

CALCIUM METABOLISM, CALCIUM CARBONATE ACCRETION AND
LIGHT-ENHANCED CALCIFICATION IN THE RED CORALLINE
ALGA, CALLIARTHON TUBERCULOSUM

by

JAMES MICHAEL LAVELLE

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A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

December 1977

An Abstract of the Dissertation of

James M. LaVelle for the degree of Doctor of Philosophy
in the Department of Biology

to be taken

September 1977

Title: Calcium metabolism, Calcium carbonate accretion and light-enhanced calcification in the red coralline alga, Calliarthron tuberculosum.

Approved: _____

Dr. Paul P. Rudy

Calcium and carbonate metabolism in Calliarthron tuberculosum (Cryptonemiales:Corallinaceae) was investigated to elucidate mechanisms underlying CaCO_3 deposition and light-enhanced calcification. Both isotopic tracer methods, using calcium-45 and C-14-bicarbonate, and non-isotopic methods, such as the measurement of calcium and total alkalinity changes in incubation media, were utilized. The results describe the influx, efflux and compartmentalization of calcium, the rates of net accretion of calcium carbonate, the influence of photosynthesis on calcification rates, the binding of calcium by organic anions and the transport of calcium, bicarbonate and organic compounds within the algal system.

The results reaffirm recent reports that calcification rates are highest and that the light-enhancement of calcification is greatest in the tips of the algal fronds. Since these tips are white (unpigmented) and have reduced (or absent) photosynthetic rates, the light-enhancement phenomenon acts at sites remote from the chloroplasts. These findings have led various authors to the conclusion

that light-enhancement must not act through simple ionic changes induced by photosynthesis. Instead, it has been speculated that compounds produced during photosynthesis participate directly in the calcification processes and that translocation of these compounds might be the mechanism of enhancement. A study of translocation in C. tuberculosum, however, supported an alternative view. It appeared that there was a general flow of materials, including not only organic compounds, but also calcium and bicarbonate, from intergeniculum to growing tip rather than the transport of specific calcification-enhancing products. These results, combined with the data on calcium fluxes, net accretion and compartmentalization of calcium within the algal system, were mostly consistent with the following hypothesis.

Calcification in the coralline algae is considered a balance between the opposing forces of CaCO_3 precipitation and dissolution. In the dark, calcification proceeds at reduced rates, possibly due to the production of H^+ by algal respiration. The rate of deposition is probably controlled by the rate at which this hydrogen ion can diffuse or be pumped out of the system. The hypothesis is supported by the instability of calcium labelling in the dark, the large sizes of the exchangeable calcium pools found in dark-incubated algae and the nature of the dependence of calcification rate on the external concentration of calcium.

In the light, through fixation of CO_2 and the uptake of H^+ by chloroplasts during electron transport, photosynthesis appears to remove metabolic constraints on the rate of CaCO_3 deposition and allow rapid mineral accretion. The hypothesis is suggested by the high

stability of calcium labelling in the light, the small size of the exchangeable calcium pools in light-incubated algae and the finding that calcification in the light is apparently limited by the solubility product of the calcite rather than some physiological or enzymatic function.

The translocation experiments suggest that the remote effects of photosynthesis may be explained by assuming that the general flow of materials from the intergenicula to the tips is the equivalent of the transport of the "calcifying milieu". This transported solution may contain, in addition to calcium and the various inorganic CO_2 species, organic acids of the TCA cycle which could serve to buffer the system and thus preserve pH and $\text{CO}_3^{=}$ concentration.

That calcification rates are low where photosynthetic rates are highest is probably an effect of overall growth characteristics. Organic growth slows dramatically in the older parts of the thallus and all available space for calcite crystals in the cell walls becomes filled. The reduced rates, then, appear to be a physical rather than a physiological phenomenon. This interpretation is supported by the finding that the degree of mineralization reaches a constant level in intergenicula adjacent to growing tips.

The literature on calcification in the coralline algae is reviewed in the light of this new support for the early and much criticised hypothesis that simple ionic considerations can be used to explain calcification in these algae. A need for further investigation is recognized and the mechanisms of algal calcification and of light-enhancement specifically are still open to some question. Areas where future study might prove particularly useful are suggested.

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VITA

NAME OF AUTHOR: James Michael LaVelle
PLACE OF BIRTH: Silver City, New Mexico
DATE OF BIRTH: February 26, 1950

UNDERGRADUATE AND GRADUATE SCHOOLS ATTENDED:

University of California, Santa Barbara
University of Oregon

DEGREES AWARDED:

Bachelor of Arts, 1972, University of California,
Santa Barbara
Master of Arts, 1976, University of Oregon

AREAS OF SPECIAL INTEREST:

Invertebrate Zoology
Environmental Physiology
Marine Ecology

PROFESSIONAL EXPERIENCE:

Physiology Trainee, Department of Biology, University
of Oregon, Eugene, 1972-1977
Teaching Assistant, Department of Biology, University
of Oregon, Eugene, 1972 and 1976
Visiting Scientist, Mid-Pacific Marine Laboratory,
Enewetak Atoll, Marshall Islands, June 1975

AWARDS AND HONORS:

University of California scholarship, 1968-1972
Bachelor's degree with high honors, University of
California, Santa Barbara, 1972
NIH Physiology Traineeship, Department of Biology,
University of Oregon, Eugene, 1972-1977

PUBLICATIONS:

Abstract--"A preliminary study of calcium deposition and accretion in the reef coral, Fungia fungites" presented to the Western Society of Naturalists annual meeting, December 26, 1975

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CHAPTER 1

INTRODUCTION

The coralline algae are those members of the Rhodophyta (Cryptonemiales: Corallinaceae) which deposit crystalline calcite (CaCO_3) in the thallus (Revelle and Fairbridge, 1957). These algae occur as encrusting plate-like forms and branching articulated forms in which segments of algal thallus arise from an encrusting base. These segments (intergenicula) are separated by non-calcified joints (genicula) which provide flexibility. On tropical coral reefs the massive deposits of the crustose corallines make important and often major contributions to total bulk production and reef building (Finckh, 1904; Emery et al., 1949; Ladd et al., 1953; Ladd et al., 1967). In temperate waters coralline algae are often locally dominant, especially in areas where one would presume a calcified thallus to be advantageous (e.g., in areas with large grazing populations, heavy wave action and/or sustained human trampling) (Littler, unpublished; Littler and Doty, 1975; Nicholson and Cimberg, 1971). In arctic waters crustose coralline algae form large calcareous banks (Foslie, 1895; Kjellman, 1883).

Despite their widespread occurrence and ecological importance, little is known of the calcification physiology of the Corallinaceae (Littler, 1973; for reviews of coralline algal biology see Blinks, 1951; Revelle and Fairbridge, 1957; Lewin, 1962; Raven, 1970; Johansen, 1973). This lack of knowledge is unfortunate since these plants represent a simple system with which to investigate biological calcification. Such investigations might be particularly applicable to the understanding of calcification in the hermatypic (= reef building) corals (Pearse, 1972). A thorough investigation of calcification processes in the Corallinaceae would have far reaching importance to the understanding of coral reef ecology, reef-coral calcification physiology and the general mechanisms of biomineralization.

Early work suggested that calcification in Corallina proceeds more rapidly in the light than in the dark (Irving and Becking, 1924). This "light-enhancement" effect has been substantiated in other Corallinaceae (Goreau, 1963; Pearse, 1972). Similar data are reported for the green calcareous alga, Halimeda (Siphonales: Codiaceae) (Goreau, 1963; Stark et al., 1969; Bohm and Goreau, 1973; Borowitzka and Larkum, 1976b). These latter are mentioned because of the paucity of work on red algal calcification and since much of the conjecture concerning algal calcification in general is based upon data from Halimeda.

The ratio of calcification rates of Corallinaceae in

the light and in the dark (the L/D ratio) has been estimated at up to 2 (Goreau, 1963). Attempts to explain this phenomenon gave rise to a simple "precipitation" hypothesis (Blinks, 1951). By removing CO_2 , photosynthesis could raise the pH at the sites of mineral deposition. Since CaCO_3 solubility varies inversely with pH, a pH rise would result in the precipitation of mineral. In this hypothesis, dark calcification would be minimal. The hypothesis, however, apparently conflicts with several observations.

First, if calcification is the result of simple precipitation, then it is not clear why all algae do not calcify (Lewin, 1962). Many algae with high photosynthetic rates grow side-by-side with the coralline algae. Second, rapid calcification of red algae in the dark has been reported (Goreau, 1963; Pearse, 1972). Third, the $^{13}\text{C}/^{12}\text{C}$ isotopic ratios for both organic (Craig, 1953) and inorganic (Craig, 1953; Keith and Weber, 1965) constituents are smaller than those of oceanic bicarbonate. The low ratio for organic material is easily explained since photosynthesis discriminates against the heavy carbon isotope (Weigl and Calvin, 1949). However, this process should leave a relatively "heavy" inorganic carbonate pool from which the CaCO_3 is deposited. The finding, instead, of a "light" mineral conflicts with the prediction of the precipitation hypothesis. Stable oxygen ratios have also been studied in the Corallinaceae (Keith and Weber, 1965). Again the inorganic

fraction ($= \text{CaCO}_3$) is light compared with seawater bicarbonate. Since respiration favors the light oxygen isotope (^{16}O), this finding suggests that a large portion of the total inorganic carbonate must be derived from respired CO_2 . Because the organic material is depleted in ^{13}C , this suggestion also fits the stable carbon isotope data.

