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TRACER STUDIES IN HYDROGEN-ADAPTED
SCENEDESMUS USING ^3H AND ^{14}C

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TRACER STUDIES IN HYDROGEN-ADAPTED SCENEDESMUS
USING ^3H AND ^{14}C

Richard Allen Goldsby

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TRACER STUDIES IN HYDROGEN-ADAPTED SCENEDESMUS USING ^3H AND ^{14}C

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I. INTRODUCTION

Scattered here and there in nature are some peculiar enzyme systems. These enzyme systems are peculiar in that they require substrates that are quite rare, curious or generally toxic to living systems. Enzyme systems capable of oxidizing molecular hydrogen,¹ thiocyanate² or carbon monoxide³ are known to occur in the lower plant phyla. Because of its close tie-in with processes of electron transport, hydrogenase, the enzyme that allows some living systems to use molecular hydrogen as a source of electrons, has been the object of considerable study.⁴ Though hydrogenase is found in many bacteria and some algae, most studies of hydrogenase and its metabolic role have been done in bacterial systems. There has been very little work using the more modern biochemical techniques to characterize the metabolic role and nature of hydrogenase found in algae. Most of what is known about hydrogenase systems in algae is found in the classic studies of Hans Gaffron.

In 1939, Hans Gaffron discovered that after a period of dark, anaerobic incubation, the green algae Scenedesmus developed hydrogenase activity.⁵ Subsequently, Gaffron and others have discovered that a number of other green algae will also develop hydrogenase activity.⁶ This formation of hydrogenase activity in systems where it is not present in an active form, is called hydrogen adaptation. Algae that have been hydrogen-adapted are capable of carrying out the following reactions;

1. Photoreduction

2. Ox-hydrogen Reaction

Gaffron, has made a thorough manometric study of the adaptation process and the adapted state in Scenedesmus. His studies of Scenedesmus adaptation may be summarized as follows:⁷

1. Complete adaptation to hydrogen requires six to eight hours of dark, anaerobic incubation.

2. Traces of molecular oxygen prevent adaptation and the adapted state is destroyed by prolonged exposure to more than 0.02 atm. of molecular oxygen.

3. Light of low intensity (100 f.c. or less) prevents the adaptation, presumably due to the formation of molecular oxygen via the Hill reaction.

4. The adapted state is destroyed by exposure to light of too high an intensity (above 200 f.c.) unless compounds such as menadione which block the Hill reaction are added.

5. Scenedesmus must be adapted for at least forty-five minutes before any measurable hydrogenase activity appears.

6. Reactions 1 and 2 are inhibited by CN, CO, N₃, and 2,4-DNP.

A great deal of information about hydrogen-adapted algae, Scenedesmus in particular, has been gleaned from the careful manometric studies of Gaffron and others. But measurements of gas uptake, however

precise, fail to answer important questions about the metabolism of hydrogen-adapted Scenedesmus. The intermediary metabolism of the hydrogen and carbon dioxide assimilated during photoreduction or the ox-hydrogen reaction is not elucidated by manometric studies. Also, the metabolism of hydrogen-adapted Scenedesmus depends primarily on the activity of the enzyme hydrogenase. The manometric assay of Scenedesmus hydrogenase which depends on measuring gas uptake during reaction 1 or 2 is not necessarily a direct measure of hydrogenase activity. In order to determine with certainty the effect of various environmental conditions on the level of hydrogenase activity, in Scenedesmus, a more fundamental and direct assay is needed.

In this study, an investigation of the metabolism of hydrogen-adapted Scenedesmus has been conducted using radioactive tracers. Carbon-14 labeled carbon dioxide has been used to trace the path of carbon in the ox-hydrogen reaction and in photoreduction. Tritium has been used to determine the fate of the hydrogen assimilated during photoreduction. This tracer has also been employed to study the generation, level, properties and mechanism of the hydrogenase formed in Scenedesmus. In order to make a direct study of the hydrogenase, use has been made of the following exchange reaction, which is catalyzed by hydrogenase:

This reaction which depends on the activation of molecular hydrogen and is carried out by all hydrogenases has been used in assays involving deuterium exchange and conversion of ortho to para hydrogen.⁸ However, the ortho-para hydrogen conversion and deuterium exchange

assay systems both require complex instrumentation and are technically difficult to carry out. For this study, a new assay system depending on the radioactivity of tritium has been developed. The assay is conducted by allowing the exchange reaction to take place under a tritium atmosphere as indicated in equation 4:

II. EXPERIMENTAL PROCEDURES

A. Abbreviations

The following abbreviations have been used:

ul wpc	microliters of wet packed cells
ml wpc	milliliters of wet packed cells
PMS	phenazine methosulfate
R _f	ratio of distance traveled by the center of the spot to the distance traveled by the solvent front
CMU	3 p-chlorophenyl, 1,1-dimethyl urea
vit K ₃	vitamin K ₃ , or menadione
cpm	counts per minute
dpm	disintegrations per minute
ATP	adenosine triphosphate
TPN	triphosphopyridine nucleotide (oxidized)
TPNH	triphosphopyridine nucleotide (reduced)
DPN	diphosphopyridine nucleotide (oxidized)
DPNH	diphosphopyridine nucleotide (reduced)
2,4-DNP	2,4-dinitrophenol
fc	foot candle

μM	micromoles
PGA	3 phosphoglyceric acid
PEPA	phosphoenopyruvic acid
sugar Mono P	sugar monophosphate
sugar DiP	sugar diphosphate

B. Manometric Studies of Adaptation and the Adapted State

A suspension of Scenedesmus obliquus grown under constant density conditions, was spun down at 600 xg and resuspended in 1 part of their own supernatant Myers media to 1 part of distilled water. A portion of the resultant algal suspension was centrifuged at 600 x g for 10 minutes to obtain the wet packed cells data, while aliquots of the remainder at pH 7 were pipetted into Warburg flasks. At this point various amounts of CMU were added to some of the Warburg flasks.

The flasks were attached to manometers, placed in the Warburg apparatus and agitated while a stream of either 96% $\text{H}_2/4\% \text{CO}_2$ or 96% air/ $4\% \text{CO}_2$ was passed through the vessels during the incubation. The incubation was carried out either in the light or in the dark. At the end of the incubation time, each flask-manometer combination was transformed into a closed system and the net rate of uptake of the 96% $\text{H}_2/4\% \text{CO}_2$ mixture or the net rate of oxygen evolution was measured at various light intensities. The temperature was maintained at 24° during incubation and measurement. The light intensities incident on the surface of the Warburg flask were measured with a photocell.

C. Studies of Adaptation and the Adapted State with Tritium

1. Plant material

Scenedesmus obliquus was grown under sterile conditions as a

photosynthetic autotroph in Myer's Medium. The temperature was 20° C and the pH was 7. Immediately before use the cells were harvested and centrifuged for 10 minutes at 5° C and 600 x g in an International Refrigerated Centrifuge. The centrifuge tube was calibrated, permitted a determination of the volume of wet-packed cells in a given volume of suspension. The packed cells were then resuspended in enough supernatant growth medium to yield the desired concentration (volume wet-packed cells/volume suspension). The suspension at pH 7 was placed in a vessel of the type shown in Figure 1. The vessel was then shaken in a constant temperature bath.

2. Kinetic Measurements of hydrogenase formation

An empty vessel of the type shown in Figure 1 was flushed with hydrogen for 2 minutes. The system (see Figure 2) was then closed and the tritium-hydrogen mixture in the reservoir was circulated through the system for 10 minutes. The algae suspension of known concentration, was then injected into the vessel and the gas mixture was allowed to recirculate for an additional 10 minutes. At various intervals after the algae were introduced, samples were withdrawn with a hypodermic syringe, and killed in sufficient methanol to yield an 80% methanol solution. The methanolic solutions were immediately flushed with hydrogen gas to remove any dissolved tritium gas. Aliquots of the methanolic solutions were then counted in the Packard Tri-Carb Liquid Scintillation Spectrometer.

3. Comparison of hydrogenase level in Scenedesmus and other systems.

Each of the organisms tested (Scenedesmus, Chlamydomonas moewusii, Chlamydomonas reinhardtii, Rhodospirillum rubrum, Rhodopseudomonas spheroides, and Chromatium) were centrifuged out of their growth media at 10° C in

Fig. 1. Photograph of incubation vessel. These vessels are made of pyrex glass and have a volume of approximately 50 cc. When necessary, the vessels are wrapped with Scotch brand plastic electrical tape to exclude light. 500 cc versions of this design were used in some of the experiments described in this paper.

