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Microencapsulation of Chloroplast Particles[1]

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ABSTRACT

Chloroplast and photosystem I particles were encapsulated in small spheres (about 20 μm diameter) with an artificial membrane built up by cross-linking amino groups of protamine with toluenediisocyanate. The artificial membrane was permeable to small substrate and product molecules but not to soluble proteins. Photosystem I activity was retained by the encapsulated chloroplast particles. Washed photosystem I particles were encapsulated with the soluble proteins, ferredoxin, and ferredoxin-NADP oxidoreductase, and the microcapsules photoreduced NADP using ascorbate plus dichlorophenolindophenol as the electron donor. The photosystem I particles were also encapsulated with hydrogenase from *Chromatium* and a very low rate of photoevolution of hydrogen was obtained. The results show that chloroplast membrane fragments can be encapsulated with soluble proteins that couple transfer reactions to the primary photochemical apparatus.

Procedures have been developed to isolate proteins or enzymes in small spherical domains (capsules) surrounded by an artificial polymer membrane (1, 6, 7). The membrane is permeable to small substrate and product molecules but not to the larger protein molecules which are held within. These procedures generally involve establishing a water in oil emulsion with subsequent formation of the polymer membrane at the emulsion interface and recovery of the capsules free from the organic phase. The size of the microcapsules can be adjusted within the range of 1 to 1000 μm by the conditions of the emulsion. Hemoglobin has been encapsulated within polystyrene or silicone membranes, in experiments designed to create a type of artificial red blood cell (5), and the normal oxygenation-deoxygenation characteristics were retained by the encapsulated hemoglobin. Catalase, urease, and lipase have all been microencapsulated in enzymically active forms (4, 5). Multienzyme systems have also been encapsulated. Microcapsules containing a muscle extract were able to break down glucose to lactic acid; similarly, alcoholic fermentation was performed by microcapsules containing a yeast extract (3).

Several advantages may result from microencapsulation. Enzyme activities may be more stable in the encapsulated particles than in free solution because the proteins are maintained in small regions at high concentration. The proteins in the capsules are also protected against the degrading action of proteinases and bacteria. The microcapsules may provide some operational advantages as well since they can be used in columns with continuous flow of the reaction medium.

The purpose of the present work was to explore possibilities of microencapsulating chloroplast particles with the retention of photosynthetic activities. Two preliminary aspects of this problem were explored. First, we wanted to learn if the basic photochemical activity of the photosynthetic apparatus would withstand the encapsulation procedures. Then, if the photochemical activity was stable, could we encapsulate soluble enzymes with the chloroplast membrane particles which would couple to the photochemical apparatus.

MATERIALS AND METHODS

Chloroplasts and PSI[3] particles were prepared by methods described by Sane *et al.* (8).

Hydrogenase was prepared from *Chromatium vinosum*, strain D. Fifty g of cells were suspended in 150 ml of 0.1 M tris buffer, pH 8, containing 3% deoxycholate and 1% cholate and disrupted by a French press at 6,000 p.s.i. The supernatant from a 10,000*g* centrifugation for 30 min was collected and ammonium sulfate was added to 20% saturation. The precipitated proteins were discarded after another 10,000*g* centrifugation for 30 min and 1 ml of 0.5 M $MnCl_2$ was added to the supernatant. The supernatant was heated to 63 C for 10 min, cooled on ice, and centrifuged again at 10,000*g* for 30 min. Solid ammonium sulfate was added to this latter supernatant to 60% saturation and precipitated proteins were collected by a centrifugation at 10,000*g* for 30 min. The pelleted proteins were dissolved in 50 ml of the tris buffer and stored until use in liquid N_2.

