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IMPROVED HYDROGEN PHOTOPRODUCTION FROM PHOTOSYNTHETIC BACTERIA AND GREEN ALGAE

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NOTICE

IMPROVED HYDROGEN PHOTOPRODUCTION FROM PHOTOSYNTHETIC BACTERIA AND GREEN ALGAE

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Photosynthetic bacteria evolve hydrogen at much higher rates than do other classes of photosynthetic microorganisms. In addition, they tolerate harsh environments, grow rapidly, and utilize both visible and near infrared light in photosynthesis. They do not split water, but this does not necessarily eliminate their potential use in future applied systems. They are easily manipulated genetically, and thus might be modified to metabolize common biomass waste materials in place of expensive defined organic substrates. Furthermore, the potential for increasing hydrogen photoproduction via genetic techniques is promising. Strains that partially degrade cellulose, have high photoproduction rates, or contain very large amounts of the enzymes associated with hydrogen metabolism have been isolated.

Green algae also produce hydrogen but are capable of using water as a substrate. For example, C. reinhardi can evolve hydrogen and oxygen at a molar ratio approaching 2:1. Based upon the effect of dichlorophenyl dimethylurea (a specific inhibitor of photosystem II, PSII) on hydrogen photoproduction in the wild type strain and upon results obtained with PSII mutants, one can demonstrate that water is the major source of electrons for hydrogen production. The potential efficiency of in vivo coupling between hydrogenase and the photosynthetic electron transport system is high. Up to 76% of the reductants generated by the electron transport system can be channeled directly to the enzyme for in vivo hydrogen production. Rates exceeding 170 μmoles of $H_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$ have been observed.

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INTRODUCTION

This paper will examine the progress we have made and would like to make in the area of microbial H_2 production using two types of organisms, photosynthetic bacteria and green algae.

PHOTOSYNTHETIC BACTERIA

Photosynthetic bacteria are not capable of using water as electron donor for photosynthesis. Instead, they utilize any of a large number of reduced carbon (or sulfur) compounds as reductant sources. In the absence of combined or free nitrogen they convert carbonaceous compounds into $H_2 + CO_2$ as indicated in Table 1. When they are incubated in the light under N-limited conditions which uncouples H_2 metabolism from growth, the bacteria act as pure photocatalysts, converting more than 98% of a given substrate, such as lactate, stoichiometrically into $H_2 + CO_2$. Presently, about 2.8% of the radiant energy is conserved as heat of combustion of the H_2 produced ($\sim$ 97% of the substrate chemical energy is conserved). The rate of H_2 production is approximately one volume of H_2 produced per equivalent volume of packed cells per minute. Cultures evolve H_2 at constant rates over several weeks as long as the substrate is replenished when depleted.

Table 2 gives some current representative rates of H_2 production from wild type, mutant, and mixed culture systems. It should be noted that much of the data are preliminary. After screening more than 100 distinct wild type strains of 13 species of photosynthetic bac-

teria, we have found that Rhodopseudomonas capsulata SCJ exhibits one of the highest rates. Through proper strain selection and environmental or genetic manipulations, the usefulness and efficiencies of photosynthetic bacteria as photoconversion agents can certainly be further improved.

At present, the cost of defined carbonaceous substrates make their photoconversion by photosynthetic bacteria economically unfeasible. If plentiful forms of biomass, such as cellulose or agar, could be metabolized by, or be made available to, these organisms, substrate costs would not be so prohibitive. Of the strains isolated so far only Rps. palustris EC has the capacity to metabolize cellulose directly. It will mediate the photoconversion of cellulose into H_2 and CO_2, but only at 1/30 of the rate that it utilizes lactate and only to a limited extent. Nevertheless, it appears possible that a more adept wild type or genetically engineered strain would make this process feasible.