A fourth argument is that calcification rates are most rapid and light-enhancement greatest in the growing tips of the articulated coralline, Bossiella orbigniana (Pearse, 1972). Since the growing tips are white and deficient in photosynthetic apparatus (see Chapter 3), it appears that light-enhancement exerts its greatest influence at sites remote from the chloroplasts. Simple precipitation predicts that just the opposite should be found.

A final argument is the suggestion that calcification may be to some degree enzyme mediated. The cell wall/matrix material from Porolithon, prepared to preserve enzyme activity, took up (bound?) more calcium ions and at a greater rate than did the same material after autoclaving (Lind, 1970). Acetoazolamide, a potent specific inhibitor of carbonic anhydrase, will cause partial inhibition of calcification in Serraticardia (Ikemori, 1970).

Although the above considerations have led most authors to reject the precipitation theory there has, unfortunately, been no cohesive hypothesis proposed to replace it. The consensus places calcification under metabolic control but

there are only vague suggestions as to the mechanisms involved (see Pearse, 1972). In fact, a careful review of the literature produces some convincing evidence that a calcification mechanism common to all calcareous genera may not exist; a variety of differences between calcification phenomena in the major calcifying groups, the Codiaceae and the Corallinaceae, are documented. Five examples are listed here.

1) It is common knowledge that green and red algae deposit different mineral types (see Revelle and Fairbridge, 1957). Halimeda and many other calcifying algae deposit aragonite while the coralline algae produce high magnesian calcite. Since green and red calcifying algae may be found in the same reef and at similar depths (though often in different habitats) mineral type does not seem likely to be a function of an environmental parameter such as temperature, salinity or light intensity. More likely crystal type is biologically controlled in at least one of the green and red algae.

2) Craig (1953), while studying $^{13}\text{C}/^{12}\text{C}$ ratios in a variety of organisms, found the inorganic carbon of Halimeda to be "heavy" as compared with seawater bicarbonate while that of Corallina was "light." This finding implies that the two algae get the bulk of their inorganic carbon for CaCO_3 deposition from different sources. The light calcite of the coralline algae suggests that organic carbon,

perhaps through respiration, contributes heavily to the total CaCO_3 .

3) Aragonite deposition by Halimeda occurs in large intercellular spaces formed by the outer cell layer, the utricles. This aragonite is not associated with an organic matrix and the algal cell walls remain free of crystals (Wilbur et al., 1969; Borowitzka and Larkum, 1976a). Calcite deposition in Corallina and Calliarthron is intimately associated with the cell walls which become impregnated with crystals. No large intercellular spaces exist in the coralline algae and the small gaps between cells are filled with an organic fibrous material onto which CaCO_3 precipitates (Bailey and Bisalputra, 1970; Lind, 1970).

4) The aragonite crystals in Halimeda form in random orientation without the presence of a matrix template (Wilbur et al., 1969). In the corallines, calcite crystals are oriented with their c-axes parallel to the long axis of the cell wall and matrix fibers (Lind, 1970).

5) Borowitzka and Larkum (1967a, 1967b) implicated the peculiar morphology of the utricles of Halimeda in the calcification processes of this algae. Since this morphology is rather uncommon, the problem of why all algae do not calcify is circumvented. Further, these data suggest that dark calcification may be due primarily to exchange or slow auto precipitation like that which occurs in calcareous sands (Weyl, 1967; Defleyes et al., 1964, 1965).

Thus, light is considered necessary for net CaCO_3 deposition. The simple "precipitation" theory is revived for this specific case. Of course, it is obvious on morphological grounds alone that Borowitzka's hypothesis is untenable for the Corallinaceae. Coralline morphology, with the exception of the non-calcified genicula of articulated forms, is not exceptional. Furthermore, crystal type and isotopic carbon ratios are not explained. If Borowitzka is right about calcification in Halimeda, then it seems obvious that an alternative mechanism must be found for the coralline algae.

It is of some interest here to mention the parallels between calcification processes in the red algae and the hermatypic corals. Pearse (1972) outlined several areas of similarity: (1) the tip of the coral branch, as the growing algal tip, is less heavily calcified than the more basal regions; (2) the tip coral polyp is white, containing very few symbiotic algae (= zooxanthellae); (3) calcification rates in the tip polyps are greater than in adjacent lateral polyps which harbor large numbers of zooxanthellae; (4) calcification rates in corals are enhanced in the light (i.e., by photosynthesis of the zooxanthellae) and; (5) the light enhancement is greatest in the tip polyps. These rather striking parallels suggest that such findings as those of Pearse and Muscatine (1971), Chalker (1975) and Crossland and Barnes (1974) should be considered in investigating coralline algal calcification. Pearse and

Muscatine provide evidence that translocated photosynthetic compounds may participate in the light enhancement mechanism in coral tip polyps. Chalker reports that hermatypic corals actively transport calcium and that this transport is activated by the energy supply from the zooxanthellae; if the energy supply is poisoned, calcification rates drop to dark levels. Crossland and Barnes have suggested a mechanism of coral calcification which involves the shuffling of NH_4^+ and HCO_3^- through the ornithine cycle and various ureides to the site of mineral deposition. The ammonia, bound in urea, is hypothesized to neutralize hydrogen ions when released by the enzyme, urease. The HCO_3^- released by the same reaction is then free to dissociate into carbonate for deposition and H^+ .

The lack of cohesive explanations for red algal calcification processes underscores our fundamental ignorance in the field. There are many unanswered questions preventing the formulation of testable hypotheses. Fortunately, there is a considerably more advanced literature on the superficially parallel calcification processes in the hermatypic corals and the calcareous green alga, Halimeda. Examination of this literature suggests ways in which the calcification processes in the red alga might be successfully explored. It is the purpose of this thesis to examine red algal calcification using extensions of techniques and concepts derived from the study of corals and

calcareous green algae.

The thesis is divided into three general parts. The first part, encompassing Chapters 3 and 4, comprises the analysis and intercomparison of several methods used for the measurement of calcification rates and the subsequent application of these methods to the analysis of calcium metabolism. The research was centered on three questions suggested by previous studies of Halimeda and Acropora:

(1) What exchange bias has been introduced into the calcification literature by the use of calcium-45 for measurement of calcification rates? (2) What relationships exist between calcium fluxes, isotopic exchange and actual net accretion of calcium carbonate? and (3) Do specific calcium pools such as those suggested in Halimeda (Stark et al., 1969; Bohm and Goreau, 1973) and Acropora (Crossland and Barnes, 1974) play a significant role in the calcification process?

The second part of the thesis is concerned with calcification processes in the growing algal tips and with the energy requirements of CaCO_3 deposition. Past investigation of Acropora (Pearse and Muscatine, 1971) has suggested that the non-photosynthetic tip polyps (tip polyps contain few symbiotic zooxanthellae) may be dependent on some translocated material for light-enhanced calcification. In Chapter 5, the translocation of material to the algal tips is investigated. The research was designed to answer three

basic questions: (1) Is the translocation of calcium, bicarbonate or some organic compound(s) from adjacent photosynthetic areas required for light-enhanced calcification in the white algal tips? (2) Can the active transport of calcium and/or hydrogen ions explain calcification phenomena? and (3) Might calcium be a substrate for an enzyme mediated calcification process?

The final section of the thesis (Chapter 6) is a preliminary biochemical investigation of red algal calcification. The study here was prompted by findings in the first three chapters. The questions asked in this final section will be made clear during the discussion of results in subsequent chapters.

To carry out the above research, Calliarthron tuberculosum was the best choice for an experimental organism. This selection offered several key advantages: (1) it is locally abundant and easily collected; (2) it has been the subject of several studies of development, growth, morphology and ultrastructure; (3) it produces relatively large uniform cylindrical intergeniculae near the branch tips; and (4) it produces and releases large numbers of spores year round in the laboratory. These characteristics ensured a constant supply of experimental material, provided a background of literature as extensive as can be found among the coralline and furnished an organism amenable to a variety of experimental manipulations.

CHAPTER 2

GENERAL METHODS

Several procedures used throughout this study are detailed here to avoid repetition in subsequent chapters. Methods used in only one part of the investigation will be dealt with separately in the methods section of the appropriate chapter.