Microencapsulation of Chloroplasts. In the encapsulation procedures reported here, the material to be encapsulated was added to a dilute solution of gelatin which was a gel during the encapsulation procedures near 0 C but was a solution at room temperature where activity measurements were carried out. One ml of 10% gelatin (isoelectric point pH 9.2) was added to 4 ml of a 40% solution of protamine which had been adjusted to pH 8 with HCl. This mixture was warmed to give a clear solution and recooled at room temperature. Five ml of a chloroplast suspension of about 1 mg Chl/ml in a buffer of 20 mM KCl, 15 mM tris-HCl, pH 7.8, were stirred into the protamine-gelatin solution. One hundred ml of dibutylphthalate, DBP, were placed in a 200-ml stainless steel beaker in a H_2O bath. The contents of the beaker were stirred with a fairly powerful, variable speed stirring unit with a stirring blade whose leading edge had been sharpened to increase shear. The chloroplast-protamine-gelatin mixture was added to the DBP while stirring (at room temperature), and the size of the droplets in the emulsion was adjusted by controlling the speed of the stirring motor. Ice was then added to the H_2O bath, and the emulsion was cooled to about 5 C. After 10 min of stirring, to establish a stable emulsion, 5 ml of toluene-diisocyanate, TDI, dissolved in 20 ml of DBP were added and stirring was continued in the ice bath for 10 to 15 hr. The TDI cross-links amino groups of the protamine and slowly builds up a polymer membrane at the emulsion interface. The capsules were examined periodically under a microscope to determine their resistance to rupture (due to pressure on a cover slip). Some

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[3] Abbreviations: PSI: photosystem I; DBP: dibutylphthalate; TDI: toluenediisocyanate; DPIP: dichlorophenolindophenol.

