

TABLE I

Conditions under which algae reduced C*O <sub>2</sub>	Velocity of rotor, r. p. s.	Time of rotation, min.	Increase in specific activity at periphery, %	Sedimentation velocity constant (× 10 <sup>-14</sup> )
4 Min. in light	2250	85	15	6.2 <sup>a</sup>
10 Min. in light	2250	90	15.8	6.1
10 Min. in light	2400	60	11.1	5.7
20 Min. in light	2000	120	12	7.5
20 Min. in dark	2250	70	16	8.6

<sup>a</sup> Sedimentation carried out in KBr solution ( $\rho = 1.22$  g./cc.).

The average of the four values (for  $\rho = 1$ ) is  $7.0 \times 10^{-14}$  c. g. s. unit. Unfortunately any trend in the velocity constants with photosynthetic conditions cannot be considered significant because of experimental error. It is of interest to compare the above values with the velocity constant of sucrose. A 1% solution of sucrose was spun for ninety minutes at 2250 r. p. s. The increase in concentration<sup>6</sup> at the periphery was 4% yielding a velocity constant of  $1.7 \times 10^{-14}$ . This is the value obtained by Leyda.<sup>3</sup> The theoretical value according to McBain<sup>3</sup> for anhydrous sucrose is  $2.3 \times 10^{-14}$ .

(6) We are indebted to Dr. W. Z. Hassid for the sucrose analyses and the chlorella cultures.

We are indebted to Professor W. C. Bray and Professor J. W. McBain for their interest and advice. Thanks are due to Mr. G. F. Nelson and Mr. G. T. Boies for mechanical assistance and many suggestions. The cooperation of Professor E. O. Lawrence and members of the Radiation Laboratory made these experiments possible. Financial aid from the Rockefeller Foundation to the Radiation Laboratory is gratefully acknowledged.

### Summary

Using radioactive carbon as an indicator the sedimentation velocity constant of the radioactive molecule(s) formed by *Chlorella pyrenoidosa* in the light and dark has been found to have an average value of  $7.0 \times 10^{-14}$ . The velocity constant for sucrose obtained under the same conditions is  $1.7 \times 10^{-14}$ . This indicates the molecular weight is about four times that of sucrose, assuming other factors equal.

The additional data necessary for a more accurate molecular weight evaluation are presented in the following paper.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND THE RADIATION LABORATORY, UNIVERSITY OF CALIFORNIA]

## Photosynthesis with Radioactive Carbon. IV. Molecular Weight of the Intermediate Products and a Tentative Theory of Photosynthesis

BY S. RUBEN AND M. D. KAMEN

It is the purpose of this paper to describe the diffusion experiments which in conjunction with the determination of the sedimentation velocity constant<sup>1</sup> make possible a calculation of the approximate<sup>2</sup> molecular weight of the primary radioactive products.

The molecular weight of a compound can be calculated from a knowledge of the diffusion coefficient and the sedimentation velocity constant. The relation is

$$M = \frac{RTs}{D(1 - \rho\bar{V})} \quad (1)$$

where  $M$  is molecular weight,  $R$  gas constant,  $T$  temperature,  $s$  sedimentation velocity constant,  $D$  diffusion coefficient,  $\bar{V}$  partial specific volume of solute,  $\rho$  density of solution. The velocity

(1) Cf. III of this series, THIS JOURNAL, 62, 3450 (1940).

(2) Due to experimental difficulties (short half-life of C<sup>14</sup>, etc.) it is not practical to obtain high accuracy but rather to set a lower limit on the molecular weight.

constant was determined with the McBain opaque ultracentrifuge.<sup>1</sup> The diffusion coefficient was measured by the sintered glass plate technique.<sup>3</sup> The sensitivity of the method was increased by the use of small volumes of solution behind the sintered diaphragm (no. 4 Jena). The cells were calibrated with 1 *N* potassium chloride and showed no streaming effects when tilted. The diffusion coefficients of sucrose and sodium chloride were measured and agreed well with the values in the literature.<sup>4,5</sup>

*Chlorella* cells, after exposure to C\*O<sub>2</sub> in the light or dark for various periods, were boiled gently for approximately one minute, centrifuged and the supernatant extract filtered through a no. 4 Jena glass filter. This filtrate was introduced by gentle suction into the diffusion cell. De-

(3) Northrup and Anson, *J. Gen. Physiol.*, 12, 543 (1929).

