Reports

Platinized Chloroplasts: A Novel Photocatalytic Material

Abstract. *Colloidal platinum was prepared and precipitated directly onto photosynthetic thylakoid membranes from aqueous solution and entrapped on fiberglass filter paper. This composition of matter was capable of sustained simultaneous photoevolution of hydrogen and oxygen when irradiated at any wavelength in the chlorophyll absorption spectrum. Experimental data support the interpretation that part of the platinum metal catalyst is precipitated adjacent to the photosystem I reduction site of photosynthesis and that electron transfer occurs across the interface between photosystem I and the catalyst. Photoactivity of the material was dependent on the nature of the ionic species from which the platinum was precipitated. All photoactive samples were prepared from the hexachloroplatinate(IV) ion, whereas samples prepared by precipitation of the tetraammineplatinum(II) ion showed no hydrogen evolution activity and only transient oxygen activity. This system is among the simplest known for photosynthetically splitting water into molecular hydrogen and oxygen.*

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Photosynthesis can be separated into light and dark reactions (*1–2*). The light reactions occur exclusively in the chloroplast membranes, where the overall process is energized. This ability of the chloroplast to serve as the photochemical "factory" of photosynthesis led to the consideration of systems that drive photoreactions other than carbon reduction.

The three-component CFH system of isolated chloroplasts, ferredoxin, and hydrogenase is one such system. In 1961 Arnon *et al.* (*3*) showed that the CFH system could photoproduce molecular hydrogen when cysteine was used as the electron source. In 1973, Benemann *et al.* (*4*) and Krampitz (*5*) presented biochemical evidence for photosystem II (water splitting)–linked hydrogen evolution. Subsequent development of instrumentation allowed the direct observation of simultaneous photoproduction of molecular hydrogen and oxygen by the CFH system (*6*). Gisbey and Hall (*7*) performed energy conversion efficiency measurements of the CFH system, and Krasna (*8*) demonstrated that platinum catalyst on asbestos can substitute for hydrogenase as the hydrogen-evolving catalyst, although it was necessary to also substitute methyl viologen for ferredoxin as the electron relay between chloroplast and catalyst. Reduced ferredoxin will not interact with platinum catalyst. Ochiai *et al.* (*9*) demonstrated that intact cells of the cyanobacterium *Mastigocladus laminosus* functioned as an anodic photoelectrode for use in a conventional electrochemical cell. An authoritative review of photobiological hydrogen production was presented by Weaver *et al.* (*10*).

In the study reported here, colloidal platinum was precipitated directly onto photosynthetic membranes in an aqueous suspension. The resulting chloroplast–colloidal platinum composition was then entrapped on filter paper. As indicated in Fig. 1, this moistened material was capable of sustained simultaneous photoevolution of hydrogen and oxygen when irradiated with visible light. Since no electron relay was added to the system and the overall reactions occurred in an immobilized matrix, it was concluded that the precipitated colloidal platinum directly contacts the reducing end of photosystem I in such a way that electron flow occurs across the biological membrane–metal colloid interface with preservation of charge continuity and catalytic activity. In addition to the special photocatalytic properties of platinized chloroplasts, the specific ionic species used to prepare the material yield information on the physicochemical properties of the photosystem I reduction site on the thylakoid membrane surface. The data in Fig. 1 were obtained by precipitating platinum from the hexachloroplatinate(IV) ion, $[Pt(Cl)_6]^{2-}$. Platinized chloroplasts prepared by precipitating platinum from the tetraammineplatinum(II) ion, $[Pt(NH_3)_4]^{2+}$, resulted in no hydrogen activity and only a transient oxygen gush. As discussed below, the presence of insoluble platinum on the entrapped filter paper composition was determined by x-ray fluorescence analysis.

Type-C chloroplasts were prepared by the procedure of Reeves and Hall (*11*). In this preparation the chloroplast envelope is osmotically ruptured, exposing the thylakoid membranes to the external aqueous medium. A solution of chloroplatinic acid (5.34 mg/ml), neutralized to *p*H 7 with NaOH, was prepared separately. One milliliter of this solution was combined with 5 ml of chloroplast suspension (containing 3 mg of chlorophyll) in Walker's assay medium (*12*). The 6-ml volume was placed in a temperature-controlled, water-jacketed chamber fitted with O-ring connectors to provide a hermetic seal and inlet and outlet ports for hydrogen flow. The mixture was gently stirred and purged with molecular hydrogen in the headspace above the liquid. The temperature of the sample was held at 21°C.