Alternatively, mixed cultures also show promise in H_2 production systems, though they are subject to problems of stability. A co-culture of Rps. sulfidophila BSW8 and an untyped non-photosynthetic marine isolate exhibiting agarase activity will evolve H_2 derived from agar for a period of 1-2 days. Mutant strains lacking secreted bacteriocins may alleviate problems of mutual intolerance.

Genetic techniques also can be employed to amplify photoconversion processes. Rps. capsulata mutant W12 is defective in its capacity to fix nitrogen (Nif^-) and only produces small amounts of H_2. Since hydrogenase is present in the organism, the nitrogenase enzyme complex is responsible for at least the majority of the H_2 evolved in the wild type (2). Strain W52, however, is defective in the uptake of H_2 (Hup^-), and the rate of H_2 production is increased relative to that of the wild type. This indicates that H_2 evolved by nitrogenase is incapable of being reoxidized in this type of mutant, and therefore, the H_2 yield is higher.

Rates of H_2 production also might be improved by creating mutants genetically derepressed for nitrogenase (Nif^c). Such a strain would permit the photoconversion of high-nitrogen biomass wastes. A partially derepressed mutant of R. rubrum has been generated by Weare (1), and although the assay conditions were not optimal, the mutant exhibited a 75% increase in H_2 production over the wild-type parent.

At present we do not know what other rate-limiting steps are involved in the photoproduction of H_2, but in vivo genetic engineering may well be used to remove them as they are discovered. One such bottleneck, especially at high intensity light, may be the amount of reaction center BChl available for photoconversion. R. rubrum mutant GF-9, unlike its parent, is lacking the large majority of its light-harvesting BChl component absorbing at 870 nm. The dominant chlorophy-

llous component is reaction center BChl which is reversibly photooxidizable and is about 8 times more prevalent on a cell mass basis than in its parent. Unfortunately, due to pleiotropic or secondary mutational effects, this mutant photoproduces H_2 only at low rates so that the effect of the reaction center enhancement is not readily ascertainable.

Figure 1 demonstrates the rationale behind a technique designed to extend the useful wavelengths of the solar spectrum. Using mixed (or separately layered) cultures of photosynthetic organisms individually absorbing different wavelengths of light, it should be possible to make biological "black bodies" absorbing all wavelengths from 350 to 1050 nm. Toward this end we are actively seeking new isolates which contain BChl b or BChl c and which also produce H_2 at rates comparable to BChl a-containing strains.

Lastly, major improvements in photosynthetic bacterial H_2 production rates could probably be obtained if hydrogenase rather than nitrogenase were used as the evolving enzyme since hydrogenase does not require the surfeit of ATP that is necessary for nitrogenase activity. Such a mechnaism would require the existance or creation of efficient electron transport coupling and reductant pressures, however. Towards this goal we have surveyed large numbers of photosynthetic bacteria to identify strains most adept in H_2 uptake activity as indicated in Table 3. One particular isolate, the BSW8 strain of Rps.

sulfidophila exhibits activities far above those for other isolates. When derepressed either by autotrophic growth on H_2 or by heterotrophic growth with prolonged anaerobic, dark incubation, activities that are 50 times higher than for example, those of R. rubrum S-1 can be achieved. Research on the feasibility of modifying Hup pathways for the photoproduction of H_2 is currently under way.

GREEN ALGAE

While photosynthetic bacteria are ideal organisms for efficient conversion of organic substrates into H_2, they cannot use water as the source of reductant. Consequently, H_2 production by photosynthetic bacteria does not result in a significant net conversion and storage of solar radiation energy into chemical free energy. In contrast, the photosynthetic apparatus of algae and higher plants contains an effective water-splitting complex associated with photosystem II(PSII) which can be coupled to hydrogenase for H_2 production with a maximum potential net energy conversion effeciency in excess of 10% (3,4). The following discussion will concentrate on defining the sources of reductant and the efficiency of coupling between the electron transport carriers and hydrogenase. We will also describe briefly some observations on the activation of the hydrogenase activity in vivo.

