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THE PATH OF CARBON IN PHOTOSYNTHESIS

BY

M. Calvin and A. A. Benson

8 March 1948

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Iowa State College	47
Los Alamos	48-50
Madison Square Area	51
Massachusetts Institute of Technology	52
Monsanto Chemical Company, Dayton	53
National Bureau of Standards	54-55
Patent Advisor	56
Library Branch (for NEPA), Oak Ridge	57
Library Branch, Oak Ridge	58-72
University of California, Radiation Laboratory	73-76
Chemistry	77
Latimer	78
Declassification Procedure	79-88

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THE PATH OF CARBON IN PHOTOSYNTHESIS

by

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March 3, 1948

ABSTRACT

The dark fixation of carbon dioxide by green algae has been investigated and found to be closely related to photosynthesis fixation. By illumination in the absence of carbon dioxide followed by treatment with radioactive carbon dioxide in the dark, the amount fixed has been increased ten to twenty fold. This rate of maximum fixation approaches photosynthesis maximum rates. The majority of the radioactive products formed under these conditions have been identified and isolated and the distribution of labeled carbon determined. From these results a tentative scheme for the mechanism of photosynthesis is set forth.

This paper is based on work performed under Contract No. W-7405-eng-48 with the Atomic Energy Commission in Connection with the Radiation Laboratory, University of California, Berkeley, California

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THE PATH OF CARBON IN PHOTOSYNTHESIS

By

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8 March 1948

In a previous note we described the effect of prior illumination on the dark reduction of carbon dioxide by green algae (*Chlorella* (1)). The essential experimental factor noted then was a large increase in the amount of carbon dioxide reduced in the dark by the algae which had been just previously illuminated in the absence of any carbon dioxide. Furthermore, some of this reduced radiocarbon was found in positions other than carboxyl groups or the number 3 and 4 carbon atoms of the hexose molecule. This evidence seemed to preclude the possibility that the dark reactions there observed could have been due only to the reversibility of the only two carboxylation reactions yet known to be reversible, namely the beta carboxylation of pyruvic acid and the beta carboxylation of ketoglutaric acid (2-4). These observations were therefore taken to indicate the ability of the green algae to accumulate a certain amount of reducing power during illumination in the absence of carbon dioxide which could later be used for the reduction of carbon dioxide. The observation by McAlister and Meyers (5) of a con-

(*) This paper is based on work performed under contract No. W-7405-Eng.-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley.

- (1). A. A. Benson and M. Calvin, *Science* 105, 648 (1947)
- (2). H. G. Wood, *Physiol. Rev.*, 26, 198 (1946)
- (3). S. Ochoa, A. Mehler, and A. Kornberg, *J. Biol. Chem.*, 167, 871 (1947)
- (4). B. Vennesland, J. Ceithmal, M. G. Gollub, *J. Biol. Chem.*, 171, 445 (1947)
- (5). E. D. McAlister and J. Meyers, *Smithsonian Inst. Pub. Misc. Collections*, 99, No. 6 (1940)

tinued uptake of CO_2 after the cessation of illumination in cases of high light intensity and low CO_2 partial pressure might well be the direct manifestation of this situation. In the observations of Hill and Scarisbrick (6-9) it was demonstrated that isolated green portions of the cell (chloroplasts and grana) can produce oxygen from water upon illumination in the presence of a suitable oxidizing agent other than carbon dioxide. It now appears clear that the path from carbon dioxide to reduced compounds, such as carbohydrates, fats, proteins, and amino acids, does not include the primary photochemical act itself.

The present note represents a more extensive study of the factors influencing the dark reduction including another organism (*Scenedesmus*) as well as the beginning of the next stage of this investigation, namely the products formed during a very short period (30 seconds) of photosynthesis. The dark uptake of C^{14}O_2 by *Chlorella* into non-volatile products as a function of time was first investigated by Ruben and coworkers. (10). These authors felt that the amount of carbon dioxide taken up reached a maximum after some 60 to 80 minutes of contact. More recently Gaffron, Fager, and Brown (11) have shown that this is not the case. By continuing the observations for very long periods, up to 12 or 15 hours, they showed a continuously increasing amount of dark C^{14}O_2 uptake. The present investigation shows (Figure 1) that this uptake curve actually consists of two distinct portions, the relative importance of which is very sharply dependent upon the prehistory of the algae. Curve A in Figure 1 represents the dark fixation of C^{14}O_2 by *Scenedesmus* which had been pretreated in the dark with carbon dioxide. Curve B, Figure 1, represents the behavior of the identical cells after they had been pre-illuminated for ten minutes in the absence of carbon

