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A HALOPHILIC GREEN ALGA ASSOCIATED WITH STROMATOLITES ON SAN SALVADOR ISLAND

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ABSTRACT

Fossil stromatolites represent the first macroscopic evidence of life. Living stromatolites are restricted to such environments as the hypersaline inland lakes found on several Bahamian Islands. Stromatolites used in this study were collected from Stouts Lake in January, 1988. Cyanobacteria dominated, although a green alga was also isolated, grown in the laboratory and found to have a remarkable tolerance for fluctuating salt concentrations.

The alga is in the order Volvocales but has not been identified. It is biflagellated with a distinctive pear shape. Optimum growth occurs at salt (NaCl) concentrations between 35 and 50 ppt. Concentrations below 10 ppt are lethal. The alga can grow slowly in saturating salt (350 ppt or 5.7 M) where it shows slow rates of locomotion as it sometimes collides with small salt crystals.

Ultrastructure of the alga was studied. It is surrounded by a thin cell wall. The nucleus is located anteriorly near the point of attachment of the flagella. A single chloroplast surrounds the nucleus and contains thylakoid membranes which form a branching, net-like pattern. An exceptionally large pyrenoid body occupies the posterior portion of the alga. Ultrastructure of algae grown at varied salt concentrations was studied but no differences were observed.

The alga's physiological adaptation to high salt concentration was determined. It produces glycerol internally to balance the high external salt concentrations. When the salt concentration was doubled, it required 90 minutes for the alga to double its internal glycerol levels.

INTRODUCTION

Fossil stromatolites represent the first macroscopic evidence of life. Stromatolites have been found in rocks formed about 3.5 billion years ago. For almost 2 billion years, stromatolites constituted the dominant form of macroscopic life on earth. Awramik and Margulis (after Walters, 1976) defined stromatolites as "organosedimentary structures produced by sediment trapping, binding and/or precipitation as a result of the growth and metabolic activity of microorganisms, principally cyanophytes".

Living stromatolites were first recognized in 1961 by Logan in Hamlin Pool, a hypersaline lagoon in Sharks Bay on the western shore of Australia and soon thereafter on Andros Island in the Bahamas. Since the early 1960's, stromatolites have been found in a wide range of fresh water and marine habitates, but most studies on living stromatolites focus on those found in Australia and The Bahamas.

On San Salvador Island, stromatolites have been found in both Stouts and Storr's Lakes. Stromatolites used in this study were collected at Stouts Lake. Those from Storrs Lake have been extensively studied by Conrad Neumann (1989).

Stromatolites initially form as a mat composed of cyanobacteria (formerly called blue-green algae). These mats form a cohesive fabric of intertwined filaments which often produce copious amounts of gelatinous material. During their formation these cyanobacterial mats trap and precipitate sediments. The metabolic activities of cyanobacteria produce carbon dioxide and thus raise the pH of their environment, an activity which precipitates inorganic materials. Conrad Neumann (1989) found that during the day, when photosynthetic levels were high, the stromatolite community raised the pH from about 8 to over 9. Such conditions resulted in the precipitation of magnesium calcite which formed the bulk of the stromatolites. These three properties of cyanobacteria - trapping, binding, and precipitating sediments - leads to the formation of stromatolites.

Cyanobacterial mats soon become impregnated and covered with sediments. Mat communities that form stromatolites must move above the sediments. Filaments of *Lyngbya* are capable of a gliding locomotion and actively

move to the top of the mat. *Cyanostylon* produces gelatinous material unidirectionally thereby lifing itself on a gel stalk above the mat. *Rivularia* forms long, phototrophic filaments that grow about the mat. A stromatolite is a complex assemblage of many cyanobacterial species capable of rising above entrapping sediments.

Stromatolites are therefore layered structures, with the topmost layer comprising the photosynthetic zone. The topmost layer is composed of *Lyngbya* which is dark brown because it produces a pigment protecting it from ultraviolet light. Below that *Microcoleus* forms a blue-green layer that accounts for most of the photosynthetic activity. Next is a salmon pink layer of the photosynthetic eubacteria *Beggiatoa*. The lowest zone of living bacteria is black because of the activities of iron sulfide, decomposing, anaerobic bacteria. Conrad Neumann (1989) found all these genera in stromatolites from Storrs Lake.

In addition to the cyanobacteria and eubacteria dominating this assemblage, there are often eucaryotic species. In stromatolites I collected from Stouts Lake, there were two species of diatoms, a nematode and a biflagellated green alga. The green alga is the subject this paper.