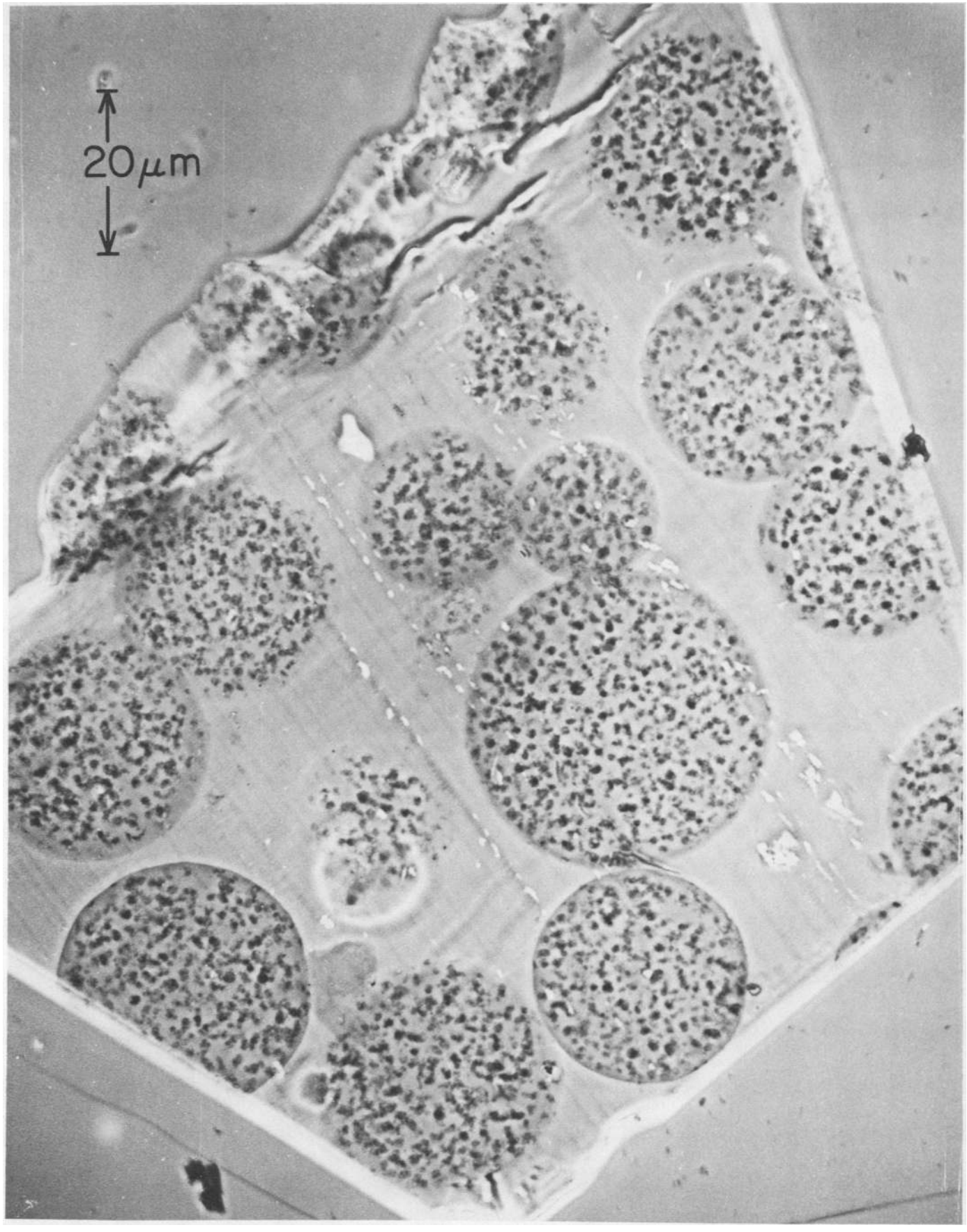

FIG. 1. Photomicrograph of microcapsules embedded in Epon.