I. Collection and Maintenance of Algae

All algae were collected from the rock substrate in Middle Cove, Cape Arago in a high tide pool. Algae were transported attached to part of the rock to the Oregon Inst. of Marine Biology in buckets of seawater and kept in running seawater aquaria under ambient temperature conditions. Light was natural, filtered through a roofing layer of translucent fiberglass. Algae to be transported to the University of Oregon (125 miles) were placed in buckets of seawater cooled with ice and aerated by dipping out beakers of water and dumping them back in each half hour during transit. At the University algae were kept at 11° C in a cold room. Illumination was provided by a bank of four high-intensity white fluorescent tubes delivering about 500

foot-candles at the water surface. The cold room was maintained on a 12/12 light/dark cycle. Seawater in the aquarium was constantly filtered and aerated. In addition, about one third of the seawater was replaced daily and the total seawater changed weekly. To remove sediment, seawater changes were made by siphoning from the bottom of the aquarium. Diatom growth was a problem and was controlled by a population of Littorina. These snails are often found browsing over the corallines in high tide pools and are effective in the control of diatom fouling. The snails do not harm mature thallus and did not suppress spore settlement and development on glass slides introduced into the aquarium. Algae were never kept more than two weeks before use in labeling experiments or three weeks before use in other phases of the study. Spores used in the study were grown entirely in the laboratory on slides or cover slips. Spore settlement occurred through the year, even in the outdoor ambient temperature and light tanks at the Institute of Marine Biology. Slides and cover slips became covered with spores when placed underneath clumps of algae for a few days. Sporelings continued to develop for 9 months in the cold room at the University of Oregon at which time the largest was six millimeters in diameter and had produced an erect branch seven millimeters in height. The sporelings were firmly enough attached to the glass surfaces to be

manipulated during experimentation on the glass slides.

II. Determination of Seawater and Algal Activities

Seawater samples containing calcium-45 were counted in either 7:2:1 toluene:methanol:Biosolv (Beckman) containing 7.5 grams per liter Beckman Fluoralloy (Fluoralloy is a commercial preparation of 28:1 Butyl-PBD:PBB) or in toluene fluor containing 7.5 grams/liter Fluoralloy mixed 2:1 with Triton X-100. The former cocktail would not accept more than 100 microliters of seawater per 10 ml of fluor and so was used only for high activity samples. The toluene-triton fluor would easily accept up to 0.5 ml of seawater and was adopted for general use for the latter part of the study.

Seawater samples containing C-14-bicarbonate were placed in vials containing 1 ml of 2-aminoethanol and 2 ml of methanol. This was mixed with 7:3 toluene:methanol fluor containing 7.5 grams/liter Fluoralloy.

Algal samples labeled with calcium-45 were rinsed five times with distilled water over a period of about 45 seconds. The algae were oven dried, weighed and placed either into polystyrene test tubes or directly into scintillation vials. Samples were dissolved in 0.5 to 2 ml of 1:1 methanol:con. HCl. Aliquots of samples dissolved in the test tubes were counted in toluene:methanol:Biosolv or toluene:triton fluor. Samples already in scintillation vials were solubilized in

toluene:triton fluor and counted directly. The latter procedure proved more reproducible and was adopted for the major part of the study.

Algal samples labeled with C-14-bicarbonate were washed once for 20 seconds with distilled water and oven dried. Some samples were also freeze-dried as suggested by Borowitzka (1976) but this procedure was found to make little if any difference in the final activities. Dried samples were weighed and placed in scintillation vials with 1 to 1.5 ml of methanol. These vials were connected to one arm of a "Y" tube connector (Figure 2-1). To the other arm of the "Y" was connected a second scintillation vial containing 1 ml of 2-aminoethanol. One half to 175 ml of concentrated HCl was injected into the vial containing the algae through the "Y" stem and the stem immediately sealed with parafilm. Diffusion and absorption of the evolved CO₂ was continued for at least 12 hours (adopted from the general procedures of Young et al., 1969 and Borowitzka and Larkum, 1976b). Controls of this method indicated that virtually 100% of the C-14 activity in samples of seawater containing a known quantity of H¹⁴CO₃ could be recovered in the 2-aminoethanol fraction. Thus no loss of activity could be attributed to leakage, retention of inorganic carbon by the algal sample or loss before the parafilm seal was complete. In addition, diffusion of C-14 activity exchanged by killed controls

Figure 2-1. Apparatus for the separation of organic and inorganic C-14 label. A "Y" tubing connector was used to join two scintillation vials. The sample was placed in one vial and one ml of 2-aminoethonal was added to the other. Seals were made with parafilm to insure airtight fits. HCl was injected into the neck the tubing connector and the neck immediately sealed with parafilm. The apparatus was placed on its side during the diffusion process and was gently shaken occasionally to promote the release of CO₂ from the sample solution.

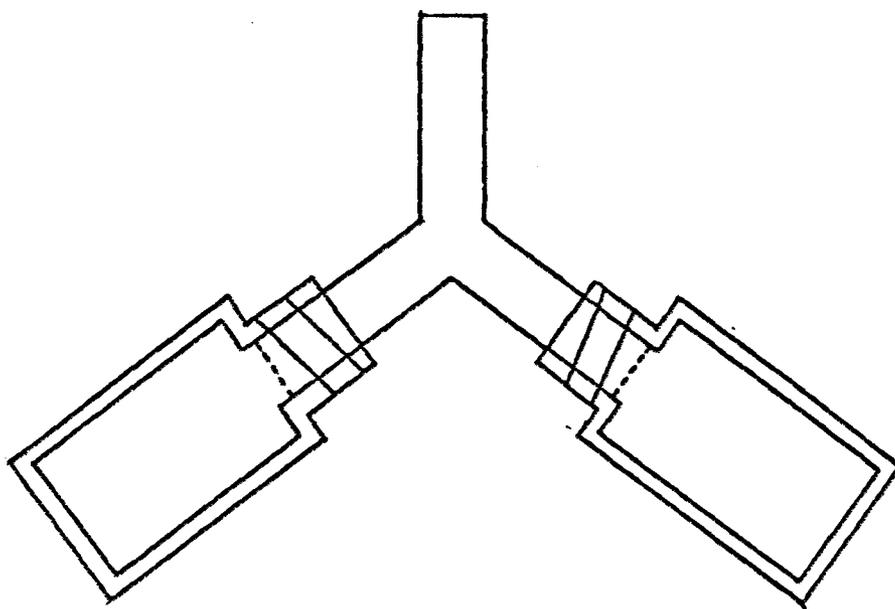


Figure 2-1.

indicated that the 12 hour diffusion period was sufficient time for 99+% of the label to be recovered in the 2-aminoethanol. Algal samples were counted as before in toluene:triton fluor. Aminoethanol samples were counted in toluene:methanol fluor.

Efficiencies of the fluors used were essentially identical in tests with aliquots of seawater or HCl:methanol containing known amounts of calcium-45 activity. No efficiency corrections were made on the data. Most of the samples were counted in an open window set for C-14 or Ca-45. For these counts quenching of the samples made little difference in the total counts and no quench corrections were made. Some counts had to be made in a window set above tritium because of a phototube malfunction. These counts were reproducible, but had to be corrected for quench. All counts from a single experiment using Ca-45 were counted on the same day to avoid the small decay effects. Counts were made on either a Beckman LS-250 or a Packard Tri-Carb Scintillation Spectrophotometer.

Activities throughout the study are presented in terms of nanograms calcium per mg alga per min. These values were calculated using equation 2.1 for Ca-45 experiments or equation 2.2 for H^{14}CO_3 experiments.

$$\text{ng Ca mg}^{-1} \text{ alga min}^{-1} = \frac{\text{CPM mg}^{-1} \text{ alga}}{\text{CPM ml}^{-1} \text{ media}} \times \text{ng Ca ml}^{-1} \text{ media} \quad (2.1)$$

$$\begin{aligned} \text{ng Ca mg}^{-1} \text{ alga min}^{-1} &= \frac{\text{CPM mg}^{-1} \text{ alga}}{\text{CPM ml}^{-1} \text{ media}} \times \text{nM CO}_2 \text{ ml}^{-1} \\ &\times 20 \text{ mg Ca nM}^{-1} \text{ CO}_2 \end{aligned} \quad (2.2)$$

III. Controls

Dead algal controls were run along with all labeling experiments. These algae were killed by the addition of 10% formalin to seawater for 4 hours. Algae killed with formalin were rinsed several times with seawater and soaked in seawater (with no formalin) overnight before use. No difference was found between controls run in the dark and those run in the light. Therefore, controls were normally run in the light as a matter of convenience.

CHAPTER 3

CALCIUM METABOLISM I. CALCIUM UPTAKE AND
NET CaCO_3 ACCRETION

Previous laboratory investigations of red algal calcification have relied heavily and often exclusively on the uptake of calcium-45 for measurement of calcification rates (e.g., Goreau, 1963; Pearse, 1972; Littler, 1973). Some doubt has recently been cast on the accuracy of the calcium isotope method by workers studying the green calcareous alga, Halimeda. Bohm and Goreau (1973) and Borowitzka and Larkum (1976b) have shown that exchange of calcium at the mineral surfaces and the binding of calcium by organic anions significantly bias calcification rates calculated from calcium-45 uptake. Unfortunately, neither of the above papers included a critical analysis of the Ca-45 method. Because of the potential uncertainties of the method, one must assume that the actual net accretion rates for CaCO_3 were unknown in any of the studies.