(4) Friedman and Carpenter, THIS JOURNAL, 61, 1745 (1939).

(5) Mehl and Schmidt, *Univ. Calif. Pub. Physiol.*, 8, 165 (1937).

pending upon the initial radioactivity, diffusion was allowed to proceed for periods of ninety to two hundred and fifty minutes. The diffusates were usually collected every twenty to thirty minutes. After filling the diffusion cells and prior to the actual diffusion measurements the diffusion cells were suspended in distilled water for approximately one-half hour in order that the proper concentration gradient be set up in the sintered disk. Table I contains a summary of the results obtained in a typical experiment. In this case the algae were exposed to  $C^{*}O_2$  for thirty minutes in the light.

Intervals, minutes	% of total diffused	Diffusion coefficient, $cm.^2/sec.$
60	16.5	$0.40 \times 10^{-5}$
30	6.5	.36
31	7.0	.40
40	7.1	.35
30	5.3	.40
30	4.2	.34
Total	221	$0.37 \times 10^{-5}$ (av.)

It is of interest to note that although more than 45% of the radioactive material has diffused out, there is no large change in the diffusion coefficient. This suggests the material may be very nearly homogeneous in respect to molecular weight.

Table II summarizes the data obtained from experiments in which  $C^{*}O_2$  assimilation was allowed to proceed in the dark or light for various periods.

Conditions under which algae were exposed to $C^{*}O_2$	Diffusion coefficient, $cm.^2/sec.$
1 15 min. in dark	$0.44 \times 10^{-5}$
2 10 min. in light	.44
3 20 min. in light	.35
4 20 min. in light	.43 <sup>a</sup>
5 30 min. in light	.37

<sup>a</sup> Diffusion carried out in KBr solution ( $\rho = 1.22$  g./cc.).

It is of interest to note that the presence of faster moving ions ( $K^+$  and  $Br^-$ ) has increased the diffusion rate of the active molecules while the presence of a non-electrolyte (glucose) was found to exert no measurable effect. It is not unlikely that the radioactive material is ionic<sup>6</sup> in which case the values in Table II are upper limits for the diffusion coefficient. The decrease in the diffusion coefficients with time of photosynthesis seems to be outside the experimental error (approximately 10%).

(6) Electrophoresis experiments are in progress to check this point.

It is apparent from equation (1) that  $\bar{V}$  must be known before the molecular weight can be calculated. Since only minute amounts are available it is impossible at the present time to measure  $\bar{V}$  directly. It can, however, be evaluated from equation (2) by changing the density of the solution and redetermining  $s$  and  $D$ .

$$\bar{V} = \frac{s_2 D_1 - s_1 D_2}{s_2 D_1 \rho_1 - s_1 D_2 \rho_2} \quad (2)$$

In one experiment the density was increased to 1.22 g. per cc. by the addition of potassium bromide. Under these conditions  $s$  was found<sup>1</sup> to be  $6.2 \times 10^{-14}$  and  $D$  to be  $0.55 \times 10^{-5}$   $cm.^2/sec.$ <sup>7</sup> These values when coupled with  $s = 7.0 \times 10^{-14}$  and  $D = 0.44 \times 10^{-5}$   $cm.^2/sec.$  for  $\rho = 1.0$  yield  $\bar{V} = 0.57$  cc./g. This evaluation of  $\bar{V}$  is not particularly accurate since it is based upon a limited number of experiments. One could instead assume  $\bar{V}$  to be about the same as for carbohydrates (which, indeed, the chemical properties suggest) whose values lie between 0.6–0.7 cc./g. (at infinite dilution). However, since the difference is slight 0.57 cc./g. will be used in the molecular weight calculation.