In the platinum precipitation step, it was determined empirically that a hydrogen incubation time of ~30 minutes was needed to obtain photoactive material. Times of 60 to 90 minutes were typically used. After incubation, the reactor chamber was opened to air and the con-

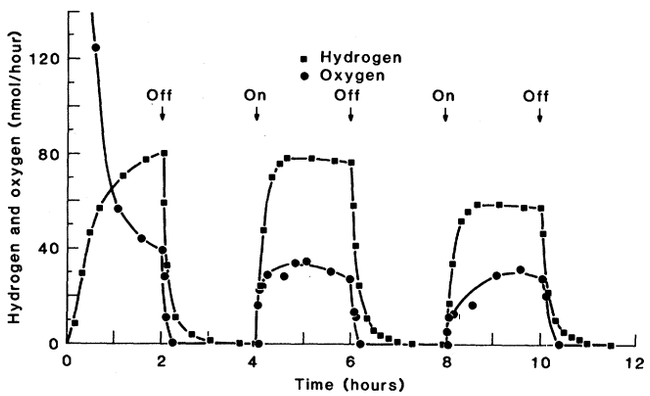

Fig. 1. Simultaneous photoproduction of hydrogen and oxygen by moistened platinized chloroplasts entrapped on filter paper. The ELH projector lamp, providing saturating illumination, was turned on at $T = 0$ and alternated with 2-hour on-off cycles as indicated. The peak oxygen rate was 7 μmol/hour.The area of the disk was 10.2 cm^2. The activity of the platinized chloroplasts was still measurable after 2 to 3 days of irradiation cycles at 10 to 20 percent of the initial rates. Greenbaum (*14*) gave a detailed description of the experimental procedure used for measuring the simultaneous photoproduction of hydrogen and oxygen.

tents were filtered onto fiberglass filter paper (AP40, Millipore). The platinum precipitation reaction had a marked effect on filtration properties. Whereas control experiments without hydrogen incubation produced a chloroplast mixture that filtered immediately, the platinized chloroplasts required a considerably longer time—typically 5 to 30 minutes. Also, the platinized chloroplasts were dark green, as opposed to the normal bright green of higher plant chloroplasts. The presence of insoluble platinum on the platinized-chloroplast filter paper composition was identified by x-ray fluorescence analysis after rinsing the filter paper in 2 liters of continuously stirred distilled water for 1 hour. Insoluble platinum was positively identified in this way for platinized chloroplasts prepared by precipitation from $[Pt(Cl)_6]^{2-}$or $[Pt(NH_3)_4]^{2+}$. As mentioned above, however, only $[Pt(Cl)_6]^{2-}$ yielded photoactive material.

The time profiles of photoactivity are presented in Fig. 1 for three light-dark cycles of 2 hours each. Figure 1 shows that the first irradiation period has a qualitatively different time profile than the subsequent two periods. In the first cycle, the oxygen profile underwent a transient gush (peaking at 7 μmol of O_2 per hour) before settling down to steady state. The hydrogen rate climbed monotonically to steady state. The time required to reach 50 percent of steady state in the first cycle was ~25 minutes, whereas the corresponding time for the second and third cycles was 5 to 8 minutes.

These patterns may be explained as follows. The transient oxygen gush initially represents the filling of reducible pools on the reducing side of photosystem II. Molecular oxygen is capable of oxidizing the components in the electron transport chain linking the two light reactions of photosynthesis, including the plastoquinone pool (*13*). The platinized chloroplasts were exposed to air during the filtration process plus the time just before insertion in the reaction chamber of the apparatus used for measuring photoactivity (*14*). Therefore, the initial oxygen gush is believed to represent the filling of the oxidized pool as well as other reducible species in the preparation.

As indicated above, the time for the hydrogen rate to reach steady state was longer for the first interval of irradiation than for the subsequent two intervals, whose time profiles were determined by the response time of the apparatus (*15*). Since the hydrogen-evolving catalyst in this preparation was elemental platinum, the observed kinetics of the first irradiation interval are interpreted to represent the reduction of the oxide layer formed on the metallic platinum during the period when the platinized chloroplasts are exposed to air, as described above. Since the platinized chloroplast remained anaerobic for the two subsequent irradiations, neither oxygen transients nor hydrogen delays were observed.

