Preparation of Metabolically Active Protoplasts from the Blue-Green Alga, Phormidium luridum 1, 2

John Biggins
Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received June 23, 1967.

Summary. A method is described for the preparation of metabolically active protoplasts from the blue-green alga, Phormidium luridum. The isolated protoplasts are stable and are capable of endogenous respiration and photoassimilation of carbon dioxide at rates not appreciably different from the untreated filaments. The protoplasts can be ruptured by dilution of the stabilizing osmoticum and, therefore, they provide a convenient starting point for the production of cell-free preparations within a biochemical reaction mixture of interest.

Our interest in the mechanism of electron transport systems in the blue-green algae prompted us to investigate respiration, photosynthesis and their interaction at the biochemical level. Many components related to the mechanisms of energy conservation in these algae are soluble proteins. They include the accessory pigments, the phycobilins (10), 3 water soluble cytochromes type c (6,7), and a factor necessary in the Hill reaction (4). Thus, the probability of losing metabolic activity on the disruption of blue-green algae for biochemical study is quite high.

A technique for the breaking of cells which avoids the use of extensive comminutive practices such as blending, grinding, extrusion and shear is the osmotic lysis of cell protoplasts. These are formed by specific removal of the cell wall by some agent (11). Although it is realized that the actual osmotic rupture of the cell envelope itself is drastic, the possibility of maintaining the integrity of internal membranes and inclusions is, however, somewhat higher than when the other more common methods of cell disruption are employed (11). Kleinschmidt, Lang and Zahn (8) showed that what appears to be the entire DNA thread of Micrococcus lysodeikticus is preserved on protoplast lysis indicating the gentleness of the technique.

Several blue-green algae are sensitive to muramidase (3,5) and Crespi, Mandeville and Kaut reported the preparation of algal protoplasts from Fremyrella diplosiphon (3). They were, however, inactive in the photoassimilation of CO2.

We have been successful in the preparation of protoplasts from Phormidium luridum which are active in both respiration and photosynthesis. This paper describes the preparation and properties of such protoplasts. The accompanying report (1) concerns photosynthetic electron transport in Phormidium luridum and substantiates our view that considerable enzymatic activity is retained in preparations of lysed protoplasts.

Methods

Algae. Phormidium luridum var. olivaceae Börsch was obtained from the Culture Collection of Algae at Indiana University. The cells were grown on Medium C of Kratz and Myers (9) and flushed with air supplemented with 4% CO2 (v/v). Illumination was provided by a bank of fluorescent tubes. Cells were harvested during the late logarithmic phase of growth.

Preparation of Protoplasts. Protoplasts were prepared by modification of the method first described by Crespi, Mandeville and Kaut (3). Phycocyanin release was used as a convenient measure of cell lysis and several buffer systems and osmotic agents were tried for effectiveness. These included phosphate, tris, glycyglycine, glucose, sucrose, Carbowax and mannitol. Mannitol-phosphate buffer was finally chosen for the incubation period for the enzyme digestion. The addition of EDTA or Mg2+ as stabilizers (11) was without effect. Ionic osmotica were not used because of possible complications in the subsequent use of the protoplasts in biochemical investigations. The following method was finally adopted: Cells were harvested and washed once in 0.5 M mannitol, 0.05 M potassium phosphate, (pH 6.8). The washed cells were resuspended in fresh mannitol-phosphate and sufficient solid egg-white muramidase (Worthington Biochemical Corporation, Freehold, New Jersey) was added to give a final

1 This investigation was supported by the National Science Foundation, GB-4046.
2 A preliminary account of this investigation was presented at the 1966 AIBS Meetings, College Park, Maryland.
FIG. 1. Phase contrast photomicrographs of Phormidium luridum. A) untreated filaments; B) C) and D) protoplasts prepared by treatment with muramidase in mannitol-phosphate buffer. The marker is 10 μ.
enzyme concentration of 0.05% (w/v). The cells were incubated at 35° for 2 and one-half hours with occasional swirling and then cooled in an ice bath. This incubation resulted in approximately 70% yield of protoplasts. Longer time periods improved the yield but also led to extensive cell rupture.