The calculated molecular weights are summarized in Table III.

Conditions under which <i>Chlorella</i> were exposed to $C^{*}O_2$	Mol. wt. <sup>a</sup>	Mol. wt. <sup>b</sup>
Dark 10 min.	1100	1500
Light 10 min.	770	1000
Light 20 min.	1200	1600

<sup>a</sup> Since no corrections for mixing in the ultracentrifuge experiments have been made these values are a lower limit.

<sup>b</sup> In an attempt to take into consideration mixing, the molecular weights have been calculated using the corresponding constants for 1% sucrose (mol. wt. = 342);  $s = 1.7 \times 10^{-14}$ ,  $\bar{V} = 0.63$ ,  $D = 0.44 \times 10^{-5}$ . It was assumed that the existence of mixing would merely multiply the right-hand side of equation (1) by a constant, which was then evaluated with the sucrose data.

Despite the high sensitivity of the radioactive indicator method the first detectable reduction product of  $C^{*}O_2$  seems definitely to be a large molecule. Furthermore, it is important to note that even in experiments in which photosynthesis was allowed to proceed with  $C^{*}O_2$  for very short periods (approximately one minute) the presence of small molecules<sup>8</sup> could not be detected. The extracted material may well be a fragment of the

(7) This value is calculated from experiment 2 (Table II) by multiplying the diffusion coefficient obtained in absence of potassium bromide by the ratio of diffusion coefficient in potassium bromide to that in pure water as found in experiments 3 and 4.

(8) Cf. paper II of this series, THIS JOURNAL, 62, 3443 (1940)

actual molecule which exists in the intact cell. Experiments with ultraviolet light (*cf.* below) lend support to this suggestion.

### Discussion

Following the ideas of Willstätter and Stoll many theories on photosynthesis have been based upon two main assumptions: (1) the formation of an addition compound between carbon dioxide and chlorophyll, (2) the subsequent photochemical reduction to formaldehyde. Although the original suggestions were advanced more than twenty years ago no experimental verification has been forthcoming. Indeed, the experiments<sup>8</sup> with C<sup>11</sup> have shown that the primary process in photosynthesis is not the formation of a carbon dioxide-chlorophyll complex but rather a conversion of carbon dioxide to carboxyl on a large molecule. The subsequent photochemical reductions do not produce formaldehyde. Despite the great sensitivity of the radioactive indicator method no active formaldehyde could be detected. The following scheme seems to be in accordance with the known facts and furthermore suggests a new mode of attack.

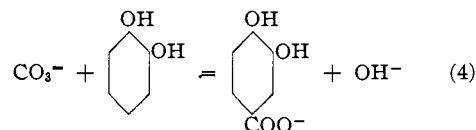
The first reaction is a thermal (non-photochemical) process (probably enzyme-catalyzed) in which the carbon dioxide is reduced reversibly to carboxyl. It can be represented simply by



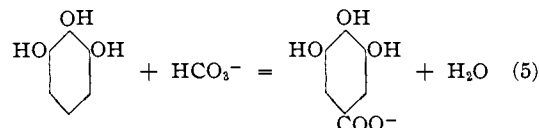
It has already been suggested by many authors<sup>9,10,11</sup> that carbon dioxide does not combine with chlorophyll but with some other substance in the cell. In addition Smith<sup>12</sup> has made a careful search for a carbon dioxide-chlorophyll complex and has shown that the results of Willstätter and Stoll cannot be interpreted as positive evidence for its existence.