Fig. 2. Schematic outline of experimental setup. A. Scharr dynapump; B. Gas sampling tube; C. Tritium reservoir; D. Vessel or vessels in series containing algae; E₁ and E₂ Traps containing distilled water. F₁ and F₂ Traps containing chromous chloride (oxosorbent); G. Rubber sleeve; H. Rubber policeman.

an International refrigerated centrifuge. The supernatant was decanted and the cells were resuspended in 0.1 M potassium phosphate buffer (pH 6.8), spun down once more, the supernatant decanted and finally resuspended in 0.1 M potassium phosphate buffer (pH 6.8). Two cc's of the suspension of each test organism was placed in a Thunberg tube. An additional Thunberg tube received 2 cc buffer and served as a blank. The Thunberg tubes were placed on a vacuum line and evacuated. Following evacuation the tubes were filled with sufficient hydrogen to bring the pressure up to 50 cm of mercury. The tubes were removed from the vacuum line, laid on their sides and incubated for 10 to 16 hours in the dark. The tubes were then placed on a vacuum line and evacuated. The tubes were then refilled to between 0.25 and 0.5 atm. of Hg with a tritium-hydrogen gas mixture. They were then placed on their sides and put into a constant temperature bath. After 3 to 4 hours of incubation under the tritium-hydrogen atmosphere, the Thunberg tubes were opened to the air and shaken. The samples were diluted up to 10 cc with H₂O and flushed with hydrogen. Aliquots were then removed for protein determination and radioactivity measurements. Radioactivity was determined by liquid scintillation. The aliquot used for protein estimation was first sonicated at 1000 kc for 3 minutes. After sonication, a portion of the aliquot was treated with the Folin phenol reagent⁹ and the optical density at 750 mμ determined on a Beckman DU spectrophotometer.

5. Measurement of the tritium-hydrogen isotope effect in D₂O

Scenedesmus and Rhodospirillum rubrum were centrifuged out of their growth media at 10° C in an International Refrigerated centrifuge. The supernatant was decanted and the cells were resuspended in 0.1 M phosphate (pH 6.8) in 99+% D₂O. The cells were allowed to stand in the

D₂O for 15 minutes and then spun down. The supernatant was decanted and the cells were again resuspended in the 99+% D₂O buffer. Two cc of the cell suspension was pipetted into Thunberg tubes of known volume. An additional tube was supplied with D₂O buffer and served as a blank. The Thunberg tubes were then placed on a vacuum line and evacuated. After evacuation, sufficient hydrogen was admitted to the tubes to yield a pressure of 50 cm of Hg. The tubes were then removed from the line, placed on their sides and incubated for 6 hours in the dark. After the incubation under hydrogen, the tubes were again placed on a vacuum line and evacuated. The tubes were then supplied with from 0.3 to 0.5 atm. of a T₂ - H₂ gas mixture. The exchange reaction was allowed to go on for three hours. At the end of the period allowed for exchange, a sample of gas was removed from each Thunberg tube. Immediately after the gas sample was taken, the Thunberg tube was opened to the air, shaken, diluted to a volume of 10 cc with distilled water and flushed with hydrogen gas. The atom percent deuterium in the gas samples was determined by mass spectrometry. The amount of tritium exchanged into the water phase was determined by counting any aliquot in the Packard Tri-carb Liquid Scintillation Spectrometer. The specific activity of tritium-hydrogen gas mixture was determined as outlined below.

5. Tritium studies of the Adapted State.

A Scenedesmus suspension was pipetted into a vessel and shaken in the dark under a stream of hydrogen for four or more hours. After adaptation, the system was closed and a tritium-hydrogen mixture was circulated through the system as described above. A hypodermic syringe was used to add chemicals to the system and to remove samples for activity determinations. The samples were killed in methanol, flushed

with hydrogen and counted by liquid scintillation.

6. Distribution of tritium label

Four cc of Scenedesmus suspension was pipetted into each of 4 vessels. The suspensions were shaken in the dark under a stream of 96% H_2 /4% CO_2 for 4 hours. Two samples were then preilluminated for 5 minutes. After preillumination, a tritium-hydrogen mixture was circulated through one of the light samples and one of the dark samples. The second light-dark pair of samples received no tritium gas but 0.5 cc of 58 mc/cc tritiated water was added. After 27 minutes of photo-reduction, 15 cc of methanol were added to each of the 4 vessels to stop the reaction. The methanol solutions of samples which had been exposed to tritium gas were flushed with H_2 and counted for tritium by liquid scintillation. The methanol solutions of all four samples were centrifuged and the supernatants decanted. The residues were extracted again with 5 cc of 20% methanol and finally with 5 cc of water. The extracts of each of the four samples were evaporated down to dryness under vacuum at room temperature. The residues were redissolved in 5 cc of 20% methanol and re-evaporated down to dryness. The residues then contained tritium only in nonexchangeable positions. The distillates from the samples that were under tritium gas were collected and counted by liquid scintillation. The residues from all four samples were dissolved in 5 cc of 80% methanol and counted for tritium.

7. General

In all experiments tritium counts were determined as disintegrations per minute by use of an internal standard.¹⁰ A sample of the tritium-hydrogen gas in the system was taken for measurement of specific activity. The specific activity was determined by converting a known volume of this gas to water over hot copper oxide. The water was collected and counted

by liquid scintillation.

D. Studies of Hydrogen-Adapted Scenedesmus
with Radioactive Carbon Dioxide

1. Plant material

Scenedesmus obliquus grown under conditions of constant temperature, pH, illumination, etc., as described by Bassham and Calvin, was used in these studies. The cells were harvested and centrifuged for 10 minutes at 5° C and 600 x g in an International Refrigerated centrifuge. The supernatant growth medium was decanted and diluted five-fold. The Scenedesmus cells were then resuspended in enough of this dilute growth medium to yield a 2.5% suspension (vol. wet packed cells/vol. suspension). Four cc of the suspension at pH 7 was pipetted into a vessel of the type shown in Fig. 1. The vessel containing the algal suspension was then supplied with a hydrogen-carbon dioxide gas mixture and shaken in complete darkness for several hours in a constant temperature bath. Before reaching the algal suspension, the H₂ - CO₂ gas mixture was deoxygenated by passage through a series of chromous chloride (oxosorbent) solutions. After the dark anaerobic adaptation to hydrogen, the vessels were swept with either 99% air/1% CO₂ or 99% H₂/1% CO₂ for thirty minutes, sealed, and then subjected to the experimental conditions described below.

2. Batch determination of CO₂ fixation in the light and in the dark at various oxygen tensions.

After adaptation, the Scenedesmus suspensions in the untaped versions of the incubation vessel were preilluminated for five minutes. After preillumination, all samples, both light and dark, were supplied

with various amounts of oxygen. This was done by injecting known amounts of air into the vessel with a calibrated hypodermic syringe. After the injection of air, 200 μ c (7.8 μ M) of $\text{NaHC}^{14}\text{O}_3$ was injected into each of the light and dark samples. The samples were then incubated with the tracer for 30 minutes and then killed by adding 16 cc of absolute methanol.

3. Batch determination of CO_2 fixation in the presence of inhibitors.

After adaptation, 0.5 cc of solutions containing 2,4 DNP, CMU or vit. K_3 were injected into the suspensions of adapted *Scenedesmus*. The light samples were preilluminated for five minutes at 116 f.c. and then 200 μ c (7.8 μ M) of $\text{NaHC}^{14}\text{O}_3$ were injected into each of the incubation vessels. After 30 minutes of exposure to tracer, 16 cc of absolute methanol was added to kill the algae and stop CO_2 fixation.

4. Time course experiments to determine the kinetics of CO_2 fixation.

Four vessels containing suspensions of adapted *Scenedesmus* were treated as follows:

Vessel 1: preilluminated at low light intensity (116 f.c.) for five minutes.

Vessel 2: hooded vessel, no preillumination, but sufficient air to yield an oxygen concentration of 1.5% was injected.

Vessel 3: preilluminated at high light intensity (3000 pf.c.)

Vessel 4: CMU added, then preilluminated at 3000 f.c.

After five minutes of preillumination, or preincubation, 200 μ c (7.8 μ M) of $\text{NaHC}^{14}\text{O}_3$ was introduced through a 1 cc hypodermic syringe. The vessel was quickly shaken and a sample of 1 cc was withdrawn and killed in 5 cc methanol. At intervals of several seconds, two succeeding samples were withdrawn from the incubation vessel with the aid of a hypodermic syringe.