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- (6). R. Hill, and R. Scarisbrick, *Nature*, 146, 61 (1940)
 - (7). O. Warburg, and W. Lüttgens, *Naturwissenschaften*, 32, 161, 301 (1944)
 - (8). S. Aronoff, *Plant Physiol.*, 21, 393 (1946)
 - (9). A. S. Holt, and C. S. French, *Arch. Biochem.*, 9, 25 (1946)
 - (10). S. Ruben, M. D. Kamen, and W. Z. Passid, *J. Am. Chem. Soc.*, 62, 3443 (1940)
 - (11). H. Gaffron, E. W. Fager, and A. Brown, Reported at Isotope Symposium, University of Wisconsin, Madison 1947.
-

dioxide. The slow terminal rise in both curves might well be due to fermentative fixation analogous to that observed in a variety of animal tissues during respiration (12,13). However, the initial extremely rapid fixation observed in Curve B (after pre-illumination) is quite obviously closely connected with the effect of the light, and its initial slope is approaching that of steady state photosynthesis. In our view, the initial rapid rise (Curve B, Figure 1) corresponds to the storage by the plant of reducing power generated during the pre-illumination period. We have examined the rate of generation of this reducing power as a function of the pre-illumination time by observing the amount of $C^{14}O_2$ fixed during a one-minute dark time. The results are shown in Figure 2. Although, in the particular set of data given in Figure 2, thirty seconds pre-illumination brings the reducing power to over 80% of its maximum value, we have observed cases in which the full maximum value is reached in the same time. It is clear that this maximum value is simply a steady state concentration of reducing power resulting from a balance between the rate of its photochemical production and the rate of its natural decay, represented by that portion of the curve in Figure 2 beyond the point at which the light is turned off. Similar curves showing the growth and decay of reducing power have been obtained with one day old chlorella cultures. However, when older (four day) cultures of Chlorella are used, the apparent rate of growth was somewhat slower, with a correspondingly more rapid rate of decay.

The maximum amount of carbon dioxide fixed is usually ten to fifteen times that fixed by the cells without pre-illumination. After complete decay, the reducing power could be repeatedly restored by illumination on the same culture.

(12). J. M. Buchanan and A. Baird Hastings, *Physiol. Rev.*, 26, 120 (1946)

(13). E. A. Evans, Jr., *A Symposium on Respiratory Enzymes*, 197, Madison 1942.

Our examination of the compounds into which the radiocarbon is fixed under a variety of conditions has been extended. This includes not only a greater variation in the fixation conditions, but also a more complete identification of the products for each condition. In Table I are shown the results of an analysis of the products obtained during a five minute dark fixation time following pre-illumination periods up to two hours.

Table I
DARK CO₂-FIXATION PRODUCTS OF CHLORELLA^a

Dark time--Five Minutes^b

PRE-ILLUMINATION ^c	NONE	5 MINUTE	60 MINUTE	120 MINUTE
Total Fixed relative units	1	~10	~10	~10
I CARBOXYLIC ACIDS in ether extract ^d	52%	21%	14%	11%
MALIC ACID ^e	16%	11.5%	7.4%	
SUCCINIC ACID ^e	5.2%	3.1%	0.5%	
II AMINO ACIDS ^f adsorbed on cation resin	31%	41%	64%	74%
III ANIONIC SUBSTANCES ^g adsorbed on anion resin	16%	29%	21%	
IV SUGARS non-ionized compounds ^h	0.45%	1.0%	0.96%	1.0%

a) One day old cultures of *Chlorella pyrenoidosa*. b) The cells were killed rapidly by adding 20% by volume of glacial acetic acid-hydrochloric acid (4:1). All radioactive products were in aqueous phase within five minutes. Cells removed by filtration. c) One cc. packed cells per 60 ml. of nutrient solution was illuminated (infra-red removed) using 17,000 lux beams from both sides. A rapid stream of helium passed through the solutions during the experiments. The maximum fixation was not diminished by illumination periods as long as 17 hours. d) Rapid continuous 15 hour extractions. e) Separated by partition chromatography on silica gel column. f) Eluted from Duolite C-3 resin using 2.5 N hydrochloric acid. g) Eluted from Duolite A-3 resin using 1.5 N sodium hydroxide. h) Effluante from both exchange resins.

