimilates bicarbonate ion while *Chlorella pyrenoidosa* does not. On the basis of the present report it seems probable that bicarbonate is acceptable to the algae in the alkaline pH region. For example, at pH 8.0, where free carbon dioxide is practically negligible, satisfactory photosynthesis was observed. At pH 8.5 and above, the alkalinity of the solution was found to have a detrimental effect on photosynthesis and growth. One can also speculate that since a "tris" buffer was employed in much of this work, carbamate may also be capable of being absorbed by the algae. Finally, the possibility exists that algae may possess two independent mechanisms for assimilating carbon: (*a*) one for carbon dioxide and (*b*) another independent path for bicarbonate ion. In an occasional run it was noted that while the algae consumed carbon dioxide normally at pH values around 7, they were refractory to bicarbonate uptake at pH 8.

Preliminary experimentation indicated that the absolute amount of sodium bicarbonate consumed by the algae over a time period was independent of the initial concentration. This was studied over the range of starting concentrations from 25 to 200 mg.

Experiments were conducted to determine whether significant amounts of glycolic acid, as reported by Tolbert and Zill (6), or other C^{14}-labeled compounds were excreted into the medium under the conditions of these studies. Tolbert and Zill used essentially weightless amounts of sodium bicarbonate while in the present study macroscopic quantities of bicarbonate, mainly nonlabeled, were employed. This precluded carbon dioxide starvation which may be responsible for glycolic acid excretion. Radioactive excretion products were not detected in our studies.

The relationship of chlorophyll content and carbon dioxide uptake was examined. Total chlorophyll and chlorophyll a were determined for both the aqueous and deuterated algae. As shown in Table I, the ratio of chlorophyll content for the deuterated to that of the aqueous algae approximates the carbon dioxide uptake relationships shown by the uptake curves. Greater differences are to be noted between the *Chlorella* cultures than between the *Scenedesmus* organisms, which is consistent with the results from the uptake studies.

The procedure described in this report for studying carbon dioxide uptake is a simple one and reproducible results within 5% were obtained when duplicate runs were conducted. Further work is currently in progress in this laboratory to extend these studies to include the effect of variation in light intensity and temperature on the photosynthesis of deuterated algae.

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Determination of Barbiturates in Urine Containing Salicylate

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A procedure is described in which urine pigments, salicylate, and other chromogenic materials which preclude spectrophotometric evaluation of barbiturate by virtue of their strong absorption in the ultraviolet region are removed from a chloroform solution by selective adsorption.

THE EXTRACTION from urine specimens of salicylate and other substances together with barbiturates has been a problem frequently encountered during toxicologic analysis. At the present time, the quantitative determination of barbiturates in biological material is best accomplished by means of ultraviolet spectrophotometry (1, 2). Analytical procedures commonly employed involve preliminary extraction of the malonylurea derivative from the specimen. by an immiscible solvent such as ether or chloroform. The spectral characteristics of the malonylurea molecule are most definitive in alkaline solution, which may be obtained by re-extraction of the barbiturate from an immiscible solvent into aqueous alkali. Normal physiological constituents which absorb strongly in the ultraviolet region and other drugs including salicylates are often extracted along with the barbiturate. The presence of these nonbarbiturate chromogens in the final alkaline extract precludes definitive spectrophotometric evaluation of the malonylurea compound unless steps are taken to remove the interference.

The attempts by various investigators to eliminate interfering substances by use of lengthy, complicated extraction procedures which resulted in loss of sensitivity, low recoveries, and failure to eliminate nonbarbiturate chromogens have been criticized (3). Differential spectrophotometric techniques (3–5) in which determination of barbiturate is based on the differences in the ultraviolet absorption spectra of strongly alkaline and pH 10–10.5 buffered solutions represent a significant improvement over older methods employing conventional spectrophotometry. It has been reported (3) that ultraviolet-absorbing substances including the drugs

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