5. The relationship between CO₂ fixation and hydrogenase activity

Scenedesmus were adapted to hydrogen along the lines set down above. Each vessel contained 3 ml of 2.5% suspension. After the adaptation, the H₂ - CO₂ atmosphere was replaced with an atmosphere containing H₂ and a trace amount of tritium gas. Sufficient air was injected into each vessel save one to yield oxygen concentrations from 0.5% up to 5%. 30 µc (25 µM) of NaHC¹⁴O₃ was added to each vessel. At this point, a 1 cc sample of the suspension was taken and killed in 5 cc of methanol. The suspension remaining in the vessel was exposed to tracer for 10 minutes and then killed by the addition of 8 cc of methanol to the vessel. The methanolic solutions were acidified with glacial acetic acid and flushed with a 96% H₂ - 4% CO₂ gas mixture to remove dissolved C¹⁴O₂ or tritium. Aliquots of the methanolic solutions were then counted for C¹⁴ and H³ in the Tri-Carb Liquid Scintillation Spectrometer.

6. General

Determinations of the total C¹⁴ radioactivity were carried out by counting aliquots of samples in the Tri-Carb Liquid Scintillation counter or by drying the sample as thin films on planchets and counting with a Scott tube. Because of its great sensitivity, the scintillation counter was especially useful in making an accurate determination of low levels of radioactivity. For most of the experiments, aliquots of the methanolic solutions were subjected to two-dimensional paper chromatography. Phenol-water was used in the first dimension and butanol-propionic acid-water was used in the second dimension. After development, the chromatograms were placed on x-ray film for a period of one to six weeks. Then the x-ray films were developed. The compounds containing C¹⁴ were identified by cochromatography with authentic markers. The relative amount of C¹⁴ in each of the labeled compounds was determined by direct counting on the paper with a Geiger tube.

III. RESULTS AND DISCUSSION

A. Studies of Scenedesmus Hydrogenase Using Tritium

1. The assay of hydrogenase

Hydrogenase, the enzyme responsible for the activation of molecular hydrogen, has been assayed by a variety of methods all of which fall into one of the three categories below:

a. Substrate reduction: some electron acceptor such as methylene blue, ferricyanide or CO_2 is reduced.

b. Hydrogen evolution: Hydrogen is evolved from some bacterial and algal systems in the presence of light.

c. Catalysis of the exchange reaction: A hydrogen isotope in one phase is exchanged with a second hydrogen isotope in another phase. Alternatively, a non-thermodynamic mixture of ortho and para hydrogen may be converted to a thermodynamic mixture in the presence of hydrogenase.

The assay methods of the first and second categories both involve measurement of the overall rate of at least two and perhaps several reactions. On the other hand, the exchange reaction is a consequence of the activation of hydrogen, a primary reaction of hydrogenase. The exchange reaction circumvents any difficulties arising from the need to activate a reducible or oxidizable substrate. Also, since the exchange reaction involves no net production or consumption of substrate, it may be used for the measurement of hydrogenase levels in vivo.

As mentioned earlier, deuterium exchange and the ortho-para hydrogen conversion reactions have been used to measure hydrogenase activity. However, these methods suffer from a requirement for complex instrumentation and are technically difficult to perform. Also, it is

difficult to take samples rapidly and measure very low levels of hydrogenase. The assay method employing tritium described in the experimental section allows rapid sampling, great sensitivity and is simply executed.

The manometric assay heretofore employed in the investigation of Scenedesmus hydrogenase has not provided the sensitivity needed to study the adaptation process in Scenedesmus. In addition, manometry, since it gives only an indirect measure of hydrogenase activity, is subject to the objections raised above.

2. The kinetics of hydrogenase appearance in Scenedesmus.

Figure 3a and Figure 3b show plots of the accumulation of tritium vs time during the adaptation of Scenedesmus under a tritium-hydrogen atmosphere. The semi-logarithmic plot shows that the increase in tritium is strongly exponential over the first 120 minutes of adaptation. The linear plot shows that during the next 120 minutes the exponential component grows weaker and a linear component grows stronger. After about 275 minutes, the rate of accumulation is quite constant and the accumulation of tritium is linear. These curves show that hydrogenase is formed in an exponential fashion. Once formed, the hydrogenase continues to exchange tritium out of the gas phase into the water phase at a constant rate. Inspection of Figures 3a and 3b shows that some hydrogenase is present in Scenedesmus after only 5 minutes of adaptation. It is also apparent that after about 3 hours, the formation of hydrogenase is essentially complete. The strongly exponential raise in hydrogenase activity suggests that during adaptation the enzyme is formed in an autocatalytic fashion. Each unit of hydrogenase formed seems to catalyze the formation of additional units of enzyme until the full complement of hydrogenase is formed.

Fig. 3a. Time Course of *Scenedesmus* Adaptation. Semi-log plot. 26 ml of Myer's medium containing 170 μl of wet packed *Scenedesmus* under 500 cc's of tritiated hydrogen gas. The temperature was 25° C and the pH 7.

Fig. 3b. Time Course of Scenedesmus Adaptation. Linear plot. Same conditions as in Fig. 3a.

3. Mode of hydrogenase formation in Scenedesmus.

In Scenedesmus the hydrogenase may be formed by modification of a pre-existing protein or by the induction of new enzyme synthesis. The addition of chloromycetin, an inhibitor of protein synthesis,¹² does not retard the rate of hydrogenase appearance during adaptation. The data supporting this conclusion are shown in Figure 4. However, dithionite, a reducing agent, markedly accelerates the formation of hydrogenase (see Fig. 6) in Scenedesmus. This finding leads to the conclusion that hydrogenase is activated in Scenedesmus by reductive modification of a pre-existing protein. It is possible that under the normal conditions of adaptation to hydrogen, the first units of hydrogenase formed supply electrons for the reduction required to produce additional units of hydrogenase. This would account for the exponential appearance of hydrogenase during the adaptation of Scenedesmus.

4. Factors affecting the generation of hydrogenase in Scenedesmus.

In his early studies, Gaffron found that at least forty-five minutes of dark, anaerobic incubation was necessary in order to have manometrically detectable amounts of hydrogenase formed in Scenedesmus. Anaerobiosis is required because molecular oxygen destroys hydrogenase. Gaffron has suggested that darkness is necessary because the small amount of hydrogenase present early in the adaptation would be rapidly oxidized by products of the Hill reaction. If Gaffron's suggestion is correct, the addition of CMU, a compound known to block the Hill reaction,¹³ might make it possible to adapt Scenedesmus in the presence of light. This experiment has been performed and the results are shown in Fig. 5 and 6.

Fig. 4. Effect of chloromycetin on the rate of hydrogenase formation in *Scenedesmus*. Gas phase: tritiated hydrogen gas (specific activity: 47 $\mu\text{c/cc}$). Solid line = chloromycetin 560mg/ml. Dashed line = no chloromycetin.

Fig. 5. The adaptation of *Scenedesmus* in the presence of light and CMU.

Fig. 6. Effect of light, CO_2 and S_2O_4 on the rate of Scenedesmus adaptation. Each vessel contained 3 cc of Myer's medium and 36 μl of wet packed Scenedesmus cells under 50 cc of tritiated hydrogen gas. Vessel 2 contained sufficient CMU to maintain a saturated solution (about 2 milligrams). Vessel 4 contained 4 milligrams of $\text{Na}_2\text{S}_2\text{O}_4$. Vessels 2 and 1 contained 24 μmoles of NaHCO_3 .

One of the curves in Fig. 6 shows the time course of hydrogenase formation in the presence of light when CMU is present. The adaptation to hydrogen proceeds but at a slower rate than under the normal dark anaerobic conditions of adaptation. CMU, though a very powerful inhibitor of the Hill reaction, does not cause total blockage of this reaction. Presumably, the small amounts of oxidized products generated by the Hill reaction are sufficient to retard the rate of hydrogenase formation. However, if the adaptation is carried on for four hours, the level of hydrogenase formed during adaptation is sufficient to maintain quite comparable levels of photoreduction whether the adaptation is carried out in light or in the dark, provided CMU is present. This is shown by the manometric data presented in Fig. 5. This figure also shows that if CMU is omitted during the four-hour incubation period, the cells do not adapt and fail to show the net gas uptake characteristic of photoreduction. It is noteworthy that CMU prevents the destruction of the adapted state by high light intensities. This protection of the adapted state against deadaptation has been seen earlier by Gaffron using Vit. K₃¹⁴ and Bishop using DCMU.¹⁵ The exchange studies and the manometric data presented in Figures 5 and 6 clearly show that Scenedesmus is readily adapted to hydrogen in the light or in the dark provided one has taken pains to block the Hill reaction.