Source of Reductant

Since the discovery of algal H_2 photoproduction by Gaffron and Rubin in 1942 (5), the source of reductant has been a matter of consider-

able controversy (3, 6, 7). The stimulation of H_2 production by glucose and other organic substrates, the insensitivity of the process toward inhibitors of PSII, the inhibition by monofluoroacetate, and the concomitant release of CO_2 during H_2 production (8-10) suggest that organic compounds are the major source of reductant. On the other hand, the simultaneous production of both O_2 and H_2, the inhibition by DCMU, and the inability of PSII defective mutant strains to evolve H_2 indicate a direct contribution of PSII and the water-splitting reaction (7, 11, 12). In our studies using Chlamydomonas reinhardi, we routinely observe simultaneous production of H_2 and O_2 in a molar ratio approximating the theoretical value of 2:1 (Fig. 2), indicating that water is the major source of reductant. This conclusion is further supported by the data in Table 4. The wild type strain produces H_2 and O_2 with a molar ratio of 1.92. The production of both gases is abolished by addition of dichlorophenyl dimethylurea (DCMU), a specific inhibitor of PSII. Moreover, mutant strain PET 20-1, with a mutational block on the reducing side of the PSII reaction center, not only failed to produce O_2 but also was unable to generate H_2 at a rate exceeding a few percent of that obtainable with wild type cells. Oxygen production is also severely inhibited in the case of mutant PET 10-1, which is blocked on the oxidizing side of PSII. However, a significant fraction of the H_2 production capacity was retained. As a result, the ratio of H_2 to O_2 increased from 1.92 (in the wild type) to 12. Furthermore, the

addition of an exogenous carbon source, sodium acetate, stimulates the production of H_2 nearly two-fold but further suppresses the residual O_2 evolving capacity of the mutant. In this case, 33 moles of H_2 were produced for each mole of O_2. Clearly, the reductant must come from organic substrates rather than water. Since H_2 production by this mutant is inhibited by DCMU, substrates must enter the electron transport system on the oxidizing side of PSII.

Efficiency of Coupling in Vivo

An important aspect of H_2 photoproduction in algal systems which has rarely been addressed is the efficiency of *in vivo* coupling between hydrogenase and the photosynthetic electron transport system. Is the activity of hydrogenase and the associated redox carrier system sufficient to process the steady state flux of reductant generated by PSII?

The data summarized in Table 5 show that when properly adapted cells of *C. reinhardi* are illuminated under saturating white light, 84 μmoles $H_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$ are observed with simultaneous production of O_2. Addition of sodium dithionite increased the production rate to 106 μmoles $H_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$ probably by eliminating the back (knallgas) reaction between H_2 and O_2 (as illustrated in Fig. 2) as well as by protecting the hydrogenase itself from inactivation by O_2. Furthermore, addition of the phosphorylation uncoupling agent, carbonylcyanide 3-chlorophenyl hydrozone (CCCP) which releases the constrains

on electron flow by the phosphorylation steps, resulted in a maximal rate of 174 μmoles $H_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$. Since in the presence of dithionite, the reaction is still highly sensitive to DCMU, H_2 production by these cells is also coupled to PSII reactions. One can estimate from the rate of H_2 production and the rate of steady state photosynthesis (115 μmoles of $O_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$ in cells from the same batch of culture) that the overall coupling efficiency between the hydrogenase and the photosynthetic electron transport system is as much as 76% of the reductant generating capacity of normal photosynthesis.