The formation of carboxyl is a probable reaction energetically and structurally and is suggested by the presence of C\* in the carboxyl group. According to thermal data for similar reactions  $\Delta H = 0 \pm 2$  kcal. Free energy values for a reaction such as (3), involving large molecules, are not available, however. For small molecules in general the free energy change is +5-10 kcal. It is of considerable interest to note that certain polyphenols (catechol, pyrogallol, etc.) can, under the proper conditions, reduce +4 carbon in reactions

similar to (3). For example at 140° aqueous ammonium carbonate and catechol react



Pyrogallol (and its isomers) are even better reducing agents, for when heated with bicarbonate solutions gallic acid (and its isomers) can be formed, *viz.*



Furthermore these reactions are reversed (*i. e.*, decarboxylation) at fairly low temperatures (~200°). Compounds of this type are the principal constituents of tannins, which occur in the majority of, if not all, plants. It is not unlikely that R may contain such polyphenols.

Experiments with ultraviolet light<sup>13</sup> (2537 Å. mercury line) coupled with results of Arnold<sup>14</sup> furnish independent evidence for the big molecule hypothesis. Arnold found with controlled dosage of *Chlorella* using the 2537 Å. mercury line it was possible to inhibit photosynthesis but not respiration. Arnold's calculated cross-section for inactivation by a one-quantum hit was larger than a single chlorophyll molecule but smaller than an individual cell. The cross-section corresponded roughly to the dimensions of a large group (approximately 2000) of chlorophyll molecules. We have found that ultraviolet light inhibits photosynthesis by preventing the dark conversion of C\*O<sub>2</sub> to carboxyl. Since the dark reaction does not involve chlorophyll, Arnold's cross-sections indicate the primary reaction of carbon dioxide must involve large molecules other than chlorophyll. This suggests the measured molecular weight of approximately 1000 is a lower limit and probably a fragment split off the true reducing agent which exists in the cell. It should be noted that the ultraviolet inhibition can no longer be used as evidence for the existence of a "photosynthetic unit" comprised of chlorophyll molecules.

There is considerable support, based upon the finding of carbon dioxide uptake by non-photosynthetic systems, etc., for the idea that the pri-

(9) Emerson, *Ergeb. Enzymforsch.*, **5**, 305 (1936).

(10) Gaffron and Wohl, *Naturwissenschaften*, **24**, 81, 103 (1936).

(11) Thimann, *Science*, **88**, 506 (1938).

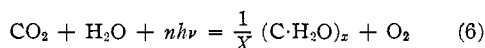
(12) J. H. C. Smith, *Plant Physiol.*, **15**, 183 (1940).

(13) *Cf.* article II of this series.

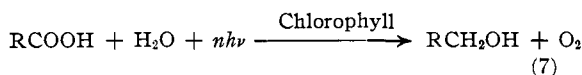
(14) Arnold, *J. Gen. Physiol.*, **17**, 135, 143 (1933).

mary reaction of carbon dioxide is widely separated from the photochemical reactions.<sup>15,16,17</sup> Carbon dioxide reduction by non-chlorophyllous organisms such as the methane bacteria,<sup>18</sup> *B. coli*,<sup>19</sup> *Clostridium sp.*,<sup>20</sup> propionic acid bacteria,<sup>21</sup> etc.,<sup>22</sup> is well known. Indeed, many published<sup>23,24,25,26</sup> and unpublished experiments on widely divergent aerobic and anaerobic systems strongly suggest that *carbon dioxide reduction may well be a general and important reaction in all living cells*. It is conceivable<sup>27</sup> that the primary reduction process of carbon dioxide may be similar in all cases, the chief difference between the photosynthetic and non-photosynthetic systems being that in the former the secondary reactions are photochemical and endothermic and in the latter, exothermic.