The data presented in Fig. 6 also show that the reduction potential of the system has a profound effect on the rate of hydrogenase appearance. A comparison of the rate of hydrogenase appearance in the presence of dithionite, a powerful reducing agent, with the rate in the

light - CMU case, relatively oxidizing conditions, shows that the lower the reduction potential of the system, the more rapid is hydrogenase formation. Fig. 6 shows that the presence or absence of carbon dioxide has no significant effect on hydrogenase appearance. Gaffron has shown that the time required to generate a manometrically detectable level of hydrogenase decreases as one increases the temperature of adaptation.

4. Factors affecting the activity of *Scenedesmus* hydrogenase.

Once the hydrogenase is formed, factors such as light, oxygen, cyanide, carbon monoxide and temperature affect its activity. Most of these factors have been studied by manometric methods.⁹ But manometry does not measure the hydrogenase directly. The manometric methods which have been used measure the rates of reduction of some electron acceptors by an enzyme system that may contain several members. As a result, the effect that one sees during manometric studies of hydrogen-adapted *Scenedesmus* may be due to changes in hydrogenase activity or merely to changes in the activity of some intermediate electron carrier. However, catalysis of the exchange reaction is a fundamental property of hydrogenase. The tritium exchange studies described below have given a quantitative measure of the effect of various environmental conditions on the hydrogenase itself.

The activity of the hydrogenase varies with temperature. Figure 7 shows a plot of the log of hydrogenase activity vs. the reciprocal of the absolute temperature. The slope of this Arrhenius plot has been used to calculate an activation energy of 7.65 kcal for *Scenedesmus*

Fig. 7. Effect of temperature on Scenedesmus hydrogenase activity. 45 μ l of wet packed cells in 30 cc of Myer's medium were adapted under hydrogen for 18 hours at 25° C and pH 7. Rates were determined under tritiated hydrogen at 12.0, 17.1, and 23.3° C.

hydrogenase. The hydrogenase of Proteius vulgaris, a bacterium, has been found to be 7.7 kcal.¹⁶

The stability of Scenedesmus to temperature has been examined and its thermal profile is shown in Fig. 8. Scenedesmus hydrogenase, like hydrogenases from other systems, exhibits remarkable thermal stability. Unlike most enzymes, this hydrogenase suffers no great loss of activity on freezing and thawing or heating at 60° C.

Earlier in vivo assays of hydrogenase activity in Scenedesmus depended on measurements of gas uptake during either photoreduction or the oxhydrogen reaction. The results in Table I show that hydrogenase is significantly inhibited by the low light intensities used to carry on photoreduction or the small amounts of molecular oxygen commonly used in the ox-hydrogen reaction. Table II shows the protective effects of PMS (phenazine methosulfate). PMS is a dye that initiates a form of cyclic photophosphorylation and blocks the Hill reaction.¹⁷ Even at the high light intensity of 2,000 f.c., PMS prevents extensive deactivation of the hydrogenase, presumably by preventing the formation of oxygen by the Hill reaction.

The inhibitory effects of cyanide and carbon monoxide on Scenedesmus hydrogenase are shown in Table III and Fig. 9 respectively. Visible light partially reverses the inhibition by carbon monoxide. The inhibition of Scenedesmus hydrogenase by cyanide and carbon monoxide shows that a heavy metal is a vital constituent of this enzyme. Mineral nutrition experiments have shown that Ankistrodesmus which is deficient in iron is unable to carry out photoreduction.¹⁸ This result has been interpreted as showing that the Scenedesmus hydrogenase

Fig. 8. Thermal resistance of *Scenedesmus* hydrogenase. 30 min. incubation with tracer. Specific activity H₂ - T₂ gas: 4.18×10^1 μ c/cc.

Table I

Effect of oxygen and light on Scenedesmus hydrogenase.

Each vessel contained 4 cc of Myer's medium and 84 μ l of wet packed Scenedesmus cells (w.p.c.). The samples were adapted for 8 hours under 96% H_2 - 4% CO_2 . The temperature was 24° C and the pH was 7. The specific activity of the gas phase after addition of tracer was 171 μ c/cc.

Condition	" μ ls" H_2 exchanged per hour per μ l w.p.c.	Percent Inactivation
Light (80 f.c.)	1.54	26
Dark	2.04	0
Dark + 0.4% oxygen added 1 hr. prior to introduction of T_2	1.74	15
Dark + 0.4% oxygen added 10 min. prior to introduction of T_2	.885	58

Table II

Protection of hydrogenase at high light intensities.

Each vessel contained 4 cc of Myer's medium and 180 μ l of wet packed Scenedesmus (w.p.c.). The samples were adapted for 11.5 hrs. under 98.5% H_2 - 1.5% CO_2 . The temperature was 23° C and the pH was 7.1. 56 μ M of PMS (pherazine methasulfate) was added 15 min prior to addition of the tracer.

Condition	Activity μ MC/ μ l w.p.c./hr	% Inactivation
Bright light (2,000 f.c.)	3.14×10^3	98.6
Dark	218×10^3	0
Bright light (2,000 f.c.) + μ M PMS	182×10^3	19.6
Dark + μ M PMS	227×10^3	0

Table III

Effect of CN^- on hydrogenase activity.

Each vessel contained 4 cc of Myer's medium containing 80 μl of wet packed Scenedesmus cells. All samples were adapted for 5 hrs under 98.5% H_2 - 1.5% CO_2 . KCN was added to vessels 2 and 3 one hour prior to introduction of the tracer. The temperature was 23° C and the pH was 7.

Vessel no.	Experimental condition	Activity in dpm/ μl w.p.c.	Percent Inactivation
1	<u>Scenedesmus</u> + No CN^-	385,000	0
2	<u>Scenedesmus</u> + 10^{-3} M CN^-	92,4000	79
3	<u>Scenedesmus</u> + 10^{-2} M CN^-	26,2000	94

Fig. 9. Effect of light on CO inhibition of *Scenedesmus* hydrogenase. 175 μ l of wet packed *Scenedesmus* cells in 35 cc of Myer's medium were adapted for 16 hours under hydrogen. 7.8 μ M of FMS were added at the close of adaptation. After 47 min exposure to tritiated hydrogen sufficient carbon monoxide was added to make the system 33% CO-67% H_2 . The temperature was 22° C and the pH 7.

has a structural requirement for iron. However, the assay (reduction of CO_2 by molecular H_2) used in these nutrition experiments did not measure the hydrogenase activity directly. Hence, the iron-deficiency experiment may mean that iron is required for some intermediate electron carrier between hydrogenase and the site of CO_2 reduction. A positive identification of the heavy metal involved in Scenedesmus hydrogenase awaits purification of the enzyme itself.

the chemical nature of Scenedesmus hydrogenase, like that of all other hydrogenases described to date, is only vaguely known. Scenedesmus hydrogenase contains a heavy metal bound to some sort of prosthetic group. The photoreversibility of the carbon monoxide inhibition of the hydrogenase indicates that this prosthetic group is capable of absorbing visible light and transferring the energy to a bound metal ion. Two commonly encountered prosthetic groups capable of playing such a role are the isoalloxazine and porphyrin systems. Claims have been made by various authors that either or both of these natural chelating systems comprise the active site of hydrogenase.^{19,20}

6. The form of reducing power supplied by hydrogenase.

Both photoreduction and the ox-hydrogen reaction involve a net uptake of molecular hydrogen. One might wonder if the system incorporates protons from the gas phase or if it merely extracts electrons from the gas phase and incorporates protons from the aqueous medium. This question has been investigated using tritium.

Studies with tritium have shown that when the tracer is supplied to hydrogen-adapted Scenedesmus as tritiated hydrogen gas, no appreciable tritium label appears in organic compounds. This is true in the light or in the dark. On the other hand, if the tracer is supplied to the

system as tritiated water, there is a light-dependent fixation of tritium into organic compounds during photoreduction. These findings are summarized in Table IV. The results show that in this system molecular hydrogen is used only as a source of electrons for cellular reductions. Whatever protons are incorporated during photoreduction come from the aqueous phase.

7. Comparison of the hydrogenase activity found in several microorganisms.

The hydrogenase activity in three green algae, Scenedesmus and two species of Chlamydomonas has been compared with the hydrogenase content of three photosynthetic bacteria. In Table V the results of this comparison are shown. There is considerable variation from one organism to another. Chlamydomonas moewusii and Chlamydomonas reinhardi are members of the same genus, but differ in hydrogenase content. This difference in hydrogenase activity from one organism to another was first studied by Rittenburg and Krasna in various bacterial systems.²¹ The difference in activity found in two such close relatives as C. moewusii and C. reinhardi are probably due to differences in the amount of hydrogenase. However, the possibility exists that there is more than one kind of hydrogenase. The differences in hydrogenase activity between the other members of the table may be due to quantitative differences in hydrogenase level or qualitative differences in hydrogenase efficiency. There is some evidence from inhibitor studies that there may be more than one kind of hydrogenase. If such is the case, it is quite possible that one kind of hydrogenase might be more efficient than another in catalyzing the exchange reaction.