After cooling, the protoplasts were separated from the untreated filaments by passage of the mixture through a column 3 x 10 cm, loosely packed with glass wool. The long, untreated filaments were held in the fibers and the small protoplasts passed through. Protoplasts were collected from this solution by centrifugation at 500 g, 4 minutes. The protoplasts were gently resuspended in fresh mannitol-phosphate and resedimented to remove excess muramidase. As described in the accompanying report, the protoplasts can be transferred at this point to mannitol-tris prior to their use in reactions where lower concentrations of phosphate are desirable.

Photoassimilation of Carbon Dioxide. Carbon dioxide fixation was measured by assaying the acid-soluble radioactivity remaining after illumination of the protoplasts or untreated filaments in the presence of H14CO3-. Saturating white light was provided by means of a General Electric 150W reflector flood filtered through 8 cm water. The reactions were carried out at 25°. Chlorophyll a was measured in 80% acetone extracts of the cells using E mM-0.033m = 82.

Endogenous Respiration. Endogenous respiration was measured in the dark at 25° using standard manometric procedures. The specific activities of the cells were first calculated on a chlorophyll basis because of the difference in weights between protoplasts and filaments. They were then converted to dry weight of the untreated filaments for comparison with other data from the literature.

Results and Discussion

Figure 1 shows phase contrast photomicrographs of the untreated filaments of Phormidium (A) and a typical protoplast preparation (B, C and D) derived therefrom. In agreement with the observations of Crespi, Mandeville and Katz (3) we found that the Phormidium sheath was removed and long strings of cells held together at the cross walls were formed after about 1 and one-half hours incubation of the cells with muramidase. Gentle swirling of the incubation mixture usually resulted in the formation of single cells (D) which were readily separated from the long untreated filaments on the glass wool column.

We found that the washed protoplasts were stable for some 4 to 5 hours if kept on ice. In the metabolic experiments described in the accompanying report (1), they were generally used within the first hour subsequent to preparation.

Figure 2 is an example of the time course of the photoassimilation of carbon dioxide by the isolated protoplasts compared with that for the untreated filaments. The observed rates for protoplasts varied between 95 and 155 μmoles CO2/mg chlorophyll/hr with 130 μmoles CO2/mg/hr as average (7 experiments). It can be seen that the isolated algal protoplast is capable of a substantial rate of photosynthesis comparable in magnitude to that of the untreated filament. The constancy of the rate for 20 minutes is an indication of the stability of the protoplasts.

Crespi et al. (3) found that protoplasts of the blue-green alga Fremyella diplosiphon were incapable of CO2-photoassimilation. They reported that this was because of the inhibitory action of the sucrose-Ficoll mixture they used as osmoticum.

The endogenous respiration of the algal protoplasts was compared with that of the untreated filaments in figure 3. Again, there was no great reduction of activity as a result of the enzyme treatment. The rate of endogenous respiration was low, 3.8 μl O2/mg dry weight/hr, and is comparable with previous reports on the respiration of blue-green algae (2,12). We were unable to detect any significant respiratory increase by the addition of glucose or acetate.

These 2 measurements suggest that, at least for a limited time period, the general metabolic activity of the cell is not altered significantly as a result of the removal of the algal cell wall. Because such protoplasts can be lysed quite simply by lowering the concentration of osmoticum, these preparations provide a valuable starting point for the production of cell-free extracts for biochemical study. In the accompanying report (1) we have utilized protoplast
lalysis as a method of preparing such extracts for the study of photosynthetic reactions. Non-cyclic photophosphorylation is a reaction which appears to be particularly susceptible to physical disruption in blue-green algae. The high rates we observe for this activity by lysed protoplasts suggest that this technique is a mild method and we foresee the usefulness of these preparations in other studies of structure and function in the blue-green algae.

Fig. 3. Endogenous respiration of Phormidium filaments and protoplasts. The reaction mixture was 0.5 M mannitol, 0.03M phosphate (pH 6.8). Filaments were 5.25 mg dry weight/ml. The activity of the protoplasts was first calculated on a chlorophyll basis and then converted to be equivalent to filament dry weight for comparative purposes.

Acknowledgment

We thank Mr. C. R. Granger of this department for supplying the photomicrographs.

Literature Cited