MATERIALS AND METHODS

The alga was grown in a standard medium for photosynthetic marine organisms (Rechcigl, 1978, p. 286, Table G). The stock culture in which the alga was grown was supplemented with either 60 or 120 ppt NaCl. Experimental media contained varing concentrations of sodium chloride. Experiments used stock cultures that were between two and three weeks old.

Ultrastructure of the alga was studied. Cultures were grown at four concentrations of NaCl: 30, 60, 120 and 240 ppt. After three weeks of growth the algal cultures were centrifuged at 2000 xg for 10 min., fixed in gluteraldehyde and osmium tetroxide, dehydrated with alcohol, sectioned and stained with salts of lead and uranium (Meek, 1976).

Growth rates of the alga were determined over a wide range of salt concentrations. Each experiment was repeated four times. Values given represent the means of these four experiments. Standard errors were never over 8% of the mean. Algal numbers were determined with a hemocytometer daily or on alternate days over a three week period. In another series of experiments, water in cultures of algae growing in 240 ppt NaCl were allowed to slowly evaporate until saturating levels of salts were reached.

The internal glycerol content of algae grown at various NaCl levels was determined (Ben-Amotz and Avron, 1973). Algae were grown in 2 l flasks for 3 weeks at NaCl concentrations of 15, 30, 60, 120 or 240 ppt. A volume of culture containing between 50,000,000 and 100,000,000 cells was centrifuged for 10 min at 2,000 xg. The supernatant was discarded and a 2% solution of trichloroacetic acid was added to the pellet to disrupt the cells. Debris was removed by again centrifuging the extract at 2,000 xg for 10 min. The supernatant was then assayed for glycerol.

One ml of 10N sulfuric acid was added to 15 ml of the supernatant. Then 5 ml of sodium periodate was added and the mixture incubated for 5 min. Five ml of sodium arsenite was added and again incubated for 5 min. This sample was diluted to 100 ml, and 1 ml was removed to a clean tube. Then 10 ml of chromotrophic acid reagent was added and the solution was placed in a boiling water bath for 30 min, then cooled. Absorbance was read at 570 nm. Standard curves were determined in each experiment. The experiment was performed four times. Values are expressed as internal molar concentration of glycerol per cell. The total number of algal cells was known, and the volume of a single cell was calculated based on the volume of an oval. The width of the oval was the average of the width at the top of the "pear" and the width at the base of the "pear" shaped cell.

The time course for change in internal glycerol levels when the NaCl concentration in which the cells were grown was increased from 60 to 120 ppt was determined. Algae were grown for 3 weeks at 60 ppt NaCl, then a 100 ml sample was centrifuged for 10 min at 2,000 xg, and the precipitate was resuspended in 120 ppt media. At 10 min intervals over a three hour period a subsample of this suspension was removed and the glycerol content was measured as described above.

RESULTS AND DISCUSSION

The alga I collected from stromatolites is in the order Volvocales but has not been identified. I have sent samples of the organism to Dr. David Starr at the University of Texas, and he has agreed to attempt an identification. He suggested the alga might belong to the genus *Dunaliella*, which is known to have a tolerance for high salt

concentrations. Other species in this genus are described as having an oval shape, but the alga used in these studies has a distinctive pear shape. It is 15 microns long, 4.5 microns at the basal end and 3.5 microns at the apical end. It has two flagella that are almost as long as the alga itself (Fig. 1).

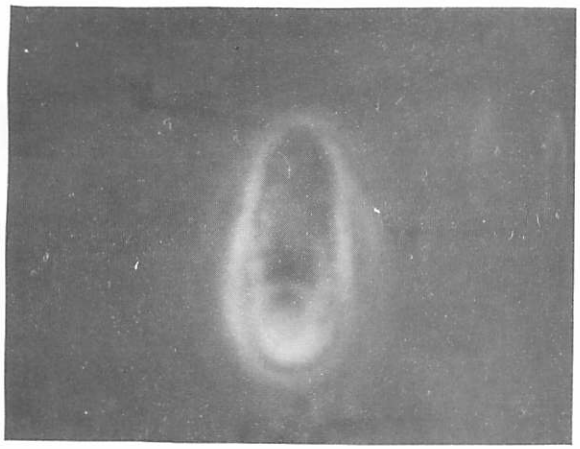

Fig. 1. The alga used in this study. It is biflagellated, with a distinctive pear shape.