It might be added that the presence of hydrogenase in C. reinhardi and R. spherioides has not been reported elsewhere. They were examined for catalysis of the exchange reaction because hydrogenase was known to

Table IV

Variation of tritium labeling with source of tritium.

Each vessel contained 81 μ l of wet packed Scenedesmus cells (w.p.c.) in 4 cc of Myer's medium. All samples were adapted four hrs under 96% H₂-4% CO₂. After adaptation, the light samples were preilluminated 5 min. Tritium gas was added to vessels 1 and 2 (final specific activity 79.5 μ c/cc) and tritiated water was added to vessels 3 and 4 (final specific activity 3 mc/cc). The temperature was 23° C and the pH was 7.

Vessel no.	Experimental condition	Tritium supplied in	μ c fixed/hr/ μ l w.p.c. x 10 ³	
			exchangeable	non-exchangeable
1	Light	gas phase	39.8	.52
2	Dark	gas phase	79.4	1.1
3	Light	aqueous phase	--	14.7
4	Dark	aqueous phase	--	8.4

Table V

Hydrogenase activity of some microorganisms.

Organism	Relative hydrogenase activity per unit of protein
<u>Scenedesmus obliquus</u>	100
<u>Chlamydomonas reinhardi</u>	25
<u>Chlamydomonas moewusii</u>	11
<u>Chromatium</u>	71
<u>Rhodospirillum rubrum</u>	8.5
<u>Rhodospseudomonas spheroides</u>	12

be present in the closely related species C. moewusii and R. rubrum.

8. The mechanism of hydrogenase action.

Many systems are known to catalyze the activation of molecular hydrogen. Systems that are capable of activating molecular hydrogen will catalyze the exchange and conversion reactions mentioned earlier. It is possible that by comparing the properties of other hydrogen activating systems with the properties of hydrogenase, some useful suggestions for the mechanism of hydrogenase action may be obtained.

The most familiar agents for hydrogen activation are the metallic catalysts such as platinum, palladium and nickel. These metals, and some of their compounds, in the finely divided or colloidal state, can catalyze the reduction of a number of substances by complex surface phenomena. Since the activation always involves two phases, hydrogen activation by surface phenomena is a form of heterogeneous catalysis. A little more than twenty years ago, Calvin discovered that cuprous acetate dissolved in quinoline catalyzed the homogeneous activation of molecular hydrogen.²² Since the discovery of the cuprous acetate-quinoline system, other workers have developed a number of other metal ion dependent systems for the homogeneous catalysis of hydrogen activation.²³ Although some of the metal ion systems require organic solvents such as pyridine or quinoline, many exhibit catalytic properties in aqueous systems.

The homogeneous systems like the heterogeneous catalytic systems function at modest pressures, usually in the neighborhood of one atmosphere. However, slightly higher temperatures (usually in the range from 80-150°C) are frequently used to obtain convenient rates in homogeneous systems. The heterogeneous catalyze H_2 - D_2 exchange and the o-p hydrogen conversion reactions in the presence or absence of solvent. On the other hand, homogeneous catalysis of hydrogen activation by hydrogenase or metal

ions requires the presence of solvent.²⁴ When the exchange of H_2 and 99+% D_2O are heterogeneously catalyzed by PtO_2 , primarily D_2 is formed.²⁵ Homogeneous catalysis by aqueous metal ion systems of the exchange between D_2 and H_2O produces a mixture of DH and H_2 . In the case of hydrogenase, the H_2 - D_2O exchange produces DH and D_2 . From the foregoing considerations it is safe to say that the enzyme or group of enzymes known as hydrogenase has little in common with the heterogeneous catalysts. However, the parallel between hydrogenase and homogeneous metal ion catalysis is marked and is sharpened by noting the effects of complexing on the catalytic efficiency of the metal ion system.

Halpern has made a survey of the effects of complexing on the rates of activation of molecular hydrogen by metal ions which catalyze the heterolytic splitting of molecular hydrogen.²⁶ He suggests that for a complex MX_n^{Z+} , a transition state of the sort shown below is formed. Here M is the metal and X is the ligand molecule or ion.

In this formulation, the transition state involves stretching of the $M-X$ and $H-H$ bonds and incipient formation $M-H$ and $X-H$ bonds. Hence the reactivity of this complex should depend directly on the strength of the $M-H$ and $X-H$ bonds but be inversely proportional to the strength of the $M-X$ bond. Halpern has found that for copper or silver, complexation with strongly binding ligands, such as ethylenediamine in the copper

case, depresses the catalytic efficiency of the system relative to that of the uncomplexed ion. When the metal is bound to relatively weakly binding ligands such as acetate, the main influence of the ligand on catalytic efficiency is that arising from differences in the strength of the X-H bond. This prediction is consistent with Halpern's finding that the enhancement of catalytic efficiency by weakly bonding ligands increases with increasing basicity. Halpern's mechanism is a concerted one in which the electron accepting metal pulls electrons from one pole of the hydrogen molecule and the electron donating ligand pushes electrons to the opposite pole.

The evidence in the Scenedesmus case and in the case of other hydrogenases does indicate that a vital part of the active center is a complexed metal ion. Rittenberg and Krasna have found that the hydrogenase of Proteus vulgaris will convert para to ortho hydrogen in H_2O but not in D_2O .²⁷ These workers have also found that when Proteus vulgaris is suspended in 99+% D_2O and allowed to exchange with a hydrogen, both DH and D_2 are formed. The amounts of HD and D_2 formed during the hydrogen catalyzed H_2 - D_2O exchange are not the same for all hydrogenase systems. Krasna has stated that these differences in the HD/ D_2 ratio indicate mechanistic differences in hydrogenase. It is possible that factors such as pH and the accessibility of the postulated enzyme hydride to solvent protons could cause alterations in DH/ D_2 ratio. These alterations would not be referable to mechanistic differences between hydrogenase systems. Mills, et al. have found that in the potassium cobaltous cyanide system where hydrogen is activated heterolytically, the exchange of H_2 and D_2O at high pH produces almost exclusively D_2 and very little HD.²⁸ It would be interesting to see if the ratio of

D₂ to HD could be increased in systems containing hydrogenase by increasing the pH.

Krasna and Rittenberg have proposed a mechanism that accounts for the above observations.²⁷ The mechanism shown below involves the formation of an enzyme hydride.

The enzyme hydride mechanism involves a heterolytic splitting of the hydrogen molecule, producing a proton and an enzyme hydride. The exchange reaction probably involves the reaction of the enzyme hydride with a proton of the solvent phase as shown below:

If the reaction is carried out using different isotopic species of hydrogen in the gas and aqueous phase, the situation becomes more complex.

If one studies the catalysis of the exchange reaction by hydrogenase using a hydrogen gas phase, and a D₂O aqueous phase the enzyme hydride formed by combination of the enzyme with molecules of the gas phase may undergo either of two reactions. The hydride may combine with the protons of the solvent phase to yield a gas molecule containing both isotopic species. Alternatively, the enzyme hydride may undergo an exchange with protons of the solvent phase to yield a hydride of the same isotopic species as the solvent phase. Subsequently the hydride may combine with protons of the solvent phase to yield an isotopically-homogenous gas molecule. These comments are summarized by the following

postulated reaction sequence. The water phase is D₂O and the gas phase is H₂.

The amounts of HD and D₂ produced by a particular hydrogenase system would depend on the rate of the reaction governed by k₃ as opposed to the rate of the reaction governed by k₂.

With hydrogen gas containing tracer amounts of tritium and a D₂O liquid phase, it is possible to measure the hydrogen-tritium isotope effect for the exchange reaction. This measurement has been carried out in Scenedesmus obliquus and Rhodospirillum rubrum. The results are shown in Table VI.