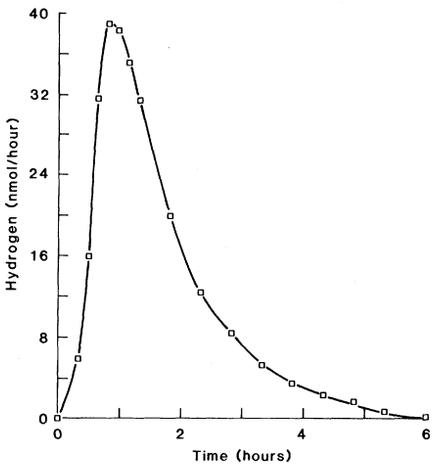

Fig. 2. Control experiment omitting the filtration step in which the platinized chloroplasts are entrapped on filter paper. Hydrogen (and oxygen) produced in the 6-ml aqueous phase is equilibrated with the carrier gas in the headspace and transported to the oxygen and hydrogen sensors downstream. The loss in activity as a function of time is interpreted as the separation of membrane and metal catalyst particle during stirring.

A novel aspect of this material is the absence of an added electron relay (such as ferredoxin or methyl viologen) to transport electrons from the reducing end of photosystem I to the hydrogen-evolving catalyst. Two control experiments were performed that emphasize this point. In the first experiment the order of adding chloroplasts and precipitation was reversed. That is, the colloidal platinum was prepared in the absence of chloroplasts (by precipitation from $[Pt(Cl)_6]^{2-}$), and then the chloroplasts were added to the aqueous phase. This mixture was entrapped on fiberglass filter paper. In spite of the physical contact between platinum colloid particles and chloroplasts, no photocatalytic activity was observed, except for a brief oxygen transient.

In the second experiment the filtration step was omitted. The 6-ml aqueous suspension of platinized chloroplasts was stirred in the photoreactor of the assay system with a Teflon stirrer. Hydrogen and oxygen photoproduced in the aqueous phase equilibrated with the carrier gas in the headspace above the liquid. The time profile for hydrogen evolution is given in Fig. 2. The oxygen profile was virtually the same as this hydrogen profile. As shown in Fig. 2, the hydrogen rate peaked at ~50 minutes and then decayed monotonically with a first half-life of ~110 minutes, a time significantly shorter than the corresponding value for the filter paper–entrapped platinized chloroplasts of Fig. 1. The loss of activity in Fig. 2 was presumably associated with the separation of chloroplast and platinum colloid particles at the photosystem I reducing site.

The only material capable of sustained photoacivity was prepared by precipitating the $[Pt(Cl)_6]^{2-}$ ion in the presence of thylakoid membranes. Platinized chloroplasts prepared by precipitating the $[Pt(NH_3)_4]^{2+}$ ion in the presence of thylakoid membranes were incapable of any hydrogen photoactivity. These results suggest that $[Pt(NH_3)_4]^{2+}$ is repelled (either Coulombically or sterically) by the photosystem I reduction site and that $[Pt(Cl)_6]^{2-}$ has a specific chemical affinity of, perhaps, an ion exchange–like nature for this site. It is known from several types of measurements, such as electrophoretic mobility studies, that thylakoid membrane surfaces bear a net negative electrostatic charge at neutral *p*H due to carboxyl groups (*16*). However, the discrete nature of the proteins associated with the membrane prevents this charge from being uniformly distributed across the membrane surface. Photosystem I is capable of donating electrons to negatively charged electron acceptors such as $[Fe(CN)_6]^{3-}$, the classic Hill electron acceptor (*17*). Moreover, as shown by Zweig and Avron (*18*) and Kok *et al.* (*19*), the methyl viologen (MV^{2+}) cation can be reduced by photosystem I. Chow and Barber (*20*) have shown that MV^{2+} accumulates in the diffuse layer of the membrane interface. A comparison of the interactions of MV^{2+} and $[Pt(NH_3)_4]^{2+}$ with photosystem I indicates that MV^{2+} can be reduced at a relatively large distance, whereas a smaller distance is required for platinum precipitation and catalytic activity from photosystem I.

In conclusion, a technique has been developed for contacting dispersed platinum catalyst with photosynthetic membranes. It may be possible to extend this technique to other catalysts for dinitrogen and carbon dioxide reduction. Fundamental studies of this nature are clearly of interest in the context of fuel and chemical synthesis from renewable inorganic resources.