Ultrastructure of the alga was studied. I shall describe the alga beginning at the apical end and proceeding distally. Two flagella are attached to the anterior portion of the alga (Fig. 2 shows one of these flagella in longitudinal section). A golgi body and an eye spot are located near the point of attachment of the flagella (Fig. 3). The nucleus is also located anteriorly just below this region (Fig. 3). A single chloroplast surrounds the distal end of the nucleus and has thylakoids with a net like arrangement with few grana (Fig. 4). The chloroplast also extends into the enlarged posterior portion, where it partially surrounds the anterior end of the pyrenoid body (Fig. 4). The posterior end of the alga contains a single, very large pyrenoid body. The anterior portion of the pyrenoid has a small diameter with only a single ring of starch grains. The posterior portion however has two large rings of starch grains (Fig. 5). About half of the total volume of the alga is occupied by the pyrenoid body.

Ultrastructure was compared for algae grown in a wide range of salt concentrations. Surprisingly, no ultrastructural differences were observed, not even in the size of the pyrenoid body. Einspahr (1988) compared ultrastructure of *Dunaliella salina* after an osmotic shock and noticed considerable shrinkage of the cells and several internal organs, as well as subtle changes in membrane architecture. The cultures used in this study experienced no osmotic shock since they had been growing for three weeks at the various salt concentrations.

Growth rates of the alga were determined over a wide range of salt concentrations. In one series of experiments, growth rates were determined at NaCl concentrations ranging between 0 and 90 ppt. Initially all cultures grew, though at varying rates. However, after about two weeks, the cultures growing at 0 and 15 ppt died. In subsequent experiments, cultures were maintained at 15 ppt NaCl. *Dunaliella* requires Na as an essential element and it seems likely that my alga also requires this element.

Growth rates were greatest at 45 ppt and less at 30, then 60 and finally 90 ppt repectively (Fig. 6). Growth rate is optimal at salt concentrations above that of oceanic waters, and the alga is halophilic and not just halotolerant.

In another series of experiments, growth rates were determined over a much wider range of salt concentrations, particularly at high salt concentrations: 35, 50, 100, 150, 200 and 250 ppt (Fig. 7). The stock culture of alga initially used in this experiment contained 60 ppt NaCl. When these cells were inoculated into concentrations of 200 and 250 ppt NaCl the osmotic shock killed the cells. Therefore, a second stock culture was maintained at 120 ppt NaCl and it was used to inoculate test cultures at the two highest NaCl levels. Even so, there was a lag period of a couple of days in the three highest test cultures before the alga began to grow at logarithmic rates. Growth rates were highest at 50 ppt again clearly indicating the halophilic nature of the algae. The alga can reach surprisingly high numbers even at the higher salt concentrations (Fig. 8). Maximum cell density (about 6,000,000 cells/ml) is reached in cultures with salt levels of 100 ppt.

Ben-Amotz and Avron (1972) studied osmoregulation in *Dunaliella* and *Asteromonas*. They found that internal glycerol was used by this species to balance high external salt concentrations. I undertook a series of experiments to determine if glycerol was also important as an osmoticum in this alga. Fig. 9 shows the concentration of glycerol within alga cells maintained at salt concentrations ranging from half that of oceanic water to seven times that level.