If one considers these data in the light of the mechanism detailed above, the exchange reaction governed by k₂ will show an isotope effect between hydrogen and tritium in the gas phase. However, once the enzyme hydride has exchanged with solvent protons in the reaction governed by k₃, the reaction governed by k₄ will show no isotope effect. The effect of the reactions governed by k₃ and k₄ is to minimize the tritium-hydrogen isotope effect on k₂. Table VI compares the rates at which hydrogen and tritium enter the aqueous phase. A combination of the enzyme hydride with the solvent via the reaction governed by k₂ leaves only one of the protons of the gas phase in solution. On the other hand, if the hydride equilibrates with solvent protons and then

Table VI

The hydrogen-tritium isotope effect

Two samples of Scenedesmus obliquus each containing 0.03 cc wpc/cc sus and two samples of Rhodospirillum rubrum, each containing 0.06 cc wpc/cc sus in 99+% D₂O were adapted and placed under an atmosphere of tritiated hydrogen gas (sp. ac. 13.2 μ c/cc or 5.12×10^{-6} cc of T₂ per cc of gas at S.T.P.). The time of exposure to the tracer was 310 minutes. The temperature was 25° C and the pH was 6.7. The gas pressure was 39 mm and each vessel contained 2.4 cc of gas at STP.

Organism	% of Gas Molecules as D ₂	DH	Fraction of Atoms present as D	Atoms of D	T incorporated in dpm	T incorporation in dpm normalized to 1 cc T ₂ /cc gas	T atoms incorporated normalized to 1 cc T ₂ /cc gas	$\frac{k_H}{k_T}$
RR I	2.9	4.1	4.97×10^{-2}	6.4×10^{18}	2.47×10^6	4.82×10^{11}	4.5×10^{18}	1.4
RR II	2.3	3.1	3.59×10^{-2}	4.6×10^{18}	1.45×10^6	2.83×10^{11}	2.7×10^{18}	1.7
Scen. I	3.6	2.6	4.84×10^{-2}	0.63×10^{19}	5.79×10^6	1.13×10^{12}	1.1×10^{19}	0.57
Scen II	4.4	3.2	6.0×10^{-2}	0.78×10^{19}	6.87×10^6	1.34×10^{12}	1.3×10^{19}	0.60

forms a gas molecule of homogenous isotopic composition, both hydrogen atoms of the gas phase are left in solution. The enzyme hydride containing the lighter hydrogen isotope should react along the path governed by k_2 faster than the hydride containing the heavier isotope. This will favor the entry of both heavy hydrogen atoms from the gas phase into the water phase. As this effect becomes more and more important, the tritium-hydrogen isotope effect in a system, as measured by the entry of protons into the aqueous phase, will decrease. Under conditions in which one increases the magnitude of k_3 the isotope effects between heavy and light isotopes should approach one. The magnitude of k_3 could be increased by increasing the pH or perhaps by a geometrical or electronic alteration of the environment around the active site of hydrogenase that would facilitate the exchange of the proton of the enzyme hydride with solvent water.

The observation that the hydrogen-tritium isotope effect in Scenedesmus differs from that observed in Rhodospirillum rubrum could be interpreted in either of two ways. The difference in an isotope effect could mean a fundamental difference in the structure of the prosthetic groups of the hydrogenase. On the other hand, structural differences in the protien that tend to facilitate or hinder the equilibration of the postulated enzyme hydride with solvent could cause marked differences in the isotope effect. These measurements of the isotope effect in hydrogenase systems are complicated by the considerations introduced above. Correspondence of the isotope effect received from hydrogenases of different systems probably indicates a marked similarity in the mechanism and surroundings of hydrogenase in these systems. The fact that the isotope effect found in Rhodospirillum is

different from that found in Scenedesmus could indicate a difference in the fundamental structure of the two hydrogenases or merely minor differences in the environment of the active sites of Rhodospirillum and Scenedesmus hydrogenase.

The mechanism involving the formation of some sort of enzyme hydride, while giving some definition to be made of hydrogenase action, does not definitely implicate any particular chemical species. The active site of hydrogenase could be metalloporphyrin, an isocalloxazine nucleus with a metal ion bound by chelation, or perhaps a complex of a heavy metal with sulfhydryl groups. The nature of the metal involved in hydrogenase is still an open question. Iron and molybdenum are frequent nominees. Complexes of cobalt and mercury have been shown to activate molecular hydrogen in aqueous solutions. At this point it must be admitted that any one of several metals may be the catalytically active metal ion in hydrogenase. In Proteus vulgaris and Scenedesmus, hydrogenase forms a complex with carbon monoxide which is photodissociable. Short of isolating a pure hydrogenase, the measurement of an action spectrum for the photoreversibility of carbon monoxide inhibition in a purified hydrogenase preparation will probably yield valuable information about the nature of the active site in hydrogenase. The sensitivity of the tritium assay system should facilitate the measurement of such an action spectrum.

B. Studies of CO₂ Fixation in Hydrogen-Adapted Scenedesmus

1. CO₂ fixation pattern during photoreduction and photosynthesis.

Two rather striking differences are observed when carbon fixation during photosynthesis and photoreduction are compared. Under identical conditions of temperature, CO₂ pressure, and light intensity, hydrogen adapted Scenedesmus fixes 2.5 times as much CO₂ as the un-adapted system. The second effect is seen in the differences in the distribution of label between photosynthesis and photoreduction. Less carbon is found in sugar phosphates in photoreduction than in photosynthesis. Instead of flowing into sugar phosphates, carbon fixed during photoreduction flows into alanine. Table VII shows the data on which these remarks are based.

Carbon dioxide fixation is known to require ATP and reduced pyridine nucleotide.²⁹ The amount of these two cofactors required will depend on the disposition of fixed carbon (see Fig. 10).

The observation that substantially more of the entering CO₂ is channeled into alanine under the reducing conditions of photoreduction, suggests that a direct or indirect reductive amination pyruvate or phosphoenol pyruvate is involved. The path of carbon from CO₂ to alanine requires less ATP than the path from CO₂ to sugar phosphates. The channeling of carbon into alanine results in a lower energy requirement for CO₂ fixation during photoreduction than during normal photosynthesis. With comparable amounts of ATP, one can fix more CO₂ during photoreduction than during photosynthesis.

2. Dark CO₂ fixation in Scenedesmus.

Table VIII compares the pattern of CO₂ fixation in the dark in

Table VII

CO₂ fixation pattern in the light.

The total fixation is expressed in cpm. The distribution of activity among the various compounds is in percentage of the soluble activity.

Gas	H ₂	Air
Total fixation in cpm	21.6 x 10 ⁶	8.8 x 10 ⁶
Sugar Mono P	15.6%	29.2
Sugar Di P	0.8	3.0
PGA	32.9	20.3
PEPA	2.6	3.5
Citric Acid	0.4	1.0
Malic Acid	6.3	2.5
Aspartic Acid	7.3	14.2
Glutamic Acid	1.2	4.5
Serine	4.3	2.2
Glycine	0.5	0.3
Alanine	26.2	11.8
Identity Unknown	1.7	7.2

1. SIMPLIFIED CARBON REDUCTION CYCLE:

2. C₁ + C₃ PATHWAY

Figure 10

Table VIII

CO₂ fixation pattern in the dark.

The total fixation is expressed in cpm. The distribution of activity among the various compounds in in percentage of the soluble activity.

Gas phase	H ₂	H ₂ + 0.1% O ₂	H ₂ + 0.5% O ₂	H ₂ + 1.5% O ₂	Air
Total fixation	1.1 x 10 ⁶	1.5 x 10 ⁶	2.9 x 10 ⁶	6.6 x 10 ⁶	0.38 x 10 ⁶
Sugar Mono P	16.3	16.1	13.6	18.6	3.5
Sugar Di P	7.1	9.7	6.2	2.6	0
PGA	6.6	7.4	18.5	23.3	17.4
PEPA	1.0	0.3	1.8	2.7	1.0
Citric Acid	1.9	0.3	0.1	0.8	9.2
Malic Acid	11.2	10.3	8.2	8.0	9.5
Aspartic Acid	1.3	0.6	1.6	3.1	37.9
Glutamic Acid	0	0	0	0.9	20.5
Serine	0.5	0.2	1.5	2.6	0
Glycine	0.6	0.2	0.4	0.9	0
Alanine	50.3	54.5	47.0	28.2	0.8
Identity Unknown	0	0	0.9	6.6	0

hydrogen-adapted Scenedesmus at various oxygen tensions with unadapted Scenedesmus under air. The quantity of CO_2 fixed is much greater under hydrogen than air. As one increases the oxygen content of the hydrogen from zero up to 1.5 percent oxygen, there is a marked increase in carbon dioxide fixation. The distribution of compounds formed under hydrogen favors the production of PGA, sugar phosphates and alanine. Under air, the bulk of the fixation is in the Kreb cycle intermediates or derivatives of citric, malic, aspartic and glutamic acids. These differences indicate rather different paths of carbon fixation in adapted and unadapted Scenedesmus. Close inspection of the Table also reveals an increase in the PGA/alanine ratio as more oxygen is added to the hydrogen.