Since all of the carbon dioxide fixation induced by pre-illumination is complete in less than two minutes (see Curve B, Figure 1), a pair of experiments of the same type described in Table I were performed in which the dark fixation time was reduced to one minute. Pre-illumination times were chosen to insure maximum fixation rates. The results of these experiments are shown in the first two columns of Table II. In addition, two very short (30 seconds) normal photosynthetic experiments were performed with the two algae. Fractionation proceeded as indicated in Table I and the results are shown in columns three and four of Table II.

Fraction I in both the one minute dark fixations and the photosynthetic fixations was shown to be 75% malic acid. Fraction II is over 95% alanine in the dark fixations by *Chlorella*. Dark fixation by *Scenedesmus* and photosynthetic fixations by both algae produced less than 15% alanine in this fraction. Selective elution of the anion exchange resin gave two fractions. The fact that III-A (ammonium hydroxide eluate) was readily rendered resistant to ammonia elution by evaporation of the eluate in the presence of air suggested that this radioactive product was a reduced form of that obtained in III-B (sodium hydroxide eluate). The radioactive substance in fraction III-B of the ten minute pre-illuminated *Scenedesmus* was identified as phosphoglyceric acid. Hydrolysis by boiling 1 N hydrochloric acid gave radioactive glyceric acid which was adsorbed on the anion exchange resin and eluted with ammonium hydroxide. The glyceric acid was identified by preparing p-bromophenacylglycerate which was crystallized to constant specific activity. Solvent distribution of the derivative as well as the free acid gave distribution coefficients identical with those of authentic specimens. The radioactive substances in Fraction III-B formed during the thirty second photosynthesis in *Scenedesmus* were likewise shown to be more than 80% phosphoglyceric acid. Fraction III-A, after air oxidation in ammonia was found to contain over 90% phosphoglyceric acid and approximately four per cent sugar

Table II
DISTRIBUTION OF C¹⁴ IN ALGAE

Alga	Chlorella ^a		Scenedesmus ^b		Chlorella		Scenedesmus	
Pre-illumination time	60 minutes		10 minute		Photosynthesizing		Photosynthesizing	
Fixation time	1 minute dark		1 minute dark		30 seconds light ^c		30 seconds light ^c	
Total C ¹⁴ Fixed. c.p.m. x 10 ⁻⁶	.96	100%	.97	100%	3.1	100%	6.2	100%
I Ether extractable acids	.13	13%	.12	12%	.078	2.5%	.64	10%
II Cationic (Amino acids)	.51	53%	.38	39%	.44	14%	.68	11%
III A. Anionic. Ammonia elutable ^d	.023	2.4%	.041	4.2%	1.19	38%	2.75	44%
III B. Anionic. Not ammonia elutable ^e	.30	31%	.41	42%	1.13	36%	1.67	27%
IV Neutral Substances (Sugars)	.0033	0.3%	.0013	0.1%	.14	4.5%	.29	4.7%

a) Chlorella pyrenoidosa, one day old cultures. b) Scenedesmus D-3, two day old cultures. c) Cells rapidly photosynthesizing were given radioactive carbonate and shaken until removed from the light beams and instantly killed. d) Adsorbed on Duolite A-3 and eluted with 1.5 N ammonium hydroxide. e) Eluted with sodium hydroxide following the previous ammonia elution.