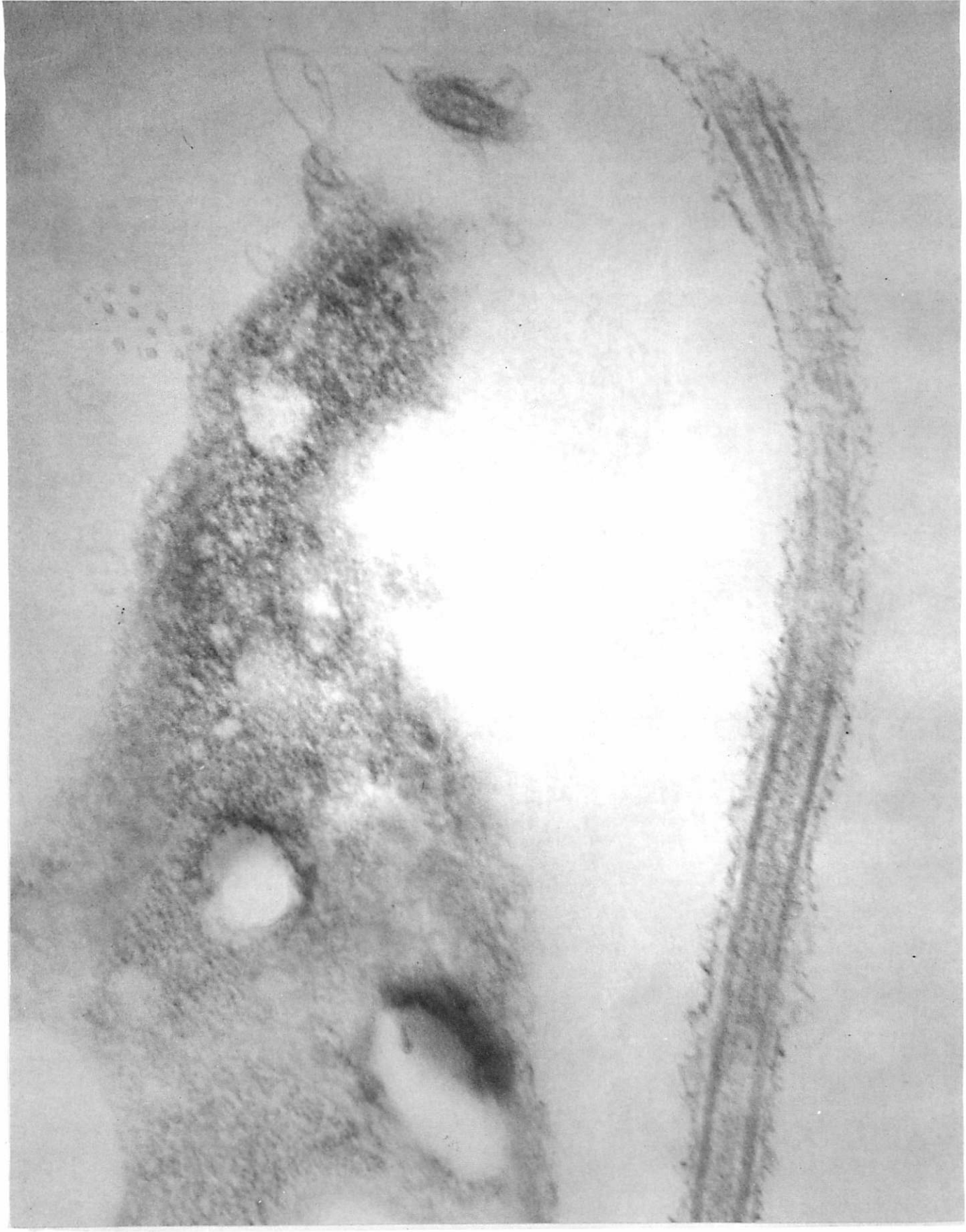

Fig. 2. Electron micrograph of the apical portion of the alga, showing one of the flagella in longitudinal section (40,000x).