Since the over-all equation for photosynthesis is



one may, without specifying the various steps involved, represent the photochemical process by the following schematic equation



The similarity between the molecular weights and other chemical properties of the molecules suggests strongly that R in (4) and (7) are identical. The  $\text{RCH}_2\text{OH}$  might add another carbon dioxide and repeat the cycle to build up long carbohydrate chains. The cyclic hypothesis has been tested in the following manner. *Chlorella* cells were exposed to 3%  $\text{C}^*\text{O}_2$  in the dark for twenty minutes. The  $\text{C}^*\text{O}_2$  was pumped out and replaced with an inactive carbon dioxide-air mixture and photosynthesis was allowed to proceed in strong illumination for twenty minutes. The barium salt, prepared as described previously,<sup>13</sup> was radioac-

tive and contained approximately 50% of the total reduced  $\text{C}^*$  present in the cells. The active barium salt was decarboxylated but no  $\text{C}^*$  (< 1%) could be detected in the carboxyl. The same experiment was performed with algae exposed to light continuously. Here again the barium precipitate was active but no  $\text{C}^*$  was in the  $\text{COOH}$  group.

This is to be expected if the process is cyclic. The  $\text{R}^*\text{COOH}$  formed in the dark is reduced to  $\text{RC}^*\text{H}_2\text{OH}$  in the light and the  $\text{RC}^*\text{H}_2\text{OH}$  takes on another carbon dioxide molecule. The newly formed carboxyl is not active since it was formed from exposure to inactive carbon dioxide. Thus the insoluble barium salt is radioactive with the  $\text{C}^*$  buried inside "R." "R" is therefore not a unique group. This experiment can by no means be considered as proof for a cyclic mechanism since it is possible the molecule (R) may possess other inactive carboxyl groups which do not enter directly into the mechanism of photosynthesis.

The energy required for (7) can be estimated from bond energies or by comparison with similar reactions to be approximately 110 kcal. per mole of carbon dioxide reduced. It may be added that formaldehyde formation requires approximately 135 kcal. the polymerization to sugar being exothermic. Thus the large molecule mechanism is advantageous not only thermodynamically but kinetically since it does not require polymerization of small molecules present in extremely low concentrations. The large molecule hypothesis suggests the simple sugars (glucose, sucrose, etc.) are formed by the splitting of the long molecular chain. Thus it is possible in certain plants that starch may be the precursor of the simple reducing sugars rather than starch being formed from the simple sugars.

This theory can only be considered tentative and its chief value may only be in that it suggests experimentation along new lines.

The production of a long-lived radioactive isotope of carbon<sup>28</sup> will make feasible a more detailed and extensive investigation. It should be possible to isolate the primary reduction product  $\text{RC}^*\text{OOH}$  and determine its structure and properties.

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- (15) G. N. Lewis, private communication.  
 (16) Van Niel, A. A. A. S. Symposium on Photosynthesis, Seattle, 1940.  
 (17) Gaffron, A. A. A. S. Symposium on Photosynthesis, Seattle, 1940; *Science*, **91**, 529 (1940).  
 (18) Barker, *Arch. Mikrobiol.*, **7**, 404 (1936).  
 (19) Woods, *Biochem. J.*, **30**, 515 (1936).  
 (20) Wieringa, *Antonie van Leeuwenhoek*, **3**, 1 (1936).  
 (21) Wood and Werkman, *Biochem. J.*, **30**, 48 (1936); **32**, 1262 (1938); **34**, 7 (1940).  
 (22) Cf. the excellent reviews by Van Niel, *Ann. Rev. Biochem.*, **VI**, 606 (1937); Gaffron, *ibid.*, **VIII**, 986 (1939).  
 (23) Ruben and Kamen, *Proc. Nat. Acad. Sci.*, **26**, 418 (1940).  
 (24) Carson and Ruben, *ibid.*, **26**, 422 (1940).  
 (25) Barker, Ruben and Kamen, *ibid.*, **26**, 426 (1940).  
 (26) Barker, Ruben and Beck, *ibid.*, **26**, 477 (1940).  
 (27) We are indebted to Professors G. N. Lewis and W. C. Bray for this suggestion.  
 (28) Ruben and Kamen, *Phys. Rev.*, **57**, 549 (1940).

Bain, G. K. Rollefson, Drs. H. A. Spoehr, J. H. C. Smith, D. C. DeVault, W. Z. Hassid and H. Gaffron for many helpful ideas and discussions. We are indebted to Professor E. O. Lawrence and the members of the Radiation Laboratory for their interest and coöperation. Thanks are also due to the Rockefeller Foundation for support to the Radiation Laboratory.