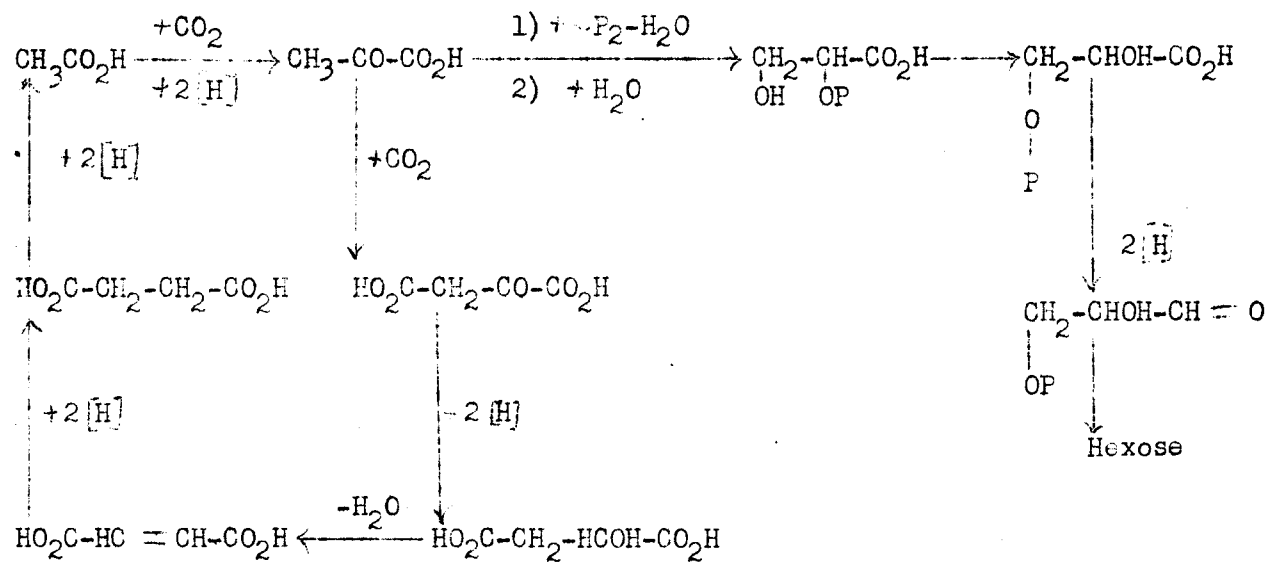
phosphate. It thus appeared that the major original component of Fraction III=A which gave rise to the phosphoglyceric acid was 3-phosphoglyceraldehyde. A subsequent experiment with *Scenedesmus* (photosynthesizing for 30 seconds) demonstrated that such is actually the case. The 3-phosphoglyceraldehyde was converted to methylglyoxal by the method of Baer and Fischer (14). The methylglyoxal 2,4-dinitrophenylosazone was found to possess 53% of the total fixed C^{14} . In separate experiments, fraction IV has been shown to be glucose and fructose in almost equal amounts.

In addition to the above described identification work, a certain amount of degradation has been performed on several of the identified compounds. Perhaps the most illuminating of these results is the distribution of C^{14} found in the hexose fraction for a number of different preparations (15). In the glucose obtained from the dark fixation in pre-illuminated *Chlorella* and from the 30 second photosynthesis of *Scenedesmus*, there was contained from 75-90% of the radioactive carbon in the 3 and 4 positions, the remainder being found in the 1,2 and 5,6 positions. In glucose obtained from barley shoots which had been photosynthesizing for one hour in $C^{14}O_2$, the distribution was 61% in the 3,4 position, 24% in the 2,5 position and 15% in the 1,6. In glucose obtained from barley which had been photosynthesizing for two hours, the distribution was 37% in 3,4, 36% in 2,5 and 27% in 1,6. In view of the presence of such large amounts of radioactive triose phosphate and phosphoglyceric acid in the very short photosynthetic experiments (30 seconds) as well as in the dark fixation, it can be taken as fairly certain that the hexose synthesis proceeds by a reversal of the usual glycolytic split of fructose diphosphate and therefore some path must be found by which the radioactivity appears first in the number 1 carbon atoms of the 3 carbon compounds and gradually spreads into the number 2 and then the number 3 carbon atom of the triose phosphate and the phosphoglyceric acid. A similar variation of the distri-

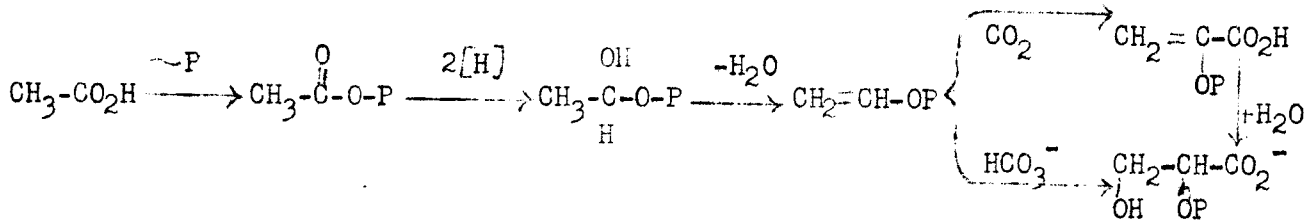
(14). E. Baer and H. O. L. Fischer, *J. Biol. Chem.*, 150, 223 (1943).