As the initial step in an extensive analysis of the light-enhancement phenomenon in the coralline algae, this chapter compares various techniques for the measurement of

calcification rates. The basic question asked in this part of the study was, "What bias has been introduced into calcification rate calculations by exchange of calcium-45 and how has this bias affected the interpretation of the previous data?"

It is shown that light-enhancement has been underestimated in previous reports, that older portions of the thallus exhibit light-enhancement to the same degree as do the growing tips and that dark calcification, though significant, has been previously overestimated.

I. Materials and Methods

A. Calcium Labelling

For calcium uptake experiments approximately 10 grams (dry weight) of alga were preincubated in 350 ml of seawater for 30 minutes. At time zero, calcium-45 in seawater was added to give a final concentration of 0.05 microcuries per milliliter. At time intervals, 1 ml samples of seawater were drawn and 100 to 500 microliter aliquots taken for scintillation counting. At the same time algal samples were taken and prepared for counting as described in Chapter 2. The total amount of algal tissue removed from any incubation was less than one percent of the total algal weight.

Short term measurements of calcification rate were

carried out with algae in small (50 ml) beakers. After a 30 minute preincubation, algae were incubated for 1 to 2 hours in seawater containing 0.05 microcuries/ml of Ca-45. At the end of the incubation seawater and algal samples were taken and treated for counting (Chapter 2). In addition, algal samples were sometimes taken for analysis of total protein and total chlorophyll content as described below.

All incubations were carried out at 11° C with illumination provided by cool white fluorescent bulbs delivering about 1000 foot-candles to the surfaces of the incubation media. Vessels were constantly aerated for agitation and to maintain pH and CO₂ concentration.

B. C-14 Bicarbonate Labelling

Algae to be labelled with H¹⁴CO₃⁻ were preincubated in small beakers containing 30 ml of seawater for 30 minutes. Beakers were stoppered and covered with parafilm. At time zero 1 ml of seawater containing 0.3 μC H¹⁴CO₃ per ml was injected by syringe through the rubber stopper and mixed by withdrawing and injecting the seawater with the syringe. After 5 minutes seawater samples were taken for initial activity. At the end of the incubation samples were collected and counted (Chapter 2). In each series of experiments total CO₂ was measured by the method of Strickland and Parsons (1965) and used to calculate the specific activity of the seawater. For incubations in which

metabolic inhibitors or artificial seawater were used, the preincubation period of 30 minutes was retained.

C. Total Alkalinity Method for Determination of Calcification Rate

Measurement of total alkalinity as described by Strickland and Parsons (1965) and using the technique of Smith (1973) was used to estimate calcification rates independently of the isotope methods. Algae were placed in incubation vessels containing 400 ml of seawater and 8-12 g (dry weight) of algae. Vessels were covered and incubated for 1 to 2 hours. Algae were removed after the incubation, washed briefly with tap water, dried and weighed. The total alkalinity (T.A.) of the seawater before and after the incubation was measured by the following technique. Twenty-five ml samples of seawater were acidified with 7 ml of 0.0100N HCl in small jars. The jars were shaken vigorously for 30 seconds then aerated with CO₂-free, water-saturated air. The sample pH was then measured to ± 0.0025 pH units. The final pH was used to calculate the total alkalinity of the sample by equation (3.1).

$$\text{T.A.} = \frac{1000}{V_s} V \times N \frac{1000}{V_s} (V_s + V) \frac{a_H}{f_H} \quad (3.1)$$

where: $f_H = 0.741$

$a_H = 10^{-\text{pH}}$

$V = \text{volume of } 0.0100\text{N HCl}$

$$N = 0.0100$$

V_s = volume of seawater

Triplicate samples were run on each incubation. The change in T.A. before and after incubation (Δ T.A.) was taken as an estimation of CaCO_3 deposition through equation (3.2).

$$\text{calcification rate} = \Delta \text{ T.A.} \times 2 \times 10^7 \times \text{algal dry weight}^{-1} \times \text{incubation time} \quad (3.2)$$

where: calcification rate: ng calcium/mg alga/minute
and 2×10^7 is the number of ng of calcium in 1 mEq

Measurements of pH which varied more than 0.01 pH units among a set of triplicate samples were discarded. Vessels were lightly agitated with a magnetic stirbar and covered with a watch glass to prevent evaporation.

D. EDTA Method for the Estimation of Calcification Rates

The absolute amount of calcium removed from seawater by the algae was measured by direct wet titration of calcium with ethylenedinitrilotetraacetic acid (EDTA) using 4-(2-hydroxy-4-sulfo-1-naphthylazo-)-2-hydroxy-3-naphthoic acid as an indicator (Pate and Robinson, 1958). The reduction in the volume of 0.0150M EDTA needed to titrate

calcium in samples taken before incubation as compared with samples taken after incubation was used to estimate the amount of calcium deposited by the alga. Incubations were similar to those described in Section A, but smaller amounts of alga (2-3 g dry weight) and seawater (80 ml) were used. Incubations were carried out for 4 hours. Incubation vessels were aerated and covered with watch glasses. A control with no alga was used to determine evaporation effects. Calcification rates were calculated according to equation (3.3).

$$\text{calcification rate} = \frac{\Delta \text{EDTA} \times 0.0150 \times 4 \times 10^7}{\text{WT}^{-1} \times \text{time}^{-1}} \quad (3.3)$$

where: ΔEDTA = change in EDTA titration volume

0.0150 = molarity of EDTA solution

4×10^7 = ng calcium/mM calcium

WT = algal dry weight

time = incubation time

E. Chlorophyll Determinations

Algal samples were frozen in test tubes cooled in a dry ice-acetone bath. These samples were immediately freeze-dried and stored in the dark in an evacuated desiccator jar at -20°C . Weighed samples were macerated in ice cold methanol and extracted in the dark at -20°C overnight. Chlorophyll absorption was measured at 578 nm

and the chlorophyll content calculated from equation (3.4).

$$\frac{\text{OD @ 578 nm}}{7.8} = \text{mg chlorophyll/ml} \quad (3.4)$$

F. Protein Determinations

Freeze-dried algal samples were macerated in 1 ml of 1N NaOH and soaked overnight. One ml of distilled water was added and the samples incubated 60 minutes at 90° C (adapted from Borowitzka, 1976). Protein was then determined on whole extracts by the method of Lowry, et al. (1951).

G. Percent Mineralization

Mineralization was measured from homogenized whole algal thallus. The homogenate was made to a known volume and 1 ml aliquots pipetted onto preweighed planchets and dried to constant weight. An identical sample of the homogenate was centrifuged and resuspended in 5.25% Na hypochlorite (Chlorox) and left to digest for 24 hours. The suspension was then pelleted and the pellet washed several times in distilled H₂O. The pellet was finally suspended in H₂O and the suspension made up to the original volume. One ml aliquots were weighed as above. The weight of the bleached sample divided by the weight of the whole algal sample gave the proportion by dry weight of the CaCO₃.

II. Results

A. Gradients in Mineralization, Total Protein, Total Chlorophyll and Calcification

The degree of mineralization gradually increases in successive segments from C. tuberculosum (Figure 3-1). The percent mineralization, based on the proportion of dry weight of CaCO_3 , increases from the tip segments where CaCO_3 accounts for 75% of dry weight to the second and third intergenicula where CaCO_3 is 88% of dry weight. The lower percent mineralization in the fourth segment was not expected. However, the variability of the data is fairly high and the values for protein and chlorophyll content for the fourth segment are consistent with a continued high degree of mineralization. The decline in mineralization for the fourth segment may be spurious.

The absolute values for the degree of mineralization presented here are about 0.8X the values given by Pearse (1972) for Bossiella orbigniana tips and about 0.95X the values for subsequent segments. These results may reflect species differences. However, it is more likely that Pearse's data overestimate the percent mineralization of the data. In her measurements she bleached whole algal segments with Na hypochlorite (commercial Chlorox) to remove organic matter, a procedure which does not remove cell wall material. When Chlorox-treated whole segments are placed

Figure 3-1. Percent mineralization of C. tuberculosum. Percent mineralization in the tips and first four intergenicula are shown. Each point is the mean of at least 12 determinations on samples of 10 to 20 segments. Vertical bars are one standard deviation of the mean.