Activation of Hydrogenase in Vivo

Hydrogen production in green algae is catalyzed solely by hydrogenase which is inactive under aerobic conditions (3,6,7). Thus, both production and utilization of H_2 by algae requires an anaerobic adaptation period of varying duration. Using washed, autotrophically grown cells of C. reinhardi, we found that detectable rates of H_2 photoproduction appeared after 45 minutes of anaerobiosis and maximal H_2 production activity was obtained after 3-5 hours of anaerobic incubation. However, if the cells were resuspended in the same buffer supplemented with sodium acetate, H_2 production can be detected within 10-15 minutes of anaerobiosis, and maximal H_2 production activity was attained in about 2 hours (see also expt. 1 of Table 6). An acceleration of the hydrogenase activation by organic substrates

can be expected if the process requires energy. Thus, one would expect the activation of hydrogenase activity to slow down if the energy production reactions of the cells were inhibited. In fact, we observed that CCCP strongly inhibits the activation of hydrogenase (Table 6, expt. 2). Similarly, sodium arsenate (an inhibitor of ATP production by both substrate level and electron transport coupled phosphorylation reactions) caused a nearly complete inhibition of the activating process (Table 6, expt. 3). It must be emphasized that both agents had essentially no effect on the hydrogenase activity of preactivated cells. These observations clearly establish the energy requirement of the hydrogenase activating process in vivo.

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Table 1. STOICHIOMETRY OF PHOTOSYNTHETIC BACTERIAL CONVERSIONS

1.	$C_3H_6O_3$ (Lactate) + 3 $H_2O \xrightarrow{h\nu}$	3 CO_2 + 6 H_2
2.	$C_6H_{12}O_6$ (Glucose) + 6 $H_2O \xrightarrow{h\nu}$	6 CO_2 + 12 H_2
3.	$C_2H_4O_2$ (Acetate) + 2 $H_2O \xrightarrow{h\nu}$	2 CO_2 + 4 H_2
4.	$C_4H_6O_5$ (Malate) + 3 $H_2O \xrightarrow{h\nu}$	4 CO_2 + 6 H_2
5.	$(CH_2O)n$ (Biomass) + $H_2O \xrightarrow{h\nu}$	CO_2 + 2 H_2

Reaction Extent:	>98% complete for example 1. (>1350 liters H_2/kg substrate)
Conversion Efficiency:	2.8% of radiant energy (97% of chemical energy) in example 1. is conserved as H_2.
Hydrogen Evolution Rates:	1 vol. H_2·equal vol. cells^{-1}·min^{-1} or 175 ml H_2·g dry wt^{-1}·hr^{-1}.

Table 2. REPRESENTATIVE RATES OF H_2 PHOTOPRODUCTION FROM PHOTOSYNTHETIC BACTERIA

Organism	Activity ($ml\ H_2 \cdot mg\ dry\ wt^{-1} \cdot hr^{-1}$)	Comments
Rhodopseudomonas capsulata SCJ	168	Lactate as C source
Rhodopseudomonas capsulata B10	124	Lactate as C-source
Rhodopseudomonas sulfidophila BSW8	106	Lactate as C-source
Rhodopseudomonas viridis NTHC 133	3	Lactate as C-source
Rhodospirillum rubrum S-1	146	Lactate as C-source
Rhodopseudomonas palustris EC	62	Lactate as C-source
Rhodopseudomonas palustris EC	2	Cellulose as C-source
Rhodopseudomonas sulfidophila BSW8 plus an unidentified marine species	6	Agar as C-source
Rhodopseudomonas capsulata W12 (B10 Nif^-)	3	Lactate as C-source
Rhodopseudomonas capsulata W52 (B10 Hup^-)	144	Lactate as C-source
Rhodospirillum rubrum S-1	4	Ref. 1
Rhodospirillum rubrum 11 C31 (S-1 Nif^c)	7	Ref. 1