(15). S. Aronoff, H. A. Barker, and M. Calvin, *J. Biol. Chem.*, 169, 459 (1947).

bution of radiocarbon in alanine spreading from the number 1 carbon atom into the other two has also been found. It is reasonable to suppose that the alanine distribution is an index of the distribution in pyruvic acid and the problem therefore becomes that of determining the path from carbon dioxide to pyruvic acid which would give this characteristic distribution. So far we have been able to account for the major fraction of the radioactive carboxylic acids formed in terms of only the 4 carbon acids. If there is any aconitic or ketoglutaric acid at all, it is small in amount. We are, therefore, led to the supposition that the three carbon compounds are produced through the four carbon dicarboxylic acids. Furthermore, the acceptors for the one or more carboxylation reactions which take place must not only be continuously regenerated, but they must be regenerated in such a manner as to contain a gradually increasing amount of isotopic carbon, relatively rapidly approaching the isotopic concentration of the carbon dioxide itself. All this can be achieved, with currently used reactions, only by the presence of what is effectively a C₂-C₁ addition. We are therefore led to the reiteration of a scheme already proposed to describe the path of the carbon atoms.



It should be realized that these are all enzymatic reactions and the precise form of the reaction species is not specified. For example the reductive carboxylation of acetate through pyruvate to phosphoglyceric acid could conceivably proceed by the following steps.



With this as a working hypothesis, specific tests are already under way. Thus, when carboxyl-labeled acetate is fed to pre-illuminated *Chlorella* for a 5 minute period in the dark, the isolated alanine contains about 35% of its radioactivity not in the carboxyl group. The presence of C^{14} in the carboxyl group of the alanine could be due to the oxidative metabolism of some of the acetate through the tricarboxylic cycle in the manner already familiar in animal tissues and yeasts (16-18) or by the direct oxidative coupling of two acetates to succinate. Also the succinic acid isolated from pre-illuminated *Chlorella* after dark fixation of C^{14}O_2 showed 2.5% of the radioactivity in the methylene groups, while the succinic acid from the aforementioned 2 hour photosynthesizing barley experiment had 37% in the methylene groups. !*

The amount of radioactivity found in any compound or carbon atom in a specific compound will depend upon the size of its actively functioning reservoir and the relative rates of all the reactions leading to and from that reservoir under any specific set of experimental conditions.

The precise nature of the reducing agent or agents cannot yet be stated, nor can we say which of the reducing equivalents required by the operation of the cycle had their origin in the photosynthetic reaction. It remains for future work to deny, modify, confirm, or extend the proposed scheme which at present appears to us the most likely path of carbon in photosynthesis.

(16). V. Lorber, N. Lifson, H. G. Wood, *J. Biol. Chem.*, 161, 411 (1945).

(17). S. Weinhouse and R. H. Millington, *J. Am. Chem. Soc.*, 69, 3089 (1947).

(18). K. Bloch, *Physl. Rev.* 27, 574 (1947).

(!) The method of succinic acid degradation is that of Benson, Bassham, and Calvin. To be published.

(*) The analysis of algae given in column I of Table I, Ref. 1, corresponds to fixation under the conditions represented by a 30 minute point on Curve A, Figure 1 of the present note. It is entirely possible that the succinic acid found therein, which was entirely carboxyl labeled, may have been formed in large part by reversibility of the Wood-Werkman reaction.