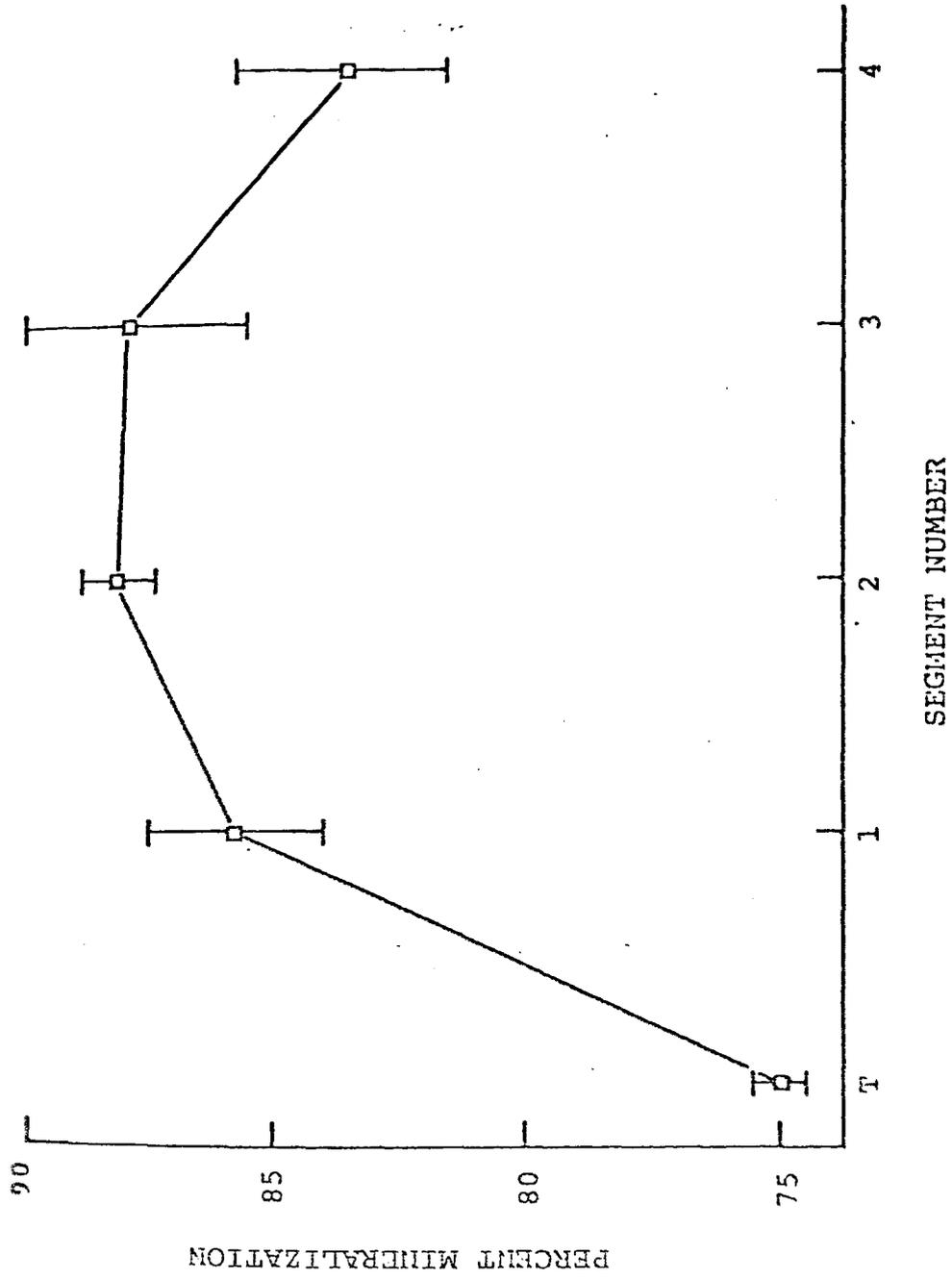


Figure 3-1.

in HCl significant amounts of organic material remain after solution of the carbonate (author's observations; see also discussion in Pearse, 1972). The data in Figure 3-1a are based on measurements of powdered material treated with hypochlorite, a technique designed to expose more organic material to the action of the bleach. This latter procedure still does not remove all of the organic matter (Bohm and Goreau, 1973) and even the values reported here are high compared with values from CO₂ evolution (Furuya, 1960). However, relative mineralization is most important for the purposes of this study so that exact absolute values are unnecessary.

The relationships among chlorophyll concentration, total protein content and dry weight are shown in Figure 3-2. The results include only the algal segments from the tips through the fourth intergenicula. The positions of subsequent intergenicula were often made ambiguous by the profuse dichotomous branching of C. tuberculosum. In addition, older segments are variable in size and shape owing to the formation of conceptacles and lateral wings (see Johansen, 1969). Such variability renders segments unsuitable for analysis.

As expected, the less heavily calcified tips contain more protein per milligram dry weight than subsequent segments. The protein:dry weight ratio declines in older segments but the gradations are not severe enough to be

Figure 3-2. Relationships among dry weight, total protein and total chlorophyll. Chlorophyll/protein and protein/dry weight (stippled bars) ratios are shown. Each point is the mean of 12 determinations on samples of 10 to 20 segment. Vertical bars represent one standard deviation of the mean.

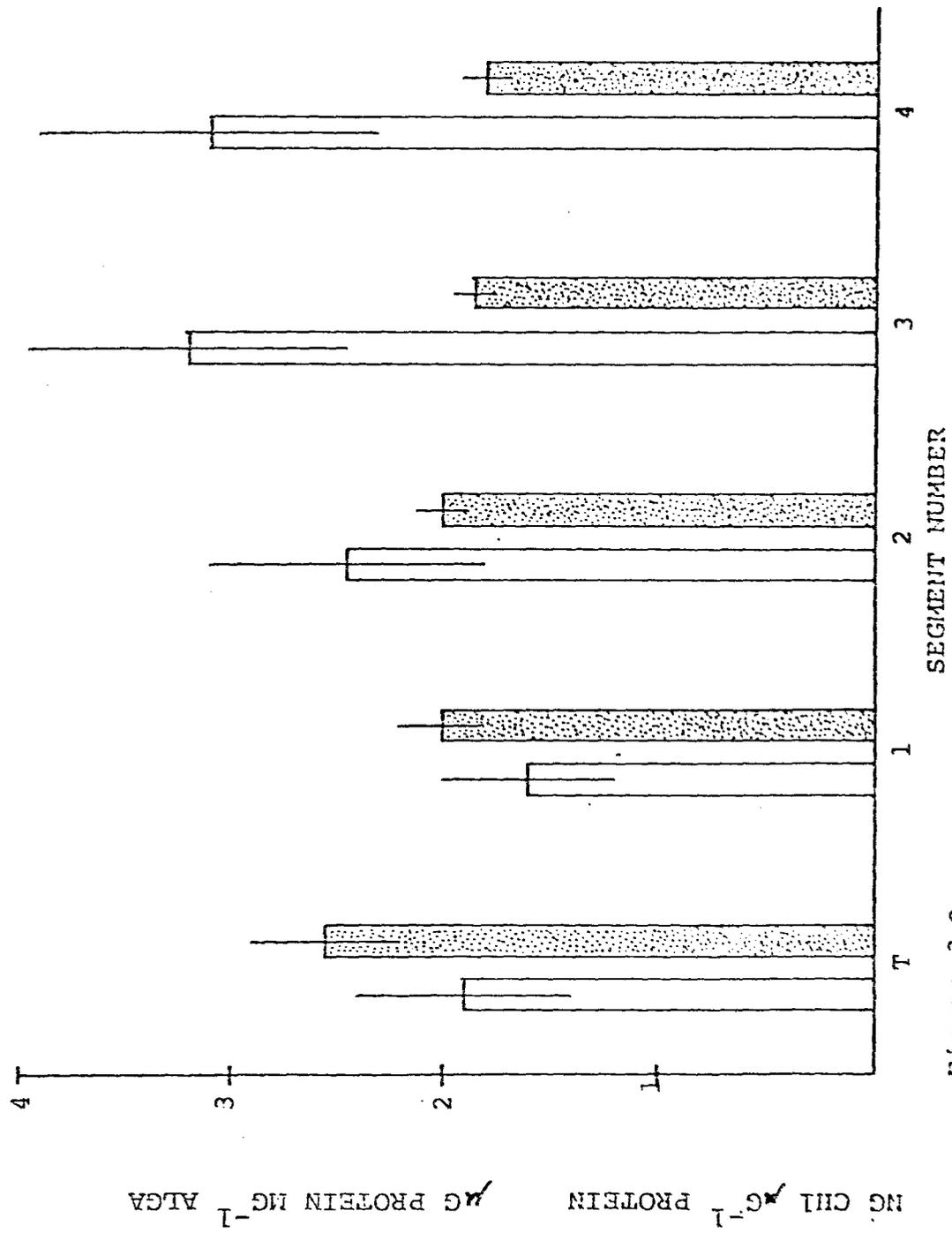


Figure 3-2.

statistically significant.

Except for the tip value, chlorophyll:protein ratios increase in progressively older segments. The tips appear to have more chlorophyll than one expects of white tissue. However, this value is largely a consequence of errors in the sampling of the tips and distortion due to expression in terms of protein content (see Discussion).