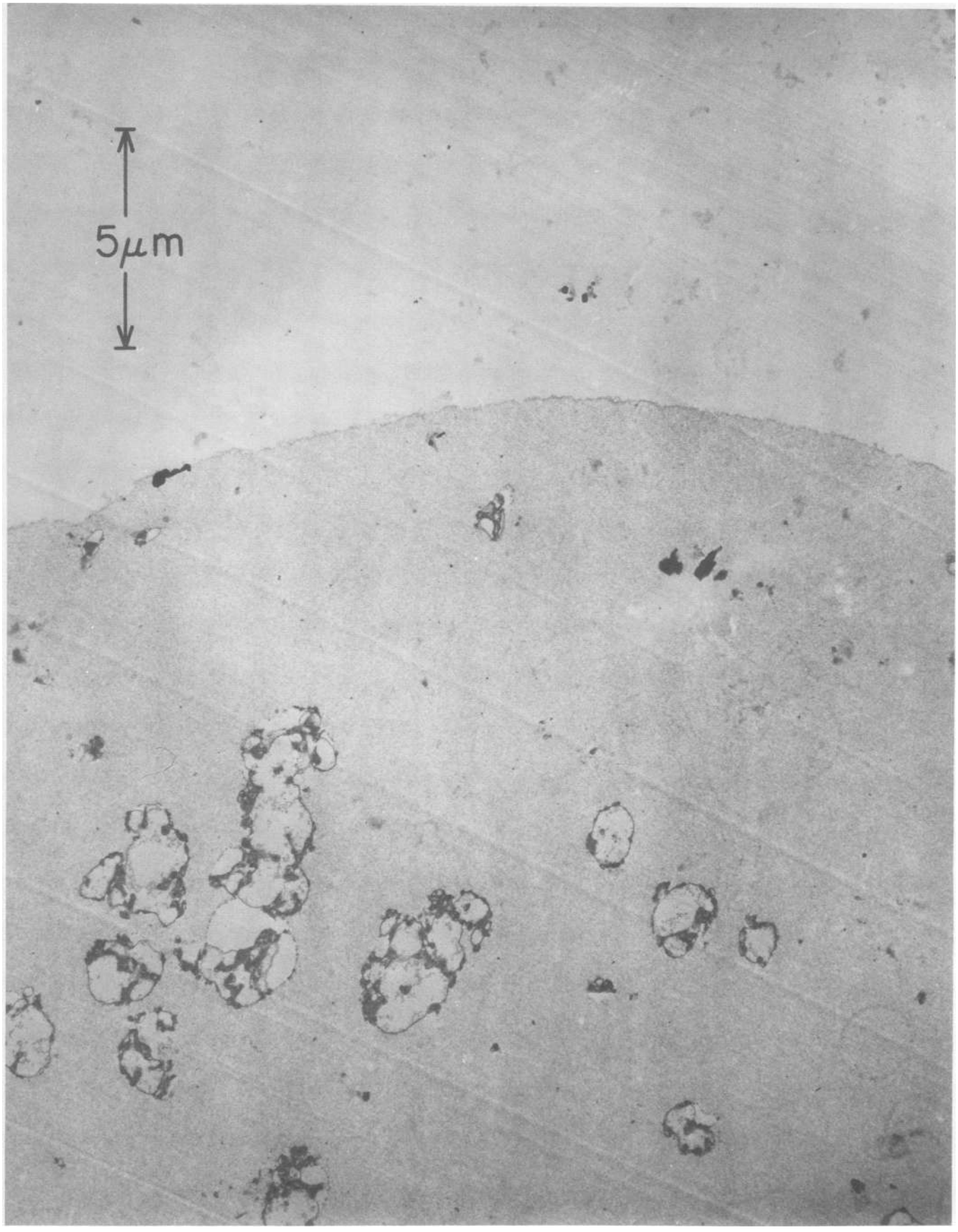

FIG. 2. Electron micrograph of a portion of a microcapsule.

experience is needed to determine when the membrane has formed to the proper extent. If the membrane is too thin, the capsules rupture during recovery, and if the membrane is too thick, diffusion appears impeded perhaps because some of the organic phase remains in the membrane.

The capsules were recovered from the DBP by centrifugation at 1000*g* for 5 min after adding 20 ml of ice-cold petroleum ether to decrease the specific gravity of the organic phase. The pellet fraction was dispersed in 20 ml of glycerol and centrifuged at 1000*g* for 10 min. Three layers were formed by this centrifugation; the organic solvent on the top, the glycerol at the bottom, and the green microcapsules in the middle. The top layer was discarded, and the layer containing the capsules was transferred to another centrifuge tube where the capsules were dispersed in 20 ml of 50% Tween 20. The capsules were collected again by centrifugation at 1000*g* for 5 min and were washed twice by suspending in 50 ml of buffer and centrifugation. The washed capsules were finally suspended in 10 ml of buffer.

Microencapsulation of PSI Particles with Soluble Proteins. PSI particles were prepared by the French press method which included differential centrifugation at 100,000*g*. One ml of the supernatant from the 144,000*g* centrifugation which contained ferredoxin, ferredoxin-NADP reductase, as well as other soluble components was added to 2.5 ml of the PSI particles (500 μg Chl/ml) plus 1 ml of 40% protamine and 0.5 ml of 10% gelatin, and this H_2O phase mixture was emulsified in 100 ml of DBP. Two ml of TDI dissolved in 20 ml of DBP were added after a stable emulsion was formed at 5 C and the capsules were collected some 15 hr later as described above.

Microencapsulation of PSI Particles with Hydrogenase. Four ml of the relatively crude hydrogenase preparation described above were mixed with 2 ml of 10% gelatin and 4 ml of PSI particles (250 μg Chl/ml). This suspension was emulsified in 100 ml of DBP and after a stable emulsion was established at 5 C, 5 ml of TDI dissolved in 20 ml DBP were added to the emulsion. The capsules were recovered after 20 hr at 5 C. Protamine was not added in this encapsulation because it appeared to inactivate the hydrogenase. The concentration of gelatin was doubled, however, and the membrane was formed by cross-linking the amino groups of the gelatin with TDI.