Table 3. REPRESENTATIVE RATES OF H_2 UPTAKE*

Organism	Growth Conditions	Activity (1 H_2·mg dry wt^{-1}·min^{-1})
Photosynthetic Bacteria		
Chromatium vinosum, Strain D	Thiosulfate heterotrophic, light	5.82
Rhodospirillum rubrum S-1	Heterotrophic, light	1.35
	Heterotrophic, light (nitrogenase induced)	0.45
	Heterotrophic, light (early logarithmic phase)	0.00
	Heterotrophic, dark, aerobic	0.00
	H_2 autotrophic, light	2.09
Thiocapsa roseopersicina	Thiosulfate autotrophic, light	3.80
Rhodomicrobium vannielii	Heterotrophic, light	0.52
Rhodopseudomonas capsulata SP7	H_2 autotrophic, light	24.95
Rhodopseudomonas capsulata SCJ	H_2 autotrophic, light	2.42
Rhodopseudomonas sulfidophila BSW8	Heterotrophic, light	4.15
	Heterotrophic, light (nitrogenase induced)	6.13
	Heterotrophic, light (dark incubated 60 hours)	103.1
	H_2 autotrophic, light	87.46
Rhodopseudomonas sulfidophila W4	H_2 autotrophic, light	4.41
Nonphotosynthetic Bacteria		
Desulfovibrio, marine sp.	Heterotrophic, dark	42.88
Clostridium pasteurianum	Heterotrophic, dark	5.42

*Unpublished results of P. F. Weaver

All reactions were measured in the dark with Warburg apparatus at 32°C and H_2 as gas phase. Cultures were in early stationary phase of growth unless otherwise noted and assayed in spent medium adjusted to pH 7.2. C. pasteurianum cells were previously frozen to -20°C. Reactions were initiated by tipping 0.3 ml of methylene blue solution (75mg/ml) into the reaction vessel.

Table 4. HYDROGEN PHOTOPRODUCTION WITH MUTANT STRAINS OF C. REINHARDI

Strains	Rate (μmoles H_2 or O_2 · mg $Chl^{-1} \cdot hr^{-1}$)		H_2/O_2
	H_2 Production	O_2 Production	
Wild Type	71	37	1.92
Wild Type + DCMU	6	0	-
PET 20-1	4.2	0	-
PET 20-1 + DCMU	4.8	0	-
PET 10-1	28	2.3	12
PET 10-1 + Acetate	46	1.4	33
PET 10-1 + Acetate + DCMU	5.2	0	-

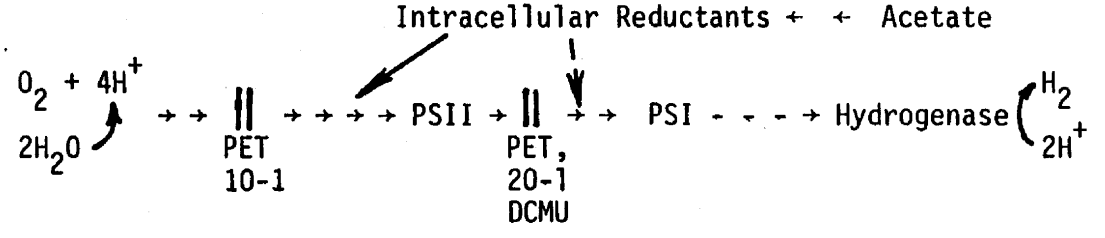

Table 5. HYDROGEN PHOTOPRODUCTION BY ANAEROBICALLY ADAPTED C. REINHARDI CELLS

Assays	Rate (μmoles $H_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$)
A: No Addition	84
B: A + Dithionite (O_2-trap)	106
C: B + 5μm CCCP (uncoupler)	174
D: C + 2μm DCMU (PSII inhibitor)	18

Note 1: Steady state rate of photosynthesis by the same culture under aerobic conditions = 115 μmoles $O_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$. Equivalent to 230 μmoles of $H_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$.

Note 2: Efficiency of *in vivo* coupling between hydrogenase and photosynthetic electron transport = 174/230 = 76%.