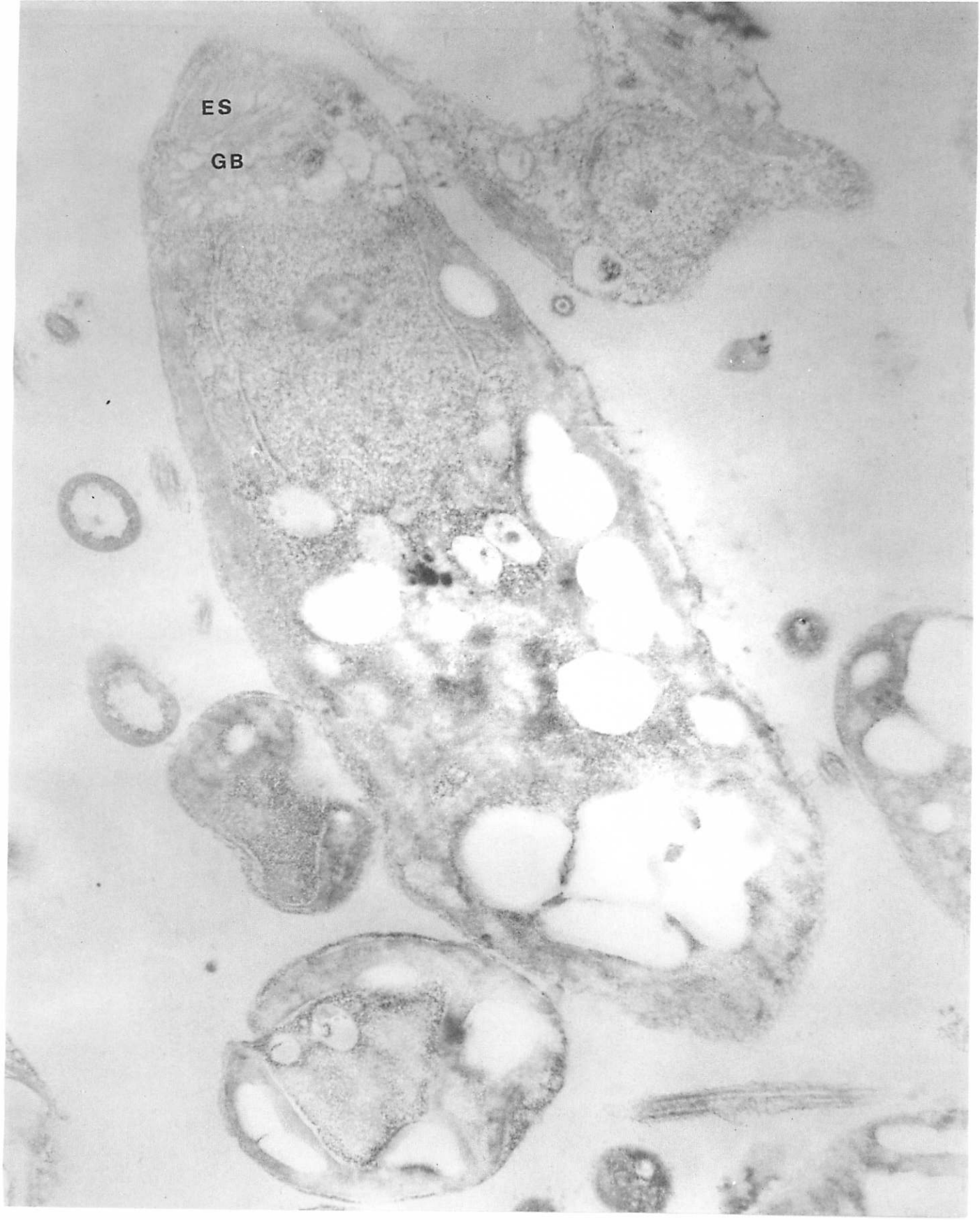

Fig. 3. Electron micrograph of the apical portion of the alga, showing a golgi body (GB) and eyespot (ES) (40,000x).