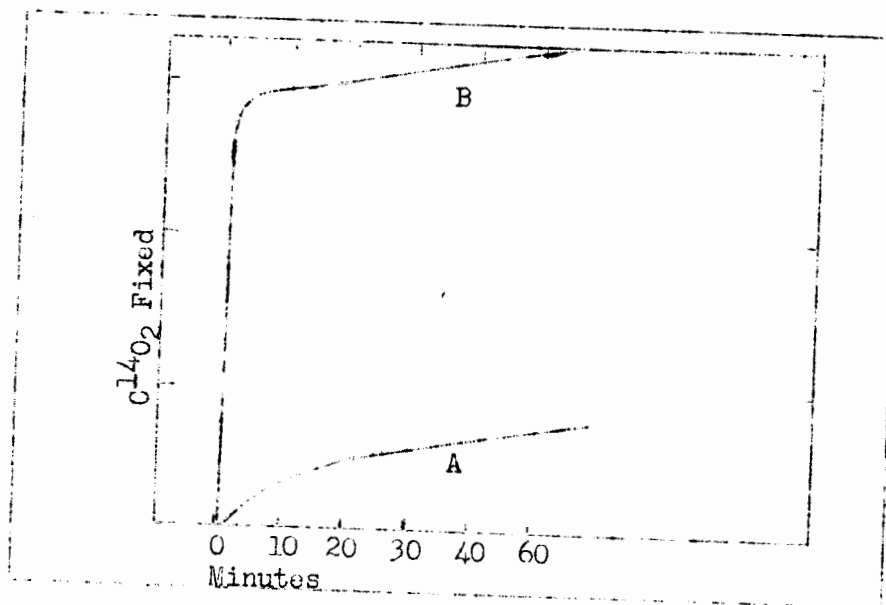


Figure 1

Dark Fixation Rate of $C^{14}O_2$ by 2-day old Scenedesmus

Curve A - Cells kept in the dark for 1 hour in 4% CO_2-N_2 and then flushed with helium for 20 minutes.

Curve B - Same cells as for curve A following a 10 minute exposure to light ($2 \times 17,000$ lux) continuing the rapid flushing with helium.

Both curves were obtained by taking aliquots from the main batch of cells and exposing them to $C^{14}O_2$ in the dark for the time period indicated.

The concentration of cells in the large vessel was 1 cc. of packed cells in 60 cc. of nutrient solution pH 6.2. The volume of the dark fixation vessels and the amount of $C^{14}O_2$ in them was such that the partial pressure of $C^{14}O_2$ was less than .2 mm.Hg.

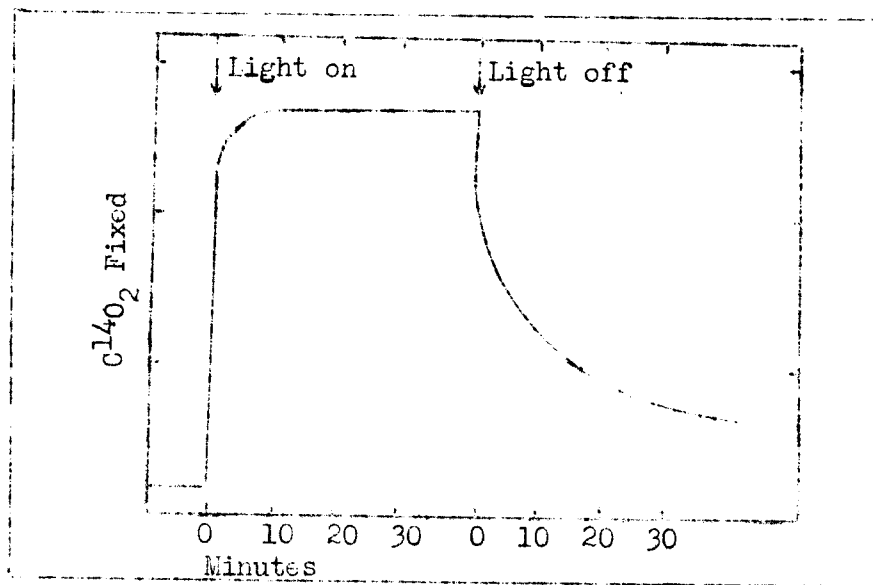


Figure 2

Effect of Pre-illumination upon 1 Minute dark Fixation (Scenedesmus)

The abscissa represents the time which the cells spent in the various conditions of light and dark as indicated, prior to the removal of an aliquot to be tested for dark fixing ability by a one minute exposure to radioactive CO_2 in the dark. The time interval between the removal of the aliquot from the illumination vessel and its coming into contact with the C^{14}O_2 was never more than 3 seconds. The ordinate represents the C^{14}O_2 fixed by an aliquot of the cells during a 1 minute exposure to C^{14}O_2 in the dark after the pretreatment shown on the abscissa.