B. Gradations in Calcification Rates

Calcification rates based upon dry weight, total protein and total chlorophyll are compared segment by segment (Figures 3-3, 4, and 5). Regardless of the method on which the rates are based, similar graphs are obtained. Thus, any of the three methods might serve for the expression of CaCO_3 deposition. However, the chlorophyll content of the tip segment is questionable and rates based on chlorophyll have little absolute significance (see Discussion). Protein-based rates, often assumed to be closely related to metabolic activity, are tedious to obtain and difficult to relate to other published data. The most acceptable choice for the expression of calcification rates is, then, based upon dry weight. For the remainder of the study, rates are reported as nanograms of calcium deposited per mg of alga per min.

Calcification rates for intergenacula (segments 1

Figure 3-3. Dry weight-based calcification rates. Calcification rates are shown for light-incubated (open circles), dark-incubated (closed circles) and killed alga (squares), in progressively older segments of C. tuberculosum. Numbers of determinations and error bars as in Figure 3-2.

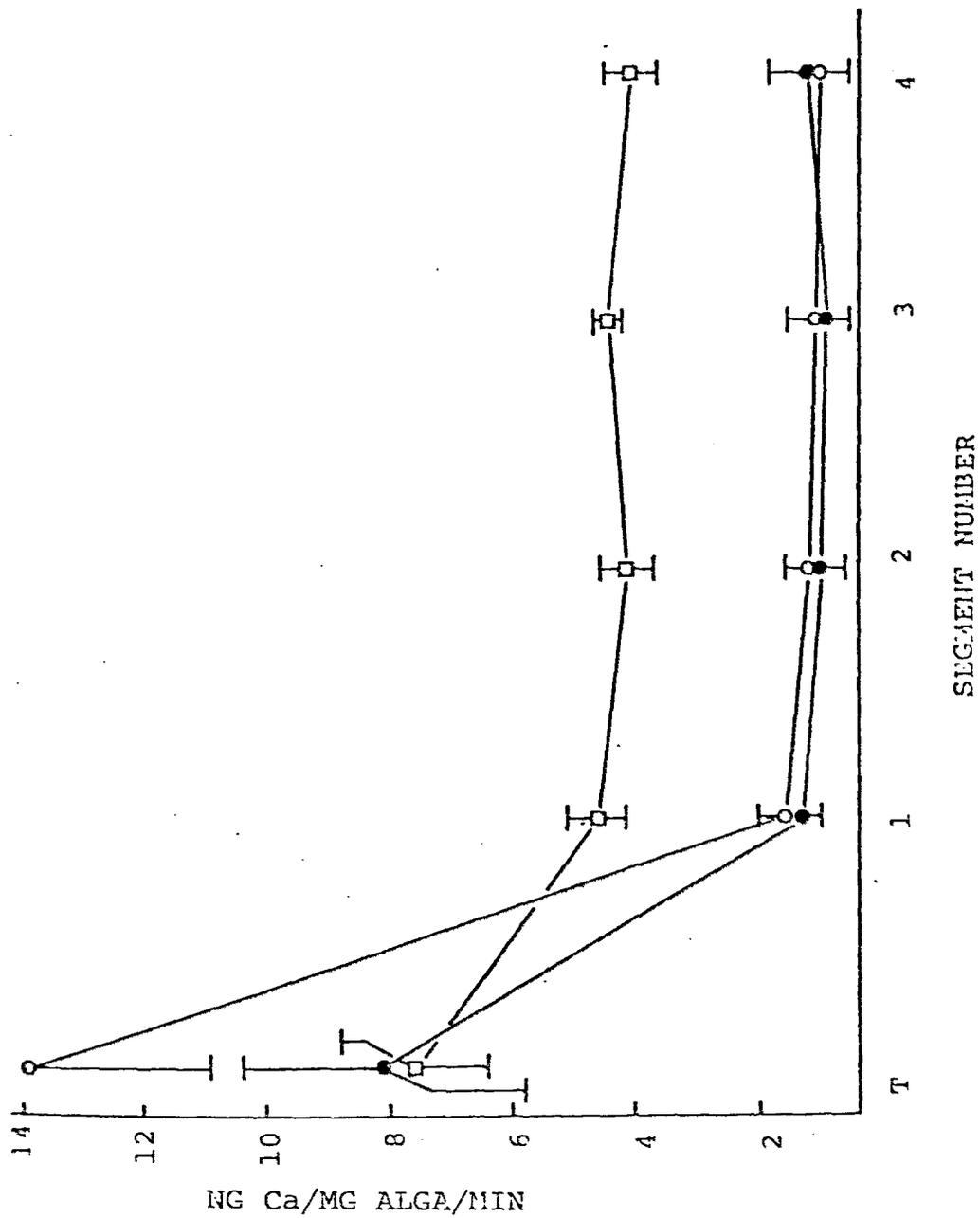


Figure 3-3.

Figure 3-4. Protein-based calcification rates.
Symbols, numbers of determinations and error bars
as in Figure 3-3.

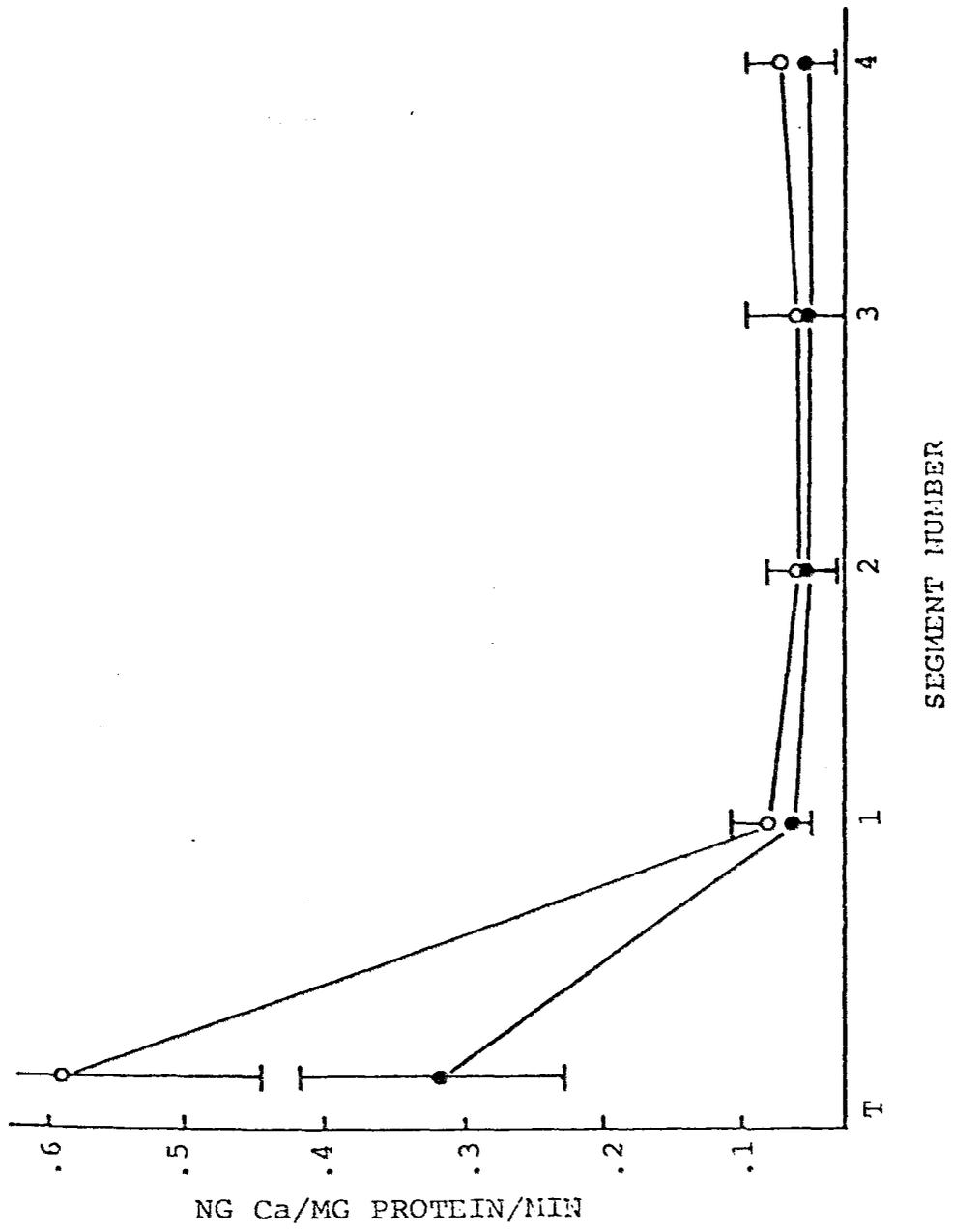


Figure 3-4.

Figure 3-5. Chlorophyll-based calcification rates. Symbols, numbers of determinations and error bars as in Figure 3-3.