Techniques. Oxygen uptake was measured with a Clark-type oxygen electrode in a 3-ml Plexiglas cuvette. The photoreduction of NADP was assayed by absorbance measurements at 340 nm. Photoevolution of H_2 was determined with a gas chromatograph. For examination of the microcapsules by electron microscopy, samples were fixed in 2% OsO_4 for 3 hr, dehydrated in acetone, and embedded in Epon 812. Sections were cut with a microtome, stained with uranyl acetate and lead citrate, and examined in a Philips EM-300 electron microscope.

RESULTS

The microcapsules, prepared by the techniques described above, appeared in the light microscope as uniform green spheres ranging in diameter from 10 to 50 μm with most capsules having a diameter of about 20 μm. A photomicrograph of the capsules embedded in Epon and sectioned for electron microscopy is shown in Figure 1. Numerous chloroplast fragments are apparent within the polymer membrane. Electron microscopy (Fig. 2) reveals a single boundary membrane without any striking details.

The photochemical activity of the encapsulated chloroplast particles was assayed as the methyl viologen-mediated O_2 uptake using ascorbate and DPIP as an electron donor system to PSI (Table I). Since the encapsulation procedures require about 15 hr at 5 C, the appropriate control is taken as chloroplast particles stored at 5 C for approximately the same length of time in the encapsulation medium (but not in contact with the organic phase). It is apparent that the photochemical activity of PSI survives the encapsulation procedures. In order to determine whether the rate of electron transport was limited by diffusion barriers, the activity of a suspension of capsules was measured after rupturing and homogenization. No diffusion limitations were apparent for the assay conditions used.

The stability of the PSI activity of the encapsulated chloroplast fragments was tested over a period of 4 weeks in a refrigerator (5 C). The activity of the encapsulated particles decayed initially (Fig. 3) but leveled off at about one-third of the initial rate, whereas the unencapsulated control decayed to zero.

Attempts were also made to encapsulate soluble proteins along with chloroplast fragments. Preliminary experiments indicated that a protein as small as horse heart Cyto *c* (mol wt 12,400) would be retained within the capsule membrane. The mixture to be encapsulated contained washed PSI particles and soluble proteins, including ferredoxin and ferredoxin-NADP oxido reductase. Activity was assayed as the photoreduction of NADP with ascorbate-DPIP as the electron donor. The activity of the washed PSI particles without the soluble proteins was negligible. The activity of the PSI particles encapsulated with the soluble proteins was present but was relatively low, 9.4 μmoles NADPH/mg Chl·hr. However, the activity of the unencapsulated control was also low, 13.6 μmoles NADPH/mg Chl·hr, because of the long incubation time in the encapsulation medium.

Attempts were also made to encapsulate PSI particles with hydrogenase using a crude hydrogenase extract from *Chromatium*. The *Chromatium* hydrogenase, which is known to be relatively insensitive to O_2 (2), was used so that the encapsulation procedures could be carried out under aerobic conditions. The hydrogenase appeared to be inactivated by the presence of protamine so the protamine was omitted and the concentration of gelatin was doubled. The assay for the photoproduction of H_2 was carried out anaerobically under argon to avoid competition

Table I. *Rates of Oxygen Uptake*

Rates of oxygen uptake mediated by methyl viologen with DPIP-ascorbate as the electron donor in the presence of 10 μM DCMU. The photoreaction was carried out with fresh chlorplasts; with chloroplasts suspended in the protamine-gelatin medium for 15 hr at 5 C (the control); with microcapsules; and with microcapsules after homogenization.

	O_2 Uptake $\frac{\mu \text{ moles } O_2}{\text{mg Chl·hr}}$	Relative Activity (%)
Fresh Chloroplasts	295	100
Reaction Mixture (1 day)	225	76
Microencapsulated	98	33
Microcapsules (broken)	99	34

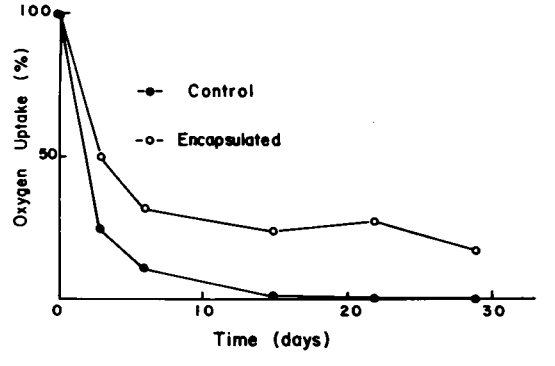

FIG. 3. Rate of O_2 uptake (as per cent of initial rate) as a function of time at 5 C.