### Summary

1. The molecular weight of the first detectable products formed in the light and dark by *Chlorella pyrenoidosa* has been found to be approximately 1000.
2. A tentative theory on the mechanism of photosynthesis is presented.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

## Oxygen Exchange between Anions and Water

BY NORRIS F. HALL AND ORVAL R. ALEXANDER<sup>1</sup>

If a water-free oxy-salt such as potassium dichromate is dissolved in a known amount of water of known abnormal density due to excess O<sup>18</sup>, and the salt and water later completely separated, a decrease in the density of the water may occur. From the weights of salt and water it is possible to calculate the density change that corresponds to complete random distribution of the heavy water-oxygen between water and salt, and hence to determine the extent to which such complete exchange of oxygen has occurred.<sup>1a</sup> Complete information on the rate and extent of exchange with various salts under different conditions should give valuable indications of the relative firmness of binding of oxygen atoms to the various other atoms in the anions, and of the exchange mechanisms.

In this paper are reported determinations of the extent of oxygen exchange between water and twenty-two inorganic oxygen compounds completely dissolved in it. The times of contact with the water varied from one to one hundred hours, and the runs were carried out at temperatures from 95–100°. Pyrex glass flasks were used to hold the reaction mixtures, and in many cases the effect of added alkali and acid on the extent of reaction was investigated. Sixteen of the compounds studied had not been previously examined from the point of view of exchange.

During the progress of the work several similar

(1) This work was supported in part by grants from the Wisconsin Alumni Research Foundation. Presented in part before the Division of Physical and Inorganic Chemistry at the Baltimore meeting of the American Chemical Society, April 6, 1939.

(1a) The assumption is made throughout this paper that the equilibrium constants of the exchange reactions are not significantly different from their "probability" values (1, 4, etc.). This assumption is justified in most cases by the limited precision of the data. Further experimental studies of this point are planned.

researches were published<sup>2-5</sup> and since its completion, a further paper<sup>6a</sup> has appeared.

More detailed studies of some of these equilibria have been published by Urey and his collaborators.<sup>7</sup>

Compared to some of the other investigators in this field we had available larger quantities of water, but its excess density was less. We were therefore constrained to attempt extremely precise density measurements (to 0.02  $\gamma d$ ),<sup>8</sup> but could use relatively large samples (40 ml.).

In order to be suitable for investigation by this method a salt must be (a) obtainable in a definite and easily reproducible state of purity, particularly as regards water content (most of our salts were anhydrous), and (b) sufficiently soluble so that the ratio of the total oxygen in the salt to that in the water may be made to approach unity. These considerations considerably limited our choice of compounds.

### Experimental

**Heavy Oxygen Water.**—This was supplied through the generosity of Professor Harold C. Urey, whose kindness is gratefully acknowledged, and was found after normalization of its hydrogen content, to have an excess density due to oxygen of 21.7  $\gamma d$ . The normalization was carried out by electrolyzing the water and combining the oxygen with tank hydrogen. Since it could not be assumed that the tank hydrogen had the same composition as that of normal

(2) Datta, Day and Ingold, *J. Chem. Soc.*, 1968 (1937).

(3) Blumenthal and Herbert, *Trans. Faraday Soc.*, **33**, 849 (1937).

(4) Titani, Morita and Goto, *Bull. Chem. Soc. Japan*, **13**, 329 (1938).

(5) Titani and Goto, *ibid.*, **13**, 667–668 (1938); **14**, 77–85 (1939)

(6) Winter, Carlton and Briscoe, *J. Chem. Soc.*, 131–137 (1940).

(6a) See also Mills, *THIS JOURNAL*, **62**, 2833 (1940).

(7) For references see Mills and Urey, *ibid.*, **62**, 1019–1026 (1940).

(8) By " $\gamma d$ " is meant a difference in density of one part per million.