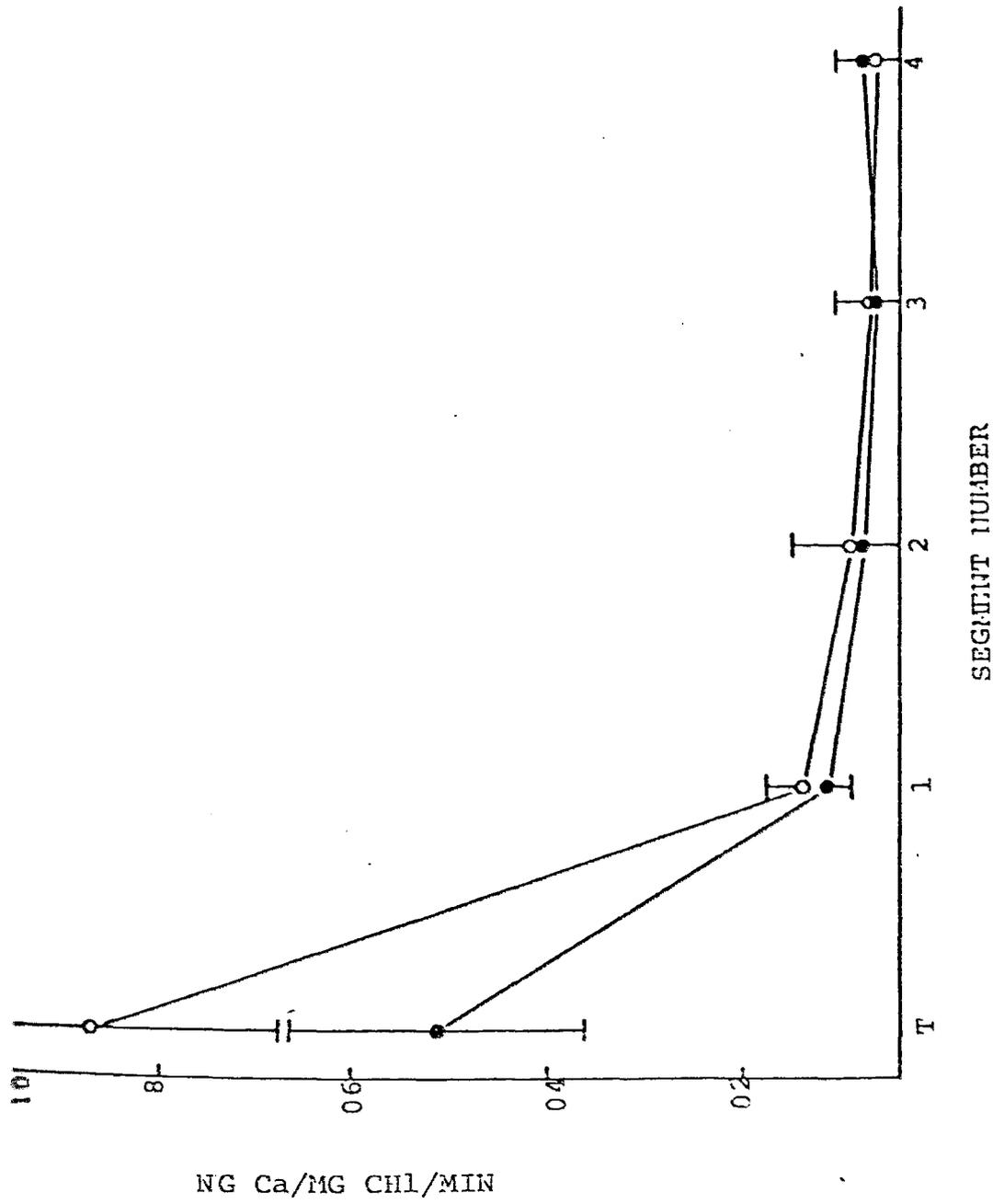


Figure 3-5.

through 4, Figure 3-3) were so similar that pooling of the data was warranted. Subsequent results are reported for the tip and the pooled intergenicula samples only.

The results demonstrate that light-enhancement is significant in the rapidly calcifying tips but apparently absent in the more slowly calcifying intergenicular (Figure 3-3). These findings are in accord with those of Pearse (1972). However, none of these data are adequately controlled. The formalin-killed alga incorporates label into the intergenicula at an unexpectedly high rate. Thus, the killed algae cannot be used as a control for exchange and binding of calcium. The data, then, may be considerably biased by exchange and binding of the calcium label.

C. Calcification Rates by Ca-45

Exchange and binding of calcium might be eliminated to some degree by measurement of calcium-45 uptake over an extended incubation period. In such experiments exchange and binding might be compensated by measuring uptake after an initial equilibration period.

Uptake of calcium-45 by light-incubated, dark-incubated and killed intergenicula is compared over a 12 hour incubation period (Figure 3-6). After an initial period of rapid uptake, the data for the live algae are approximately linear. Regression of a line along this linear portion of the curves produces uptake rates of $1.5 \pm .25$ and 0.9 ± 0.13

Figure 3-6. Ca-45 incorporation into intergenicula of C. tuberculosum. Uptake of calcium is shown for light incubated (open circles), dark-incubated (closed circles) and killed intergenicula (squares). Each point is the mean of 4 to 8 samples from duplicate vessels from 3 separate experiments. Percent standard errors ranged from 0.3 to 12%, with the smallest errors occurring in the killed samples and in the samples late in the incubations.

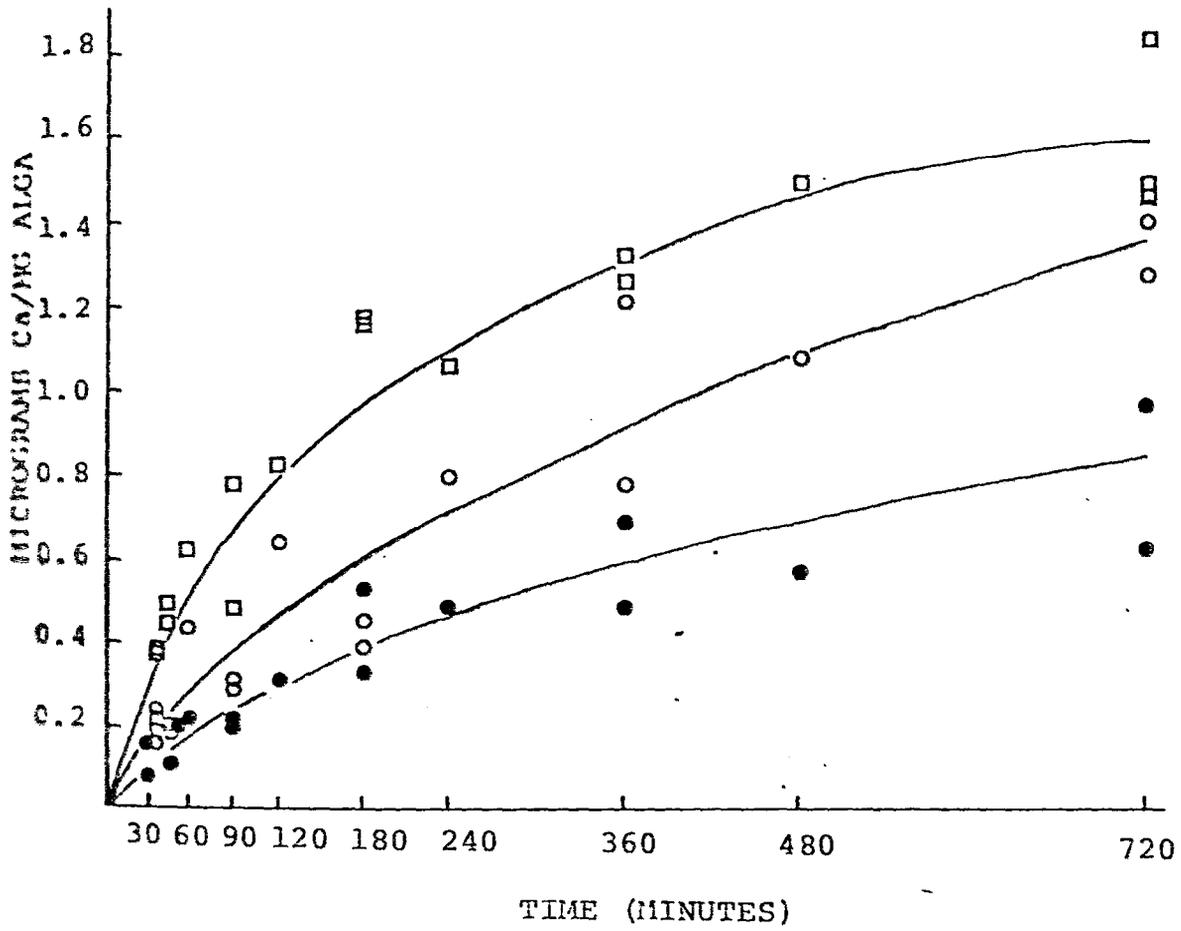


Figure 3-6.

ng Ca mg⁻¹ alga min⁻¹ (+ the standard error) for alga incubated in the light and dark respectively. Similar regression of the data from the tip samples (Figure 3-7) yields rates of 18.6± 4.1 and 6.8± 1.4 ngCa mg⁻¹ alga min⁻¹. (Regressions were run between the arbitrarily chosen times of 60 and 480 minutes.)