Table 6. EFFECT OF ACETATE AND UNCOUPLERS ON THE ACTIVATION OF HYDROGENASE

Anaerobic Incubation Conditions		Rate of H_2 Photoproduction (μmoles $H_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$)	
		1.5 Hr. Anaerobiosis	4.0 Hr. Anaerobiosis
Expt. 1.	A: In PO_4 Buffer (60 mM, pH 7.4)	24	91
	B: A + 10 mM Sodium Acetate	82	116
Expt. 2:	A: In PO_4 Buffer (60 mM, pH 7.4)	31	130
	B: A + 2.5 μM CCCP	4.5	14.5
	C: A + 5 μM CCCP	1	8.8
	D: A + 10 μM CCCP	0.25	0.9
Expt. 3:	A: In Tris-Buffer (60 mM, pH 7.6)	45	144
	B: A + 5 mM Sodium Arsenate	0.4	0.6

Experiments 1-3 were performed with three different batches of autotrophically grown cells on separate dates. In each case the cells were harvested and incubated anaerobically under the same conditions. The cell density during incubation and assay was kept within the range of 20 - 30 µg Chl per ml.

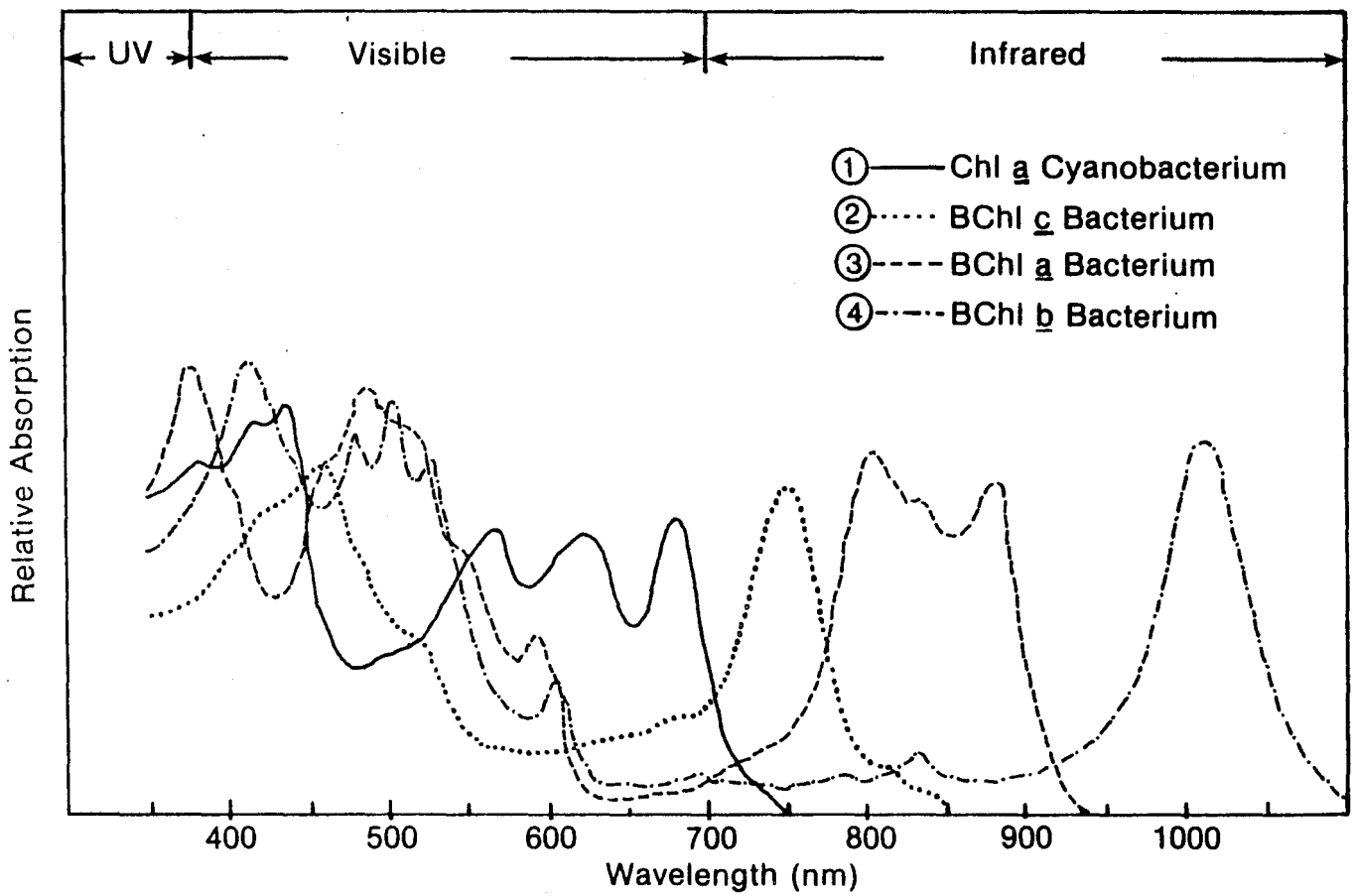

