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OXYGEN EVOLUTION FROM BROKEN THYLAKOIDS FUSED WITH LIPOSOMES

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SUMMARY: Thylakoid membrane fragments obtained by hypotonic disruption of spinach chloroplasts in cold distilled water were fused with egg leicthin liposomes and tested for oxygen evolution in the presence of ferricyanide. Stability of the oxygen evolving complexes to ageing was demonstrated to be greatly enhanced by the lipsomes. Although phosphatidyl choline liposomes increased stability of oxygen evolution, phosphatidyl ethanolamine liposomes completely destroyed it. An enclosed membrane was apparently important for oxygen production. Fusion of thylakoids to liposomes may serve as a method for eventual extraction and isolation of the elusive oxygen evolving complexes.

Photosynthetic pigments and visual chromotophores have been incorporated into planar bilayer lipid membranes (BLM) (1,2) and more recently into spherical bilayer lipid membranes (liposomes) (3,4). These artificially constituted lipid bilayers are the most realistic approach to biological membrane models (5,6). In the case of photosynthetic pigments the aims have been to study pigmented bilayers for the understanding of certain aspects of quantum conversion and oxygen evolution (7). Most of these investigations have measured physical properties resulting from light-initiated charge separation across the pigment membrane.

Of particular interest here was the recent report by Toyoshima et al. (8) of oxygen evolution when chlorophyll-containing liposomes were irradiated with actinic light. Our attempts to duplicate these experiments have met with failure. Using a system similar to Toyoshima et al., but employing stringent temperature controls, we readily measured oxygen uptake as the vesicles became irreversibly photooxidized (9). Since in our hands the approach of using cholorphyll-containing lipid extracts for liposome formation did not generate oxygen, a different approach was chosen. We report here the effect on oxygen evolution when broken chloroplast thylakoids are either fused with

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preformed liposome or else are incorporated with phospholipids during the process of liposome formation.

MATERIALS AND METHODS: Chloroplasts were isolated from fresh spinach (5) and were stored at -65°C. Typically, the chloroplasts from 240 g of spinach were used for each set of three experiments. Each experiment contained about 7.6 mg of chlorophyll as determined by the method of MacKinney (10). The chloroplasts were broken by blending at top speed in an ice cold Sears Blender in 200 ml of distilled water and ice for 5 minutes followed by sonication in ice for 10 minutes at the 50% power setting with a Branson Model W 140 D Sonifer Cell Disruptor. The material was centrifuged at 8300 RPM in a Servall Model SS-1 Centrifuge for 5 minutes to remove unbroken chloroplasts. The green supernatant was then made to 10 mM potassium ferricyanide and adjusted to pH 7.5. These green borken chloroplast thylakoids were either: (a) added to preformed liposomes described earlier (9) (with 0.3 g of phospholipid in 30 ml of 10 mM tricine, 5 mM potassium ferricyanide, pH 7.5) or else (b) added to flasks containing 0.3 g of dried phospholipids and were directly incorporated in the process of liposome formation. For the experiments involving addition of preformed liposomes, 50 ml of the broken thylakoids were mixed with 10 ml of liposomes and the 60 ml sample was tested for oxygen. For the experiments incorporating the broken thylakoid directly into liposomes, 30 ml of the chloroplast material and 0.3 g of the phospholipids were used to make the liposomes. To this, 30 ml of 10 mM tricine, 5 mM potassium ferricyanide, pH 7.5 buffer was added and the 60 ml sample was tested for oxygen. The samples were purged with nitrogen and sealed, adjusted to 25.0°C, and illuminated as previously reported (9). The light intensity at the center of the sample chamber was 1.7×10^5 erg $cm^{-2}sec^{-1}$. Oxygen was measured as previously described on a YSI Model 53 Oxygen Monitor (9).

RESULTS AND DISCUSSION: Figure 1 shows the oxygen evolution at each step in the isolation procedure of the broken chloroplast thylakoids. In all experiments controls without ferricyanide were run and in no case could oxygen be detected. We attribute the large increase in oxygen evolving capacity upon sonication (Fig. 1) to the formation of closed vesicles from the broken material. The sonicated material was found to be 9 % as active as the intact thylakoids. The further decrease in oxygen upon centrifugation is probably due to removal of unbroken thylakoids and larger vesicular particles.

The supernatant from the centrifugation was mixed with preformed egg lecithin liposomes. After mixing, the broken thylakoid material-egg lecithin liposomes were stored at room temperature in the dark. At various times samples were withdrawn, purged with nitrogen, sealed, irradiated with white light for 15 minutes and monitored for oxygen. The oxygen evolved during the first 5 minutes was recorded for each experiment (Fig. 2). Without addition of liposomes, the broken chloroplast material was not stable and its ability