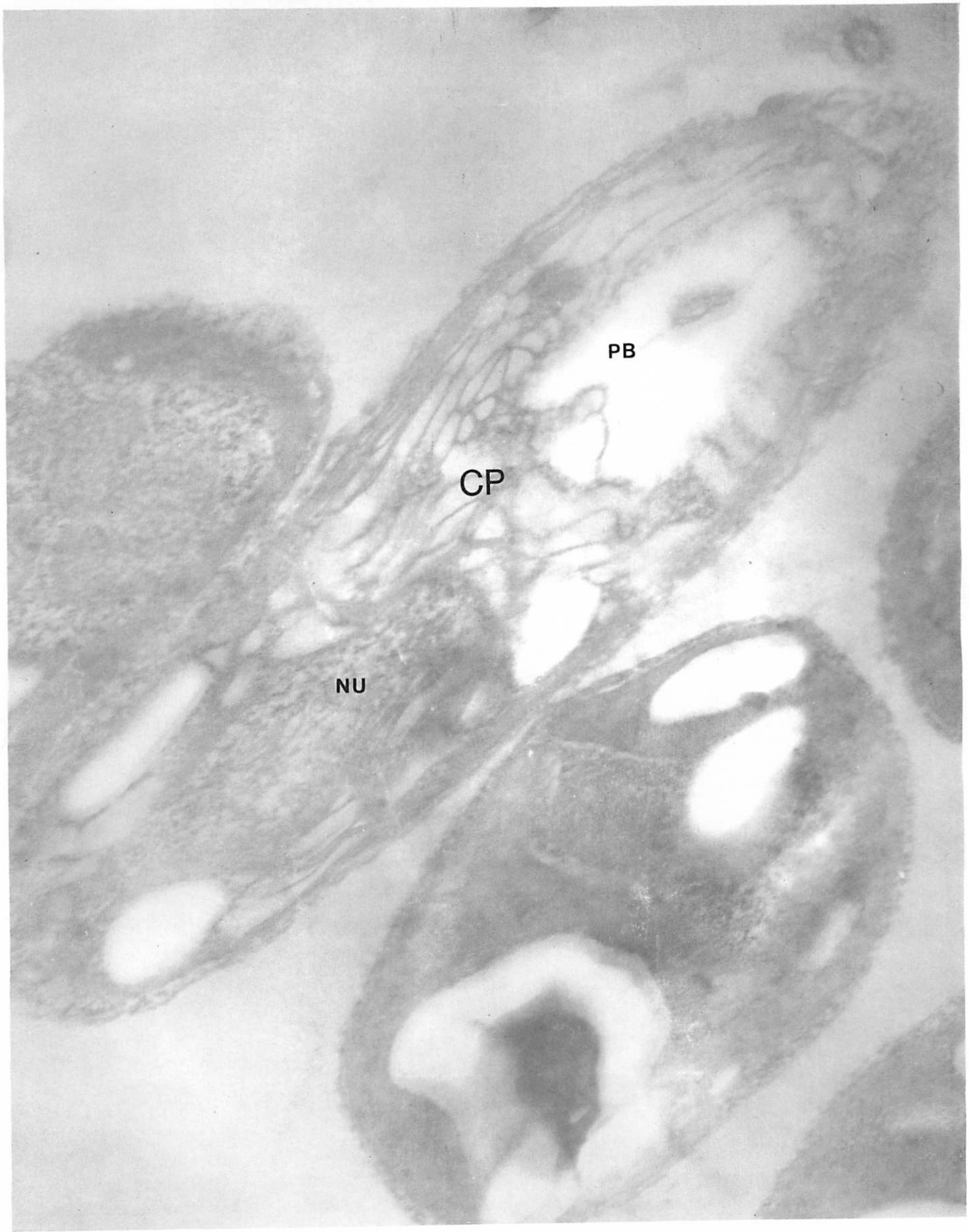

Fig. 4. Electron micrograph of the median portion of the alga, showing nucleus (NU), chloroplast (CP), and pyrenoid body (PB) (40,000x).

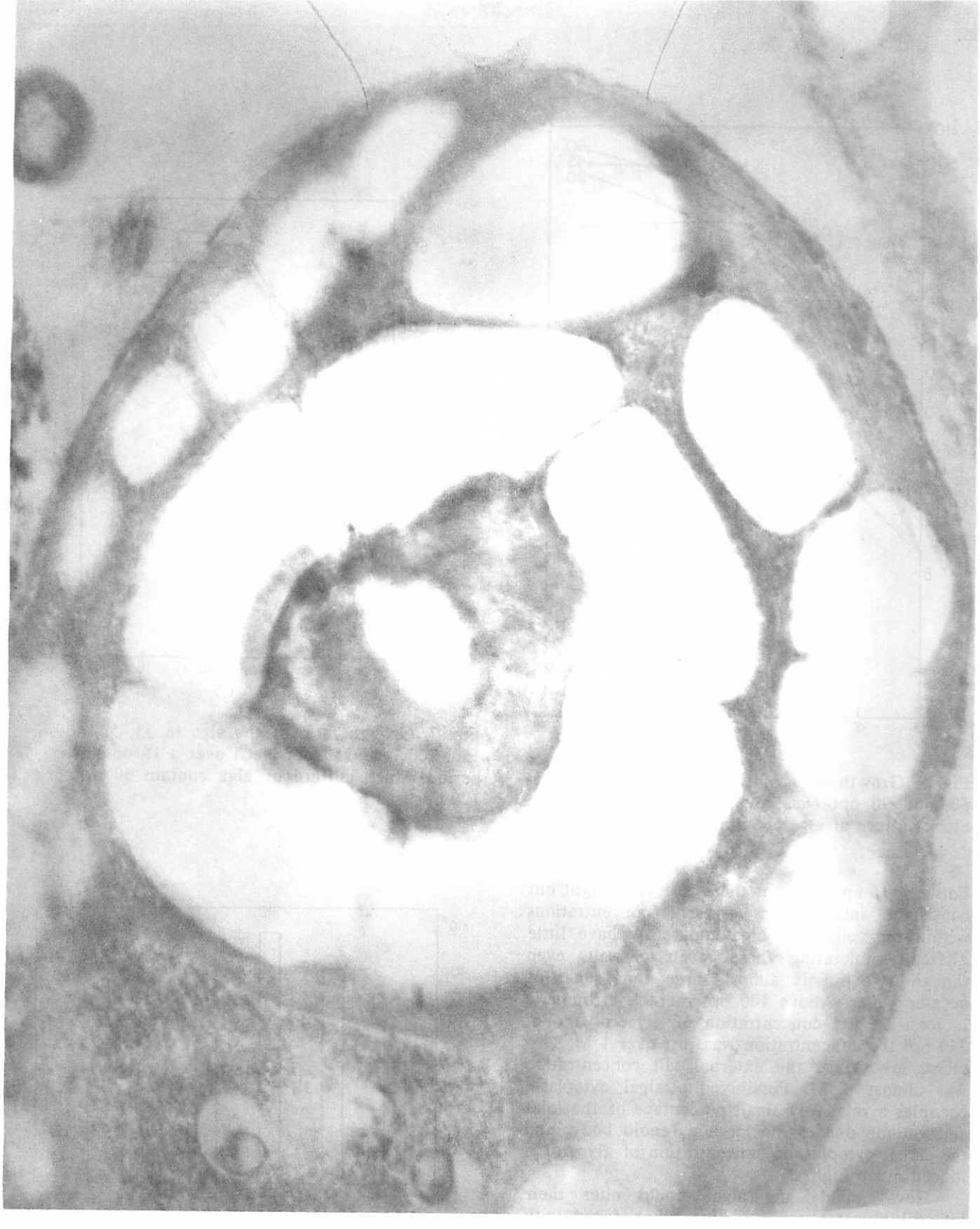

Fig. 5. Electron micrograph of the basal portion of the alga, showing the pyrenoid body composed of two concentric rings of starch grains (60,000x).

