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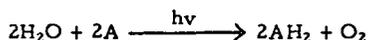
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BIOPHOTOLYSIS OF WATER TO HYDROGEN AND OXYGEN

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The concept of the biophotolysis of water with the formation of oxygen and hydrogen is the bringing together of two biological fields of scientific endeavor, each of which has made phenomenal progress during the last decade or two. The two areas of progress referred to are: (1) a greater understanding of the molecular events which occur in photosynthesis, and (2) a greater understanding of molecular events in microbial metabolism. Although thermodynamically feasible, heretofore not much thought has been given to the possibility of biophotolysis.

The photosynthetic apparatus which consists of two photosystems operating in series can, by capturing two quanta of radiant energy, place an electron from the water-oxygen couple (+0.8 volts pH 7.0) to a negative value as much as -0.7 volt which is 0.3 volts more negative than the hydrogen electrode. A minimum of eight quanta of radiant energy are required for the following photosynthetic equation:



where A is an electron acceptor (Hill reagent). A variety of compounds may serve as Hill reagents. For the purpose of employing these photosynthetic electrons for the reduction of protons to hydrogen by the action of a bacterial hydrogenase, the acceptor must have an oxidation-reduction potential near the potential of the hydrogen electrode and in its reduced state serve as a substrate for the hydrogenase. Several anaerobic non-photosynthetic and photosynthetic bacteria form hydrogen during some of their metabolic processes. Usually the immediate precursor of electrons for the reduction of protons as catalyzed by these hydrogenases is reduced ferredoxin, a small molecular weight iron-sulfur protein. A similar species of protein although smaller in molecular weight is in the normal pathway of electron transfer in the photosynthetic apparatus. In this latter case the reduced ferredoxin reduces

triphosphopyridine nucleotide (TPN) which in turn is the source of electrons for the reduction of carbon dioxide to plant materials. With some anaerobic bacteria it has been demonstrated that reduced TPN can reduce ferredoxin with sufficient kinetics in spite of the unfavorable thermodynamic barrier of approximately 0.1 volt. The electrons of reduced ferredoxin are the precursors of hydrogen as catalyzed by the bacterial hydrogenase.

The first experiments which demonstrated the biophotolysis of water to oxygen and hydrogen consisted of a two-stage reaction mixture. The first stage employed the classical method of San Pietro and colleagues who demonstrated that spinach chloroplasts could photosynthetically reduce TPN by the oxidation of water, providing ferredoxin was present. Five μ moles of TPNH were prepared and added to the second mixture containing the components listed in Table 1.

Table 1

HYDROGEN FORMATION FROM TPNH
-- NO GENERATING SYSTEM

	<u>per ml</u>
Tris buffer pH 7.6	100.0 μ moles
Glutathione (SH)	2.0 μ moles
TPNH	5.0 μ moles
Na pyruvate	20.0 μ moles
DPN	1.0 μ moles
Lactic dehydrogenase	9 units
<i>C. kluveri</i> hydrogenase	0.4 ml
Ferredoxin	500 μ g

Total volume 10 ml, argon atmosphere, temp. 35°C

	<u>Hydrogen evolved</u> (μ moles)
15 min	3.2
30 min	3.2
45 min	3.2

The important components for this discussion are TPNH, the bacterial hydrogenase preparation from *Clostridium kluveri* and buffer. At the bottom of the table is given the quantity of hydrogen evolved from TPNH. Control experiments demonstrated that hydrogen evolution was totally dependent upon the presence of TPNH. The quantity of hydrogen is less

than the TPNH added but it could be shown that the activity of the hydrogenase was lost shortly after the beginning of the experiment. A general characteristic of hydrogenases is that they are labile, particularly to the presence of oxygen. In this case, although the experiment was conducted under an atmosphere of argon, the oxidized TPN formed from the oxidation of TPNH to hydrogen inactivated the enzyme.

In an attempt to overcome the inhibition by TPN of hydrogenase activity and the thermodynamic barrier between TPNH and the hydrogen electrode in these anaerobic bacteria, we investigated the probability of employing photosynthetic bacteria. Photosynthetic bacteria although they cannot use water as an electron source use reduced organic or inorganic substances for photosynthetic electron donors and in many cases can form hydrogen photosynthetically from these substrates. Rhodospseudomonas capsulata, a photosynthetic bacterium, can form copious quantities of hydrogen from malate under anaerobic conditions providing no elemental nitrogen or fixed forms of inorganic nitrogen are present. The photochemical systems present in spinach chloroplasts are also labile, having a short-lived mechanism for forming TPNH. The photosystems present in the blue-green alga, Anacystis nidulans, were therefore employed. Freshly harvested cells of the alga are impermeable to most organic substrates. Lyophilization under appropriate conditions render the cells permeable to several substances including TPN and photosystems I and II remain active and reasonably stable. These cells were employed to prepare reduced TPN (TPNH) photosynthetically, using water as the electron donor. No reduction of TPN occurred in the dark or in the presence of 1,1 dimethyl-3-(3,4 dichlorophenyl) urea (diuron), a specific inhibitor for photosystem II.

Lyophilized cells of R. capsulata are permeable to reduced TPN and contain a very active malate dehydrogenase. These cells by well known biochemical reactions can reduce oxalacetate to malate with the reduced TPN. In contrast to freshly harvested cells the lyophilized cells cannot photosynthetically form hydrogen from malate. With a mixture of freshly harvested cells and the lyophilized cells, small quantities of oxalacetate and substrate amounts of reduced TPN (obtained photosynthetically with the algae) hydrogen was obtained photosynthetically in quantities equivalent to the amount of reduced TPN added. For example, with 10 μ moles of TPNH, 20 mg (dry weight) freshly harvested R. capsulata, and 5.0 mg lyophilized cells in a total volume of 2 ml under an atmosphere of argon and exposed to 22,000 lux of white light, 10 μ moles of hydrogen were obtained in 45 minutes.

The events may be summarized as follows:

Water + algae + TPN + light \longrightarrow TPNH + oxygen

TPN + oxalacetate + lyophilized R. capsulata \longrightarrow TPN + malate

Malate + fresh R. capsulata + light \longrightarrow hydrogen + oxalacetate

Omitting the microorganisms and electron carriers, the sum of the equations become:

Water + light \longrightarrow hydrogen + oxygen

In a one-stage experiment, by combining the following components: 40.0 mg Anacystis nidulans, 2.5 μ moles TPN, 50.0 mg (dry weight) freshly harvested R. capsulata, 12.0 mg lyophilized R. capsulata in a total volume of 5 ml under an atmosphere of argon with 175,000 lux of white light, 2 μ moles of hydrogen were formed in 30 minutes. Apparently the hydrogenase of the photosynthetic bacterium is sufficiently stable to the oxygen liberated by the photosynthetic activity of algae to form hydrogen from the photosynthetically formed TPNH.

The experiments cited here as well as other conclusively show that hydrogen can be formed by the biophotolysis of water.