These results can best be understood by a consideration of the principal mechanisms used by plant systems to fix CO_2 . When there is an adequate supply of ATP and reduced pyridine nucleotide, the CO_2 is assimilated via the carbon reduction³⁰ cycle (see Fig. 10). On the other hand, CO_2 may carboxylate pyruvate to yield malic acid (shown in Fig. 10). This carboxylation, if followed by a decarboxylation of malic acid is merely an exchange reaction. Such an exchange would result in incorporation of label in malic acid with very little net CO_2 fixation or consumption of reduced pyridine nucleotide. Under air, the supply of reduced pyridine nucleotide is rapidly utilized by respiration, leaving little available for carbon dioxide reduction. With these observations in mind, a low level of CO_2 fixation would be expected under air. A pyruvate carboxylation - malate decarboxylation would provide the observed labeling pattern of malic acid and products desirable from malic acid.

On the other hand, under hydrogen, the supply of reduced cofactors is plentiful. The channeling of carbon dioxide through PGA into alanine would provide a source of ATP in addition to the ATP produced by normal fermentation processes. The presence of ATP and reduced pyridine nucleotide would permit operation of the carbon reduction cycle. When oxygen is introduced, more energy is made available to the system through respiration. The increased ATP production permits greater CO₂ fixation. As one introduces oxygen, the level of reduced cofactor is somewhat diminished. This should result in an increase in the level of the oxidized carboxylation product, PGA, and a decrease in alanine.

Over a narrow range of oxygen concentrations, the dark CO₂ fixation in hydrogen-adapted Scenedesmus increases as the partial pressure of oxygen increases. However, oxygen is a potent inhibitor of hydrogenase, the bridge by which electrons enter the system from hydrogen. As the oxygen tension is raised, the hydrogenase activity is decreased. The results of the double labeling experiment shown in Figure 11, demonstrate that the loss of hydrogenase activity with increasing oxygen is paralleled by a decrease in the capacity of the system to assimilate CO₂.

3. Kinetic experiments outlining the path of carbon in hydrogen-adapted Scenedesmus.

Until now, the discussion has assumed that when adequate amounts of reduced pyridine nucleotide and ATP the fixation proceeds along the path of the carbon reduction cycle. The necessary cofactors have been shown to be generated during photosynthesis.³¹ The path of carbon during photosynthesis has been shown to follow the carbon reduction

Fig. 11. The effect of oxygen on CO₂ fixation and hydrogenase level during the ox-hydrogen reaction.

cycle.³² However, the published data on the path of carbon during photo-reduction and the ox-hydrogen reaction argues that CO_2 enters primarily by a carboxylation reaction which yields malic acid rather than PGA.³³ However, the data on which the argument is based are questionable.

The experimental conditions under which these data were obtained make their interpretation quite difficult. During these experiments, at the end of the fixation period, the hydrogen-adapted algae were not killed anaerobically, but were exposed to air for 3 to 5 minutes prior to killing. This radical change from a reducing to an oxidizing environment will affect the distribution of labeled compounds in a unpredictable manner.

The experiments presented in this section are intended to answer these two questions. What are the first stable products of CO_2 fixation in hydrogen-adapted Scenedesmus, and to what compounds are these first, stable products transformed? The answers to these questions are provided by following the time course of CO_2 fixation. The kinetic experiments in Figures 12, 13, 14, 15, 16, and 17, clearly show that when an energy source and reducing power are provided, most of the carbon is fixed along the broad outlines of the carbon reduction cycle.

However, between the broad outlines of the carbon reduction cycle some notable differences are seen between photosynthesis and either photoreduction or the ox-hydrogen reaction. During photosynthesis at low or high light intensity (Fig. 12 and 14) CO_2 enters via carboxylation of RuDP to form PGA and then flows primarily into sugar phosphates. During photoreduction at low light intensity or the ox-hydrogen reaction, The CO_2 appears to enter mainly via RuDP carboxylation to form PGA. From PGA the carbon now flows into both sugar phosphates and alanine.

Fig. 12. Kinetics of CO_2 fixation during photosynthesis at low light intensity. The gas phase was air and the light intensity was 1250 lux.

Fig. 13. Kinetics of CO₂ fixation during photoreduction at low light intensity. The light intensity was 1250 lux and the gas phase was hydrogen.

Fig. 14. Time course of CO_2 fixation during photosynthesis at high light intensity. The gas phase was air and the light intensity was 32,000 lux.

Fig. 15. Kinetics of CO_2 fixation under hydrogen at high light intensity. The gas phase was hydrogen and the light intensity was 32,000 lux.

Fig. 16. Time course of CO_2 fixation by hydrogen-adapted Scenedesmus at high light intensity in the presence of CMU. The gas phase was hydrogen, the light intensity was 32,000 lux and the CMU concentration was 1×10^{-4} M.

Fig. 17. $C^{14}O_2$ fixation pattern during the ox-hydrogen reaction. The gas phase was hydrogen + 1.5% oxygen. The fixation took place in the dark.

Here, as in the experiments described earlier, the reducing conditions provided by hydrogenase stimulate the PGA to alanine pathway. The carboxylation reaction leading to malic acid also plays a more prominent role in photoreduction and the ox-hydrogen reaction than in photosynthesis.

The kinetic studies under hydrogen at high light intensities shown in Fig. 15 and 16 differ in the path followed by carbon from PGA. In the case where no CMU is added, carbon flows from PGA into sugar phosphates. However, when CMU is present, the flow of carbon from PGA shows the split between sugar phosphates and alanine characteristic of photoreduction. It was shown earlier that unless the Hill reaction is blocked, the hydrogenase is destroyed at high light intensities. When the hydrogenase has been inactivated, the system is indifferent to the potentially reducing conditions afforded by a hydrogen atmosphere. On the other hand, when CMU, a potent inhibitor of the Hill reaction, is added, photoreduction takes place at the high light intensity of 32,000 lux.

4. Inhibitor studies.

In hopes of learning more about the processes which supply the reducing agent and ATP needed for CO₂ fixation, the system was studied with a number of inhibitors. The inhibitors used were CMU, menadione, and 2,4-dinitrophenol. The herbicide CMU, is a potent inhibitor of the Hill reaction. In green plants, the Hill reaction is necessary for the generation of the reduced cofactors and ATP needed to carry on photosynthesis. Menadione, or vit. K₃, is also an inhibitor of the Hill reaction.³⁴ However, Vit. K₃ is also known to induce a different

mode of photophosphorylation in green plants.³⁵ Work in isolated chloroplasts³⁶ has shown that under normal conditions, light causes the following reaction:

This is called non-cyclic photophosphorylation, but if small amounts of Vit. K₃ are added to the system, light causes this reaction:

This is called cyclic photophosphorylation.

The non-cyclic phosphorylation couples the Hill reaction to the production of TPNH and ATP, the two co-factors needed for CO₂ fixation. On the other hand, the cyclic phosphorylation produces only one of the essential cofactors for carbon fixation.

2,4 dinitro phenol has been shown to act as a general uncoupler of oxidative³⁷ and photophosphorylation.³⁸ 2,4 DNP will block any process that is dependent on ATP generated by electron transport processes such as photosynthesis and respiration.

The effect of CMU, Vit. K₃ and 2,4 DNP on CO₂ fixation during photosynthesis, photoreduction, ox-hydrogen reaction, and respiration are shown in Tables IXa and IXb. All three inhibitors cause a virtual stoppage of CO₂ fixation during photosynthesis. Photoreduction shows a much greater resistance to these inhibitors. The dark fixations shown in Table IXb are unaffected by CMU. However, 2,4 DNP and Vit. K₃, potent uncouplers of oxidative phosphorylation, cause inhibition of CO₂ fixation during the ox-hydrogen reaction and during respiration.

Table IXa

Effect of inhibitors on CO₂ fixation during photosynthesis and photo-reduction. Light intensity: 1250 lux. Preincubation with inhibitors: 60 min. Time of incubation with tracers: 30 min.

Gas Phase	Inhibitor	C ¹⁴ O ₂ fixed in cpm x 10 ⁻⁶	Percent Inhibition
Hydrogen	None	18.1	--
Hydrogen	Vit. K ₃ (saturated solution)	5.9	65
Hydrogen	CMU (1 x 10 ⁻⁴ M)	9.7	45
Hydrogen	2,4-DNP (10 ⁻³ M)	7.2	60
Air	None	3.8	--
Air	Vit. K ₃ (saturated solution)	0.08	98
Air	CMU (1 x 10 ⁻⁴ M)	0.3	92
Air	2,4-DNP (10 ⁻³ M)	0.05	99

Table IXb

Effect of inhibitors on ox-hydrogen and respiratory CO₂ fixation.