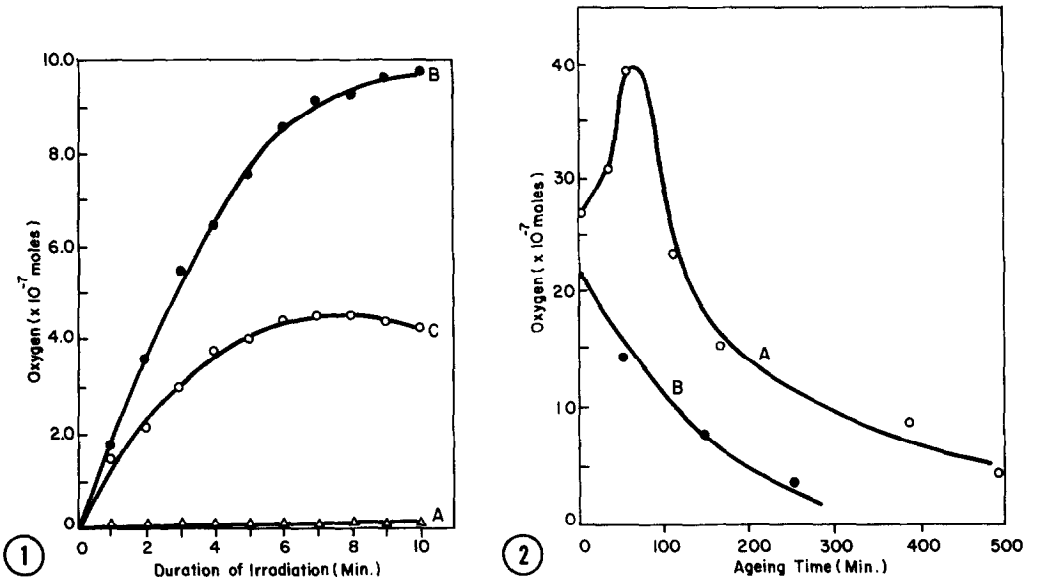

Figure 1. Evolution of oxygen during each step in the preparation of the broken chloroplast thylakoids (see text for details); (A) after blending in water and (B) after sonication and (C) after centrifugation. Illumination was with white light at 1.7 x 10^5 ergs $cm^{-2}sec^{-1}$.

Figure 2. Evolution of oxygen from broken chloroplast thylakoids with (A) and without (B) added egg lecithin liposomes. The samples containing 50 ml of the broken chloroplast material and 10 ml of liposomes were stored in the dark and were tested for oxygen at various times. The points represent the amount of oxygen generated during the first 5 minutes of irradiation.

to evolve oxygen readily diminished. However, when egg lecithin liposomes were added, the ability of the particles to evolve oxygen was greatly enhanced and the stability of the particles was extended for several hours. Considerable oxygen could be recorded even after the particles had been standing for 8 hours at room temperature. Upon mixing, the liposomes and broken chloroplast thylakoids did not fuse instantly as shown in Fig. 2. Instead, the ability of the material to evolve oxygen increased for the first hour as the fusion progressed.

In the process of liposome formation with broken thylakoids (Fig. 3), the material was mixed for 30 min and sonicated an additional minute. The material was then allowed to stand for 30 min before oxygen was tested. The oxygen

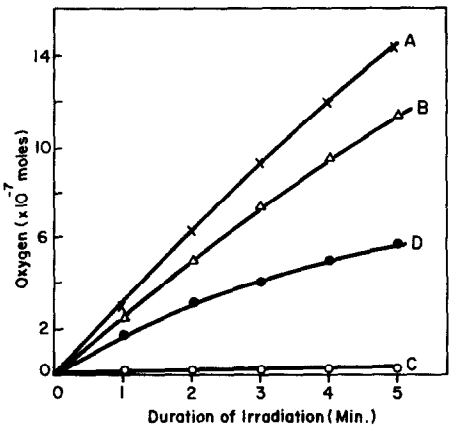

Figure 3. Evolution of oxygen when broken chloroplast thylakoids were incorporated with phospholipids in the process of liposome formation. Thirty ml of broken chloroplast material was added to each of 4 flasks containing; (A) 0.3 g of egg lecithin, (B) 0.3 g of asolectin, (C) 0.3 g of phosphatidyl ethanolamine and (D) no added phospholipid. Liposomes were prepared as previously described (9).

evolving complexes were therefore aged at least 80 min before oxygen was measured. When the broken chloroplast material was mixed with the phospholipids before liposome formation, a similar increase in stability of the oxygen evolving complexes was measured (Fig. 3). Phosphatidyl ethanolamine (PE) was shown to completely destroy the oxygen evolving capacity. Phosphatidyl choline (PC) was best at preserving the stability of the oxygen generation while asolecitin (about equal mixture of PE and PC) was intermediate between the PC and PE. This series, PC > asolectin > PE is similar to that reported for the phospholipid protection of the irreversible photo-oxidation of chlorophyll in organic solvents (11).

The results presented here indicate the possible requirement for a sealed vesicle in the process of oxygen evolution (7). The fact that sonication increased the oxygen evolving capacity of the broken chloroplast material and the incorporation of this material into liposomes increased the stability of the oxygen evolving complexes is consistent with this. Also it was noted that addition of 1% of sodium cholate or Triton X100 totally destroyed the oxygen evolving capacity of the material as the vesicles were solublized.

The large increase in stability of oxygen evolution upon incorporation into egg lecithin liposomes may be used as a novel method for isolation and purification of the oxygen evolving complexes. The great instability of this material has been a major stumbling block in its isolation. We suggest that prior incorporation into egg lecithin liposomes may allow for the easier purification of this unstable material. Such studies are currently underway.

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