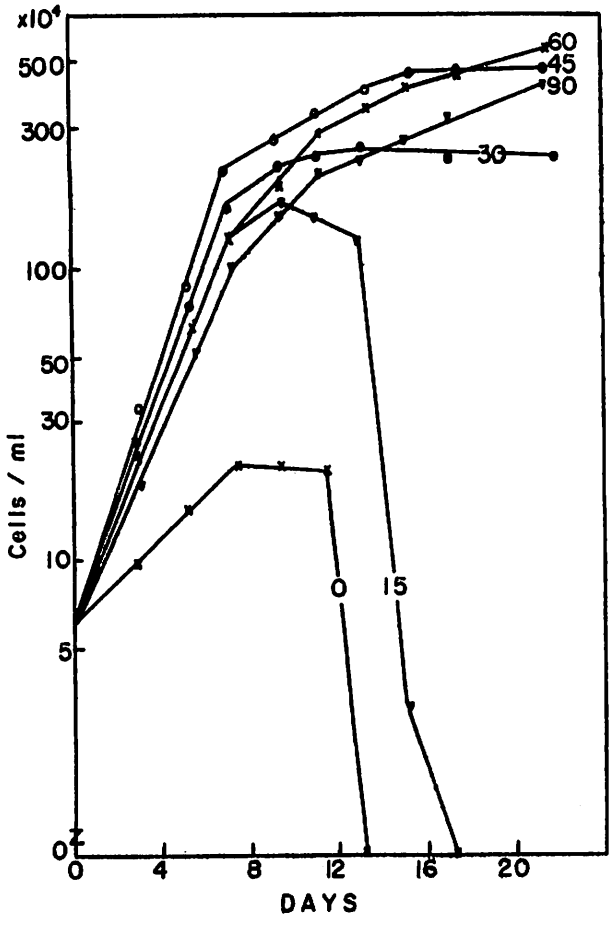

Fig. 6. Growth rates of the alga in 0, 15, 30, 45, 60, and 90 ppt NaCl over a three week period. Stock culture of alga contained 60 ppt.

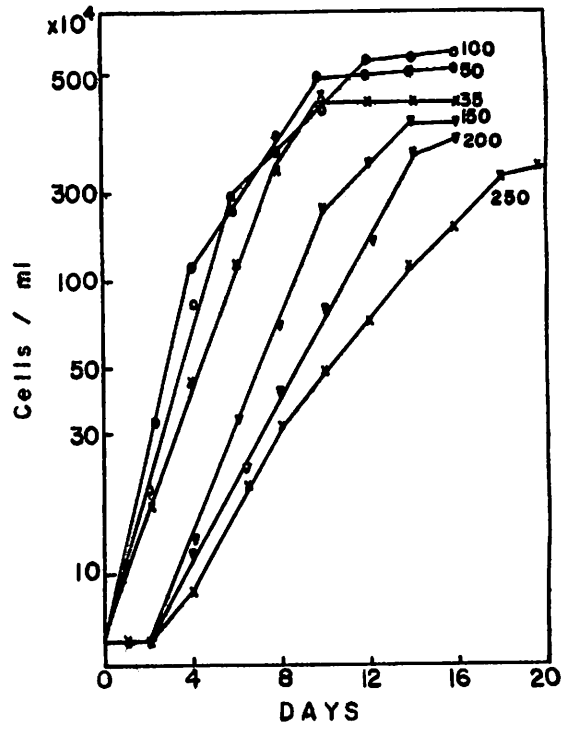

Fig. 7. Growth rates of the alga in 35, 50, 100, 150, 200, and 250 ppt NaCl over a 15 or 20 day period. Stock culture of alga contain 50 ppt or 120 ppt (see text).

Between 15 and 100 ppt there is a very slight but consistent increase in glycerol concentrations within the cells. The alga apparently have little difficulty tolerating these levels of salt, even though 100 ppt is almost three times that of oceanic water. Above 100 ppt there is a dramatic increase in the concentration of glycerol and at 240 ppt the concentration was just over 1 M. This seems low, since the external salt concentration was almost 4 M. Possibly the algal cytoplasm occupies a relatively small percentage of the total cell volume due to the large pyrenoid body, and the actual cytoplasmic concentration of glycerol is indeed much higher.