Preincubation with inhibitors: 60 min. Time of incubation with tracer:
30 min.

Gas Phase	Inhibitor	% Inhibition
Air	Vit. K ₃ (sat. solution)	97
Air	CMU (1 x 10 ⁻⁴ M)	0
Air	2,4-DNP (10 ⁻³ M)	70
H ₂ + 1.5% O ₂	Vit. K ₃ (sat. solution)	80
H ₂ + 1.5% O ₂	CMU (1 x 10 ⁻⁴ M)	0
H ₂ + 1.5% O ₂	2,4-DNP (10 ⁻³ M)	98

The respiratory activities of Scenedesmus under air or under hydrogen containing traces of oxygen would oxidize reduced cofactors with concomitant production of ATP. However, in the presence of respiratory electron transport coupled phosphorylation uncouplers such as 2,4 DNP or Vit. K₃, the oxidation of reduced cofactors is not accompanied by ATP production and CO₂ fixation suffers.

In the case of photosynthesis, the profound inhibitory effects of CMU, Vit. K₃ and 2,4 DNP can be explained of their effects on the Hill reaction and photophosphorylation. Blocking the Hill reaction with CMU prevents the generation of TPNH and ATP as shown in equation 5. Although Vit. K₃, unlike CMU, does not block all forms of photophosphorylation, it does prevent the generation of TPNH. As pointed out earlier, both TPNH and ATP are necessary for CO₂ fixation. Failure to supply one or both of these essential cofactors results in a loss of capacity for carbon fixation. Dinitrophenol very probably exerts its inhibitory effect in all of the experiments of Tables IXa and IXb by uncoupling phosphorylation.

During photoreduction in the presence of Vit. K₃, CO₂ fixation proceeds though with considerable decrease in efficiency. One would expect CO₂ fixation to proceed under the conditions of photoreduction, even with Vit. K₃ present. The cyclic photophosphorylation shown in Equation 6, which is induced by Vit. K₃, could provide ATP. Hydrogenase would supply electrons to reduce the needed cofactors for carbon fixation.

It is surprising that CO₂ fixation proceeds when photoreduction is carried out in the presence of CMU. CMU blocks the Hill reaction and consequently non-cyclic photophosphorylation. CMU does not induce

any cyclic forms of photophosphorylation. It is true that hydrogenase would allow the system to use the reducing power of molecular hydrogen. But to reduce CO_2 there must be a source of reduced pyridine nucleotide and ATP. Some recent experiments,^{39,40} delineating more clearly the role of light in generating ATP may provide the explanation for failure of CMU to completely inhibit CO_2 fixation during photoreduction.

From experiments in chloroplasts and other systems, green plant photosynthesis has been shown to depend on at least two different light reactions.⁴¹ This multiple wavelength dependence is schematized below:

In isolated chloroplasts, the relative quantum yield for oxygen evolution is greatest for light in the neighborhood of 640 $\text{m}\mu$. The relative quantum yield for TPN reduction and ATP formation is greatest at a longer wave length. The role of the first ($h\nu_1$) light reaction is to supply the energy for extracting an electron from water. The second ($h\nu_2$) light reaction pulls the electron through a phosphorylating step or steps and makes possible the reduction of pyridine nucleotide. If the first reaction is blocked by the addition of CMU, the second will not normally take place. However, if an electron donor of proper reducing potential, such as reduced dichlorophenol indophenol, is added

to the system, the second reaction generating ATP and TPNH will take place.

Considered in light of these latest findings, the failure of CMU to block CO_2 fixation during photoreduction may be understood. CMU blocks some stage in the first light reaction that normally produces electrons for the second phosphorylating light reaction. However, in hydrogen-adapted Scenedesmus, light does produce ATP. One is led to the conclusion that under these conditions, molecular hydrogen, through hydrogenase, is capable of supplying electrons to the phosphorylating sites of the second light reaction. It is quite possible that during normal photoreduction, the hydrogenase supplies a sizeable portion of the electrons used during the second photoreaction.

IV. SUMMARY

The radioactive tracers ^3H and ^{14}C have been used to study the generation, properties and metabolic implications of *Scenedesmus* hydrogenase. The use of tritium in the exchange reaction catalyzed by hydrogenase makes it possible to make a direct, in vivo estimate of hydrogenase activity. Aside from the advantages of great sensitivity enjoyed in radioactivity measurements, the tritium exchange assay can be used to evaluate hydrogenase activity in whole cells without disturbing the metabolism of the organism. A quantitative and qualitative study of carbon fixation in hydrogen-adapted *Scenedesmus* reveals the path of carbon from CO_2 to stable organic compounds. Since CO_2 fixation requires ATP and reduced pyridine nucleotide, the quantity of CO_2 fixed under a variety of conditions is an indication of the relative level of these two cofactors.

The kinetics of hydrogenase formation show that the full complement of hydrogenase is present after about three hours. Even after only five minutes of incubation under hydrogen, detectable amounts of hydrogenase are present. The studies with dithionite have shown that lowering the reduction potential of the system accelerates the formation of *Scenedesmus* hydrogenase. The same observation has been made for the formation of the hydrogenase of *Proteus vulgaris*.⁴² The sensitivity of the rate of hydrogenase formation to the reduction potential of the system indicates that the active enzyme is probably formed by reductive modification of a pre-existing protein. The kinetics of hydrogenase formation suggest that its formation is an autocatalytic process. Units of

hydrogenase formed earlier appear to facilitate the formation of subsequent units, perhaps by providing additional reducing power for the modification of an inactive form of the enzyme.

Adapted Scenedesmus has a higher hydrogenase activity than the Chlamydomonas algae and more than representative members of two bacterial families. Scenedesmus hydrogenase exhibits the sensitivity to carbon monoxide and molecular oxygen shown by the hydrogenases found in bacterial systems. This algal hydrogenase also shows a sensitivity to cyanide seen in some, but not all, bacterial systems.^{43,44} The sensitivity of Scenedesmus hydrogenase to CO and CN are strong indications that a heavy metal prosthetic group is a critical component of the enzyme structure. The partial photoreversibility of the CO inhibition by visible light indicates that the prosthetic group binding the heavy metal is a chromophore capable of absorbing visible light.⁴⁵ An experiment with labeled hydrogen has shown that Scenedesmus supplies electrons not protons from molecular hydrogen.

In the CO₂ fixation experiments, the presence of active hydrogenase increased the quantity of carbon fixed and caused alteration in the path of fixation. The kinetic experiments seen earlier showed that if an energy source is provided, CO₂ is fixed primarily along the outlines of the carbon reduction cycle. This is true during either photoreduction or the ox-hydrogen reaction. However, the presence of the additional reducing power afforded by active hydrogenase diverts the flow of carbon from PGA. Instead of flowing from PGA to sugar phosphates, under hydrogen a sizable portion of the carbon is diverted to alanine. An enzyme that synthesizes alanine from pyruvic acid,

NH_3 and reduced pyridine nucleotide has been found in bacteria.⁴⁶

Such a pathway from PGA to alanine would require less energy than the path from PGA to sugar phosphates. It is quite possible that such a pathway is made possible by the additional reducing power available in hydrogen-adapted Scenedesmus.

The ability of hydrogen-adapted Scenedesmus to use light to fix CO_2 when the Hill reaction has been blocked by CMU was particularly interesting; the earlier discussion cited the recent evidence indicating that the Hill reaction is a light-driven reaction that generates electrons for a second ATP-TPNH producing light-driven reaction. The fact that photoreduction, an ATP requiring process, proceeds even in the presence of CMU indicates that hydrogenase can supply electrons to sites of the phosphorylating light reaction. It is quite possible that even in the absence of CMU the hydrogenase competes with the Hill reaction in supplying electrons for the phosphorylating light reaction. This would result in a lower quantum requirement for photophosphorylation during photoreduction than during photosynthesis.

Clearly, hydrogenase has a profound effect, both quantitative and qualitative, on metabolic pathways. The mechanisms by which hydrogenase extracts electrons from molecular hydrogen and donates them to the cellular economy are not understood. Until the prosthetic group of hydrogenase is identified, an understanding of hydrogenase action will be out of the question. One of the primary reasons why the prosthetic group of hydrogenase is not known lies in the unavailability of a pure enzyme. Although hydrogenase has been known for more than thirty

years,⁴⁷ at this writing enzymologists have been unable to isolate hydrogenase. Though much is known about its effects and gross characteristics, the real nature of hydrogenase remains a mystery.

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