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A New Class of Steroids Inhibits Angiogenesis in the Presence of Heparin or a Heparin Fragment

Abstract. *Steroids that lack glucocorticoid or mineralocorticoid activity were found to inhibit angiogenesis in the presence of heparin or specific heparin fragments. This newly discovered steroid function appears to be governed by distinct structural configurations of the pregnane nucleus. These compounds are here named angiostatic steroids.*

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In an earlier study, we found that cortisone or hydrocortisone when administered with heparin or a heparin fragment inhibited the growth of new capillary blood vessels in the chick embryo, in the rabbit cornea, and in some mouse tumors (*1*). Dexamethasone appeared to have little or no anti-angiogenic activity in the presence of exogenous heparin, even though its glucocorticoid activity is about 30 times that of hydrocortisone. This apparent paradox suggested that the heparin-dependent anti-angiogenic function of these corticoids (corticosteroids) could be independent of their glucocorticoid function. We subsequently tested this hypothesis by substituting 11α-hydrocortisone (epicortisol) for hydrocortisone. Epicortisol is the biologically inactive stereoisomer of hydrocortisone (*2*). The α-position of the 11-hydroxyl group eliminates glucocorticoid and mineralocorticoid activity. Epicortisol retained anti-angiogenic activity in the presence of heparin when tested in the chick embryo (*3*).

We now show that there is a minimum essential structure for a new class of steroids which inhibit angiogenesis in the presence of heparin or a fragment of heparin that has no anticoagulant activity. Within this classification are steroids for which heparin-dependent anti-angiogenesis is the only recognized biological function. This new steroid function appears to be independent of glucocorticoid and mineralocorticoid activity.

All compounds were tested for their capacity to inhibit angiogenesis in the chorioallantoic membrane of the 6-day shell-less chick embryo. Each steroid, with and without heparin, was dissolved or suspended in 10 μl of methylcellulose (0.45 percent) which was then air-dried to a disk of 2 mm in diameter and implanted on the chorioallantoic membrane (Fig. 1). An optimum heparin concentration (Hepar, Inc.) was found to be 50 μg per 10 μl in the presence of hydrocortisone-21-phosphate (Sigma) (60 μg per 10 μl) (*4*). This combination was used as a positive control. A dose-dilution curve (1 to 200 μg) was generated for each test steroid in the presence of heparin. Each steroid was also tested alone at 200 μg, and alone at the concentration that had been most effective in the presence of heparin. The end point for angiogenesis inhibition was an avascular zone of 4 mm in diameter or greater when the embryo was examined 48 hours after implantation of the test compound (Fig. 2). At least 20 embryos were examined for each concentration of a steroid. Thus, 30 to 35 embryos received implants for each concentration, and 180 to 210 embryos were used for each steroid, not including the embryos used for the hydrocortisone-heparin controls. Throughout the year required for this study, the maximum anti-angiogenic activity of the hydrocortisone-heparin controls varied no more than 5 percent from week to week. Anti-angiogenic activity for each steroid was determined from its dose-response curve (Fig. 3).

At its optimum concentration, hydrocortisone (with heparin) produced avascular zones in 49 to 57 percent of the embryos. Higher concentrations (70 to 200 μg) resulted in reduced inhibition of angiogenesis and the appearance of an angiogenic reaction on the chorioallantoic membrane. This mild angiogenic reaction at high concentrations was increased in the absence of heparin but was not observed with steroids that lacked glucocorticoid activity. Neither heparin alone nor any steroid alone inhibited angiogenesis, with the exception of epicortisol, which at its highest dose (200 μg) displayed less than one-tenth of the anti-angiogenic activity that it produced with heparin.

The anti-angiogenic activity of epicortisol in the presence of heparin increased in a concentration-dependent pattern up to 200 μg. Maximum anti-angiogenic activity was about 28 percent that of hydrocortisone, while glucocorticoid and mineralocorticoid activity were completely lacking (Fig. 3). When the 11-hydroxyl group was absent, as in cortexolone, peak anti-angiogenic activity was retained at about 24 percent of hydrocortisone activity (Fig. 3). The glucocorticoid and mineralocorticoid activities of cortexolone are negligible. When both the 11-hydroxyl group and the 21-hydroxyl group were absent, as in 17α-hydroxyprogesterone, peak anti-angiogenic activity remained approximately equivalent to that of hydrocortisone (Fig. 3). This compound has essentially no glucocorticoid or mineralocorticoid activity. However, when the 17-hydroxyl group was absent, as in corticosterone, maximum anti-angiogenic activity