How quickly the algae could alter their internal glycerol concentration when faced with an abrupt increase in external salt concentration was determined (Fig. 10). As already mentioned, more than a doubling of external salt levels

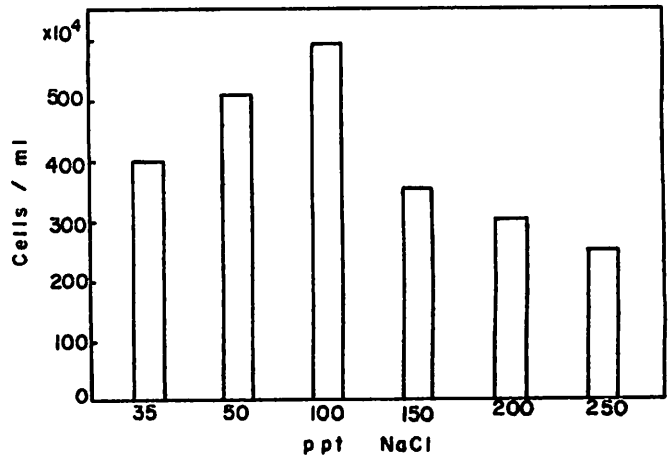

Fig. 8. Maximum cell density (in cells/ml) of the alga grown in several concentrations of NaCl.

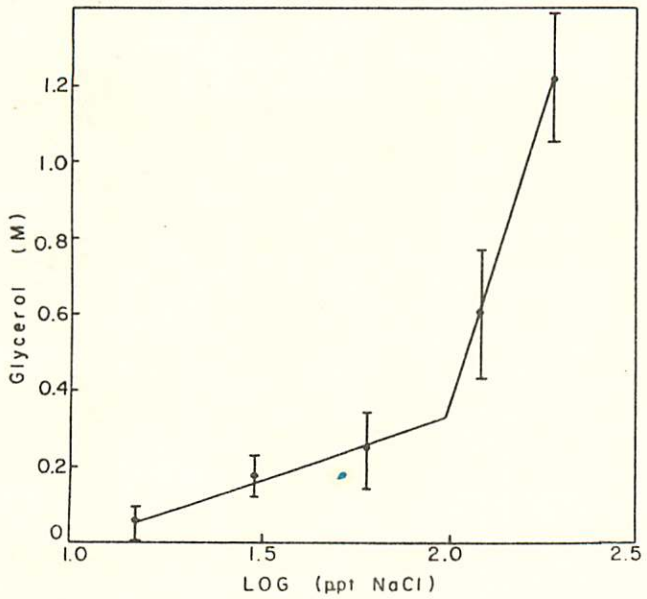

Fig. 9. Internal glycerol concentration of cells grown for three weeks in several concentrations of NaCl.

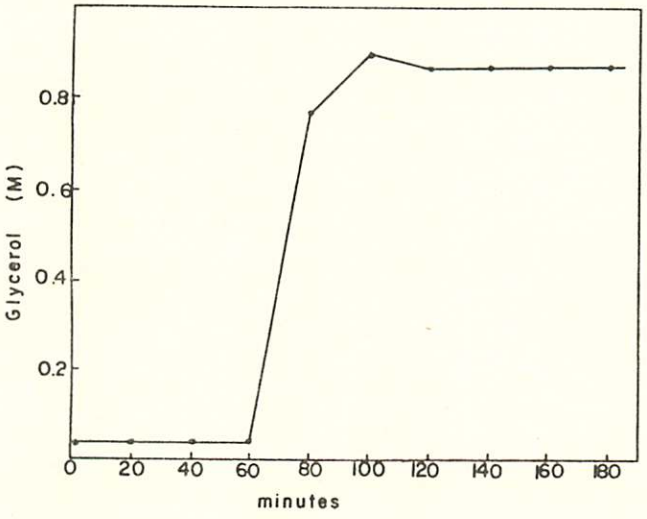

Fig. 10. Time course for increase in internal glycerol concentration over a three hour period. Initially, the algae were grown for three weeks at 60 ppt NaCl. At time zero on the graph, the external concentration of NaCl was doubled to 120 ppt.

proved fatal to the algae. Therefore, external salt was doubled from 60 ppt to 120 ppt. For the first 60 min there was no change in glycerol level. This lag period was followed by a dramatic increase in glycerol level over about a 15 min period. Glycerol levels remained fairly constant over the remainder of the experimental period.

The alga described in this paper grows in the inland lakes of San Salvador and is therefore faced with hypersaline conditions which can change dramatically. The alga is particularly remarkable in that it can tolerate a range of salt concentrations from about 15 ppt to saturated salt solution of 350 ppt. Its ability to produce glycerol, probably from materials stored in its large pyrenoid body, accounts for this salt tolerance. The alga is well adapted to live in the inland lakes of San Salvador Island.

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