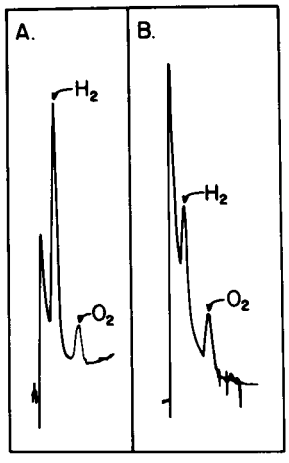

FIG. 4. Gas chromatograph of a calibration mixture of 10 μl of H_2 in 1 ml of argon (A) and a 1-ml sample taken from the reaction flask after 20 min of irradiation (B).

by O_2 for the reducing power generated by light. Methyl viologen (10 mM) was present in the medium to mediate electron transfer from the PSI particles to the hydrogenase and ascorbate-DPIP was present as the electron donor. One ml of the gas phase above the reaction mixture was sampled periodically by syringe with a gas replacement technique and injected into a gas chromatograph. Figure 4 shows the results from the gas chromatograph, first with a calibration run with 10 μl of H_2 in 1 ml of argon (Fig. 4A) and then with a 1-ml sample of gas from the reaction vessel after 20 min of illumination (Fig. 4B). In each case, the first peak is an injection artifact, the second peak is due to H_2, and the third due to a small contamination by O_2. The results show that 2.3 μl of H_2 were evolved during the 20-min irradiation period giving an average activity of 1.0 μmole H_2/mg Chl · ml.

DISCUSSION

The work reported here was designed to demonstrate whether photosynthetic electron transfer reactions could be obtained with microencapsulated chloroplast fragments. The primary photochemical activity survives the encapsulation procedures and persists in the capsules with reasonable stability. The next step of encapsulating the chloroplast fragments with soluble enzymes which can utilize the reducing power generated by PSI to form stable products, such as NADPH or H_2, was also demonstrated but with activities which were disappointingly low. The soluble enzymes appeared to be inactivated appreciably by the encapsulation procedures. Nevertheless, we regard the overall performance of the encapsulated systems as promising.

The microencapsulation of chloroplast fragments presents unique problems which have not been encountered with soluble enzymes and proteins. A number of organic solvents such as benzene which have been used for the organic phase in the encapsulation of enzymes cannot be used with chloroplast fragments because they extract the Chl. DBP or mineral oil which can be used with chloroplasts are relatively difficult to remove after the capsule membranes have formed. Polar organic solvents such as alcohol or acetone which would be effective in stripping off the DBP cannot be used with chloroplasts because of their action on the Chl. The removal of DBP from the microcapsules is very important because the capsules tend to aggregate in buffer if it is not removed, and small amounts of DBP remaining in capsule membrane would probably inhibit the diffusion of the water-soluble reactants and products through the membrane.

The primary photochemical apparatus, *i.e.* the reaction center complex with its associated antenna Chl, appears to be quite stable once it is removed from proteinases and other catabolic enzymes that are a part of the dynamic interactions of the living cell. Microencapsulation will further protect the isolated photosynthetic apparatus against the action of hostile enzymes and bacteria as well as keep that apparatus in close contact with selected electron transport enzymes. Maintaining the soluble proteins at relatively high concentrations and away from the action of degradatory processes should enhance the stability of these proteins as well. More work is needed, however, to optimize the encapsulation procedures or to discover other techniques that will not deactivate the soluble enzyme system.

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