FIGURE 1 RELATIVE ABSORPTION SPECTRA OF VARIOUS PHOTOSYNTHETIC MICROORGANISMS

Mixed or layered cultures of various organisms might lead to the more complex use of sunlight between 350 and 1050 nm.

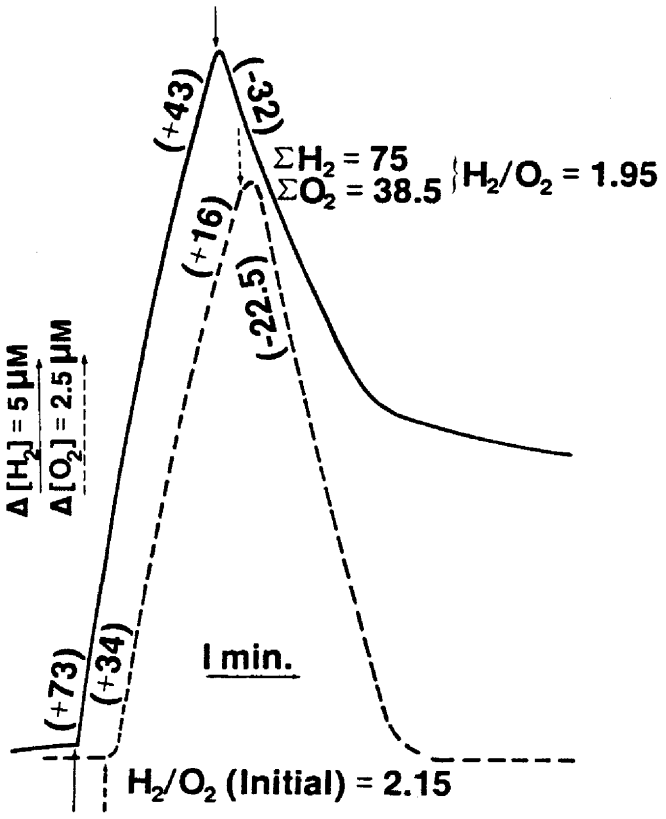

FIGURE 2. SIMULTANEOUS PRODUCTION OF H_2 AND O_2 BY C. REINHARDI
H_2 and O_2 production were assayed polarographically with two Clark Electrodes in a gas-tight reaction vessel at 25°C. The Pt electrode was polarized negatively for O_2 or positively for H_2 against the Ag/AgCl electrode. The sample contained anaerobically adapted (3 hr.) cells suspended in 60mM PO_4 buffer (pH=7.4) at a cell density equivalent to 14 µg Chl/ml. The traces for H_2 and O_2 are offset slightly for clarity. Numbers in the parentheses indicate the rate of net production or uptake of the gases in µmoles·mg $Chl^{-1} \cdot hr^{-1}$.