The killed control algae showed continued uptake of radiocalcium over the entire incubation period (Figure 3-6). In the intergenicula the killed samples took up more label than did the light-incubated or dark-incubated samples. Again, it is emphasized that the killed alga cannot be used as an exchange control.

D. Calcification Rates by Non-Isotopic Methods

The net accretion of CaCO₃ was measured by two independent methods (Table 3-1). The total alkalinity (T.A.) method makes use of the fact that calcification processes alter the alkalinity of the surrounding seawater in a stoichiometrically predictable fashion (see Smith, 1973). Measurement of calcification related T.A. changes can thus be used to estimate net calcium carbonate accretion. The second method employs the direct wet determination of calcium removal from seawater by means of titration of calcium with EDTA (Pate and Robinson, 1958).

Neither of these methods is sufficiently sensitive to allow the measurement of calcification rates in even

Figure 3-7. Ca-45 incorporation into tip segments of *C. tuberculosis*. Symbols are as in Figure 3-6. Each point is the mean of 4 to 8 samples from duplicate vessels from 3 separate experiments. Percent standard errors ranged from 2 to 18%, with the smallest errors occurring in the killed samples and in the samples late in the incubations.

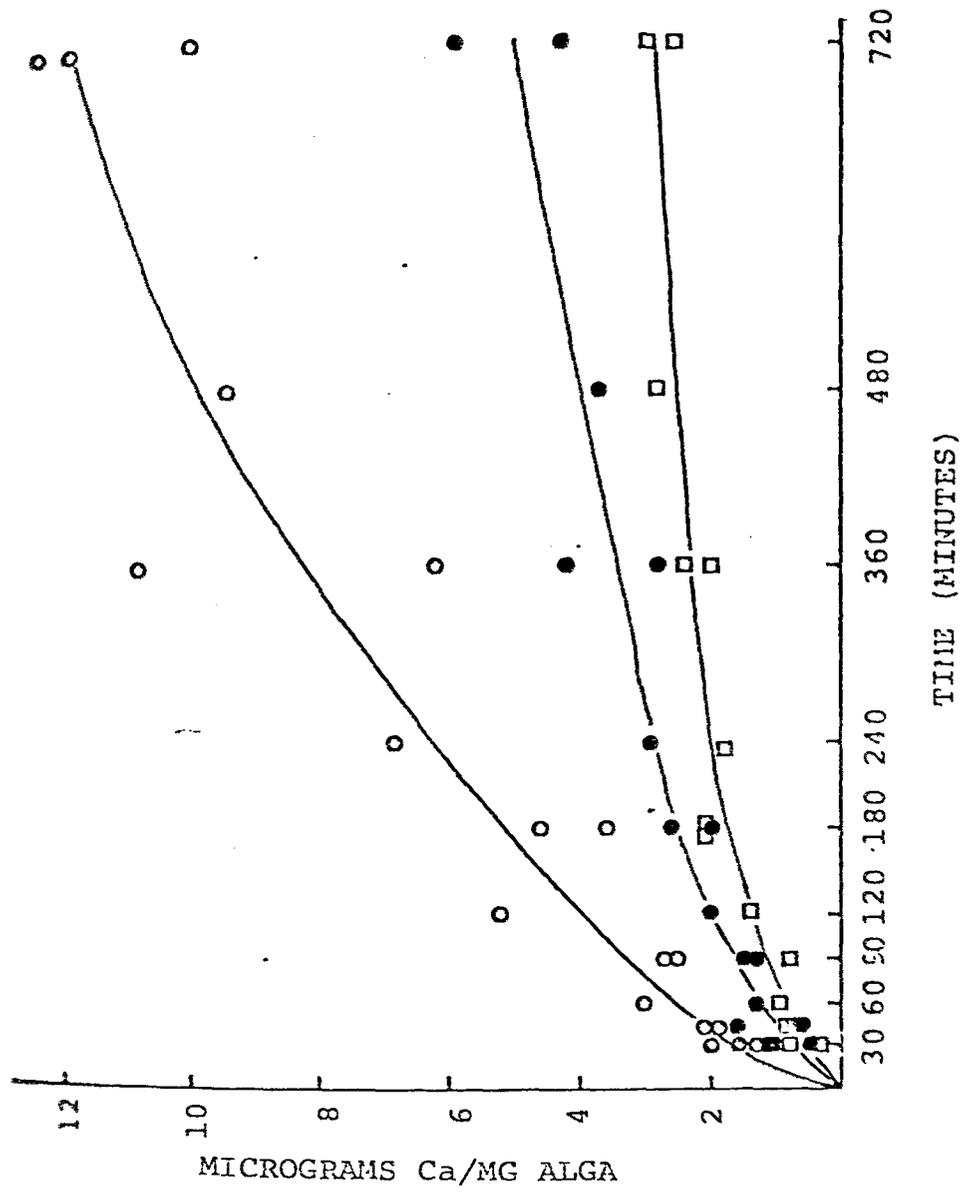


Figure 3-7.

Table 3-1

Net accretion of CaCO_3 determined by the EDTA titration and total alkalinity methods.

Treatment	Net Accretion	Method Used
Light incubation	1.09+-0.56	EDTA
Dark incubation	0.40+-0.08	EDTA
Killed alga, light incubation	0.20+-0.12	EDTA
Light incubation	1.35+-0.33	T.A.
Dark incubation	0.58+-0.25	T.A.
Killed alga, light incubation	0.39+-0.57	T.A.

Net accretion is expressed as nanograms of calcium deposited per milligram of alga per minute. Values are the means of triplicate measurements on 12 samples from two experimental series plus or minus the standard deviation.

hundreds of tips alone. Furthermore, the isolation of so many tips without causing injury is a formidable problem. Thus these direct measures were applied only to whole algal fronds. Since the total tip biomass was always negligible compared with the total mass of the alga in any incubation, the values reported here are comparable to the values obtained from measurements on intergenicula in the isotope experiments.

Both methods produce similar results. The rates from the T.A. experiments were consistently higher than the corresponding values from EDTA titrations, but the overall values were not significantly different. For light-incubated alga, the net accretion rates compare well with the uptake of Ca-45 by the intergenicula, the Ca-45-based rate being 1.11X the T.A.-based rate. The differences are not statistically significant. However, in the dark, the Ca-45-based rates significantly overestimate net accretion. The Ca-45-based rate for dark intergenicula is 1.6X the T.A.-based value and 2.25X the EDTA-based rate. Although a large amount of label is incorporated into the alga in the dark only half represents accretion of CaCO_3 .

The net accretion data from killed controls suggest that the alga may be losing mineral through dissolution. The rates of dissolution obtained, however, do not vary significantly from zero ($p > .1$). The CaCO_3 of the killed alga must be considered to be in equilibrium. Still, the

data from CaCO_3 uptake demonstrate the rapid incorporation of calcium into the killed thallus. This is strong evidence that formalin fixation effectively increases the amount and/or accessibility of the exchangeable algal calcium.

The above results highlight the fact that Ca-45 uptake is unsatisfactory for the determination of calcification rates in the coralline algae.

E. Calcification Rates by $\text{H}^{14}\text{CO}_3^-$

The use of C-14-bicarbonate to measure calcification rates is apparently not subject to the binding errors which affect the Ca-45 method. In addition, free $\text{H}^{14}\text{CO}_3^-$ incorporated into the thallus may be largely removed by freeze-drying (Borowitzka and Larkum, 1976b). This technique could provide a sensitive concurrent measure of both CaCO_3 deposition and photosynthesis.

Bicarbonate-based calcification rates are apparently less affected by exchange and binding than the Ca-45-based rates (Figure 3-8). It is interesting that while Ca-45 incorporation greatly overestimated "calcification" in the killed controls (Figure 3-6), uptake of $\text{H}^{14}\text{CO}_3^-$ into killed sclerogenicula is less than that of live alga. Borowitzka and Larkum (1976b) suggest the use of C-14-bicarbonate in place of Ca-45 as the routine laboratory method for measuring calcification rates.

Figure 3-8. Uptake of $H^{14}CO_3$ into the inorganic carbon fraction. Open symbol tips samples; closed symbols intergenicular samples. Circles are the light-incubated samples, squares the dark-incubated samples and triangles the killed samples. Each point is the mean of 8 determinations on 2 duplicate samples from two separate experiments. Percent standard errors ranged from .2 to 9% for the intergenicular samples and from 2 to 15% for the tip samples. Lines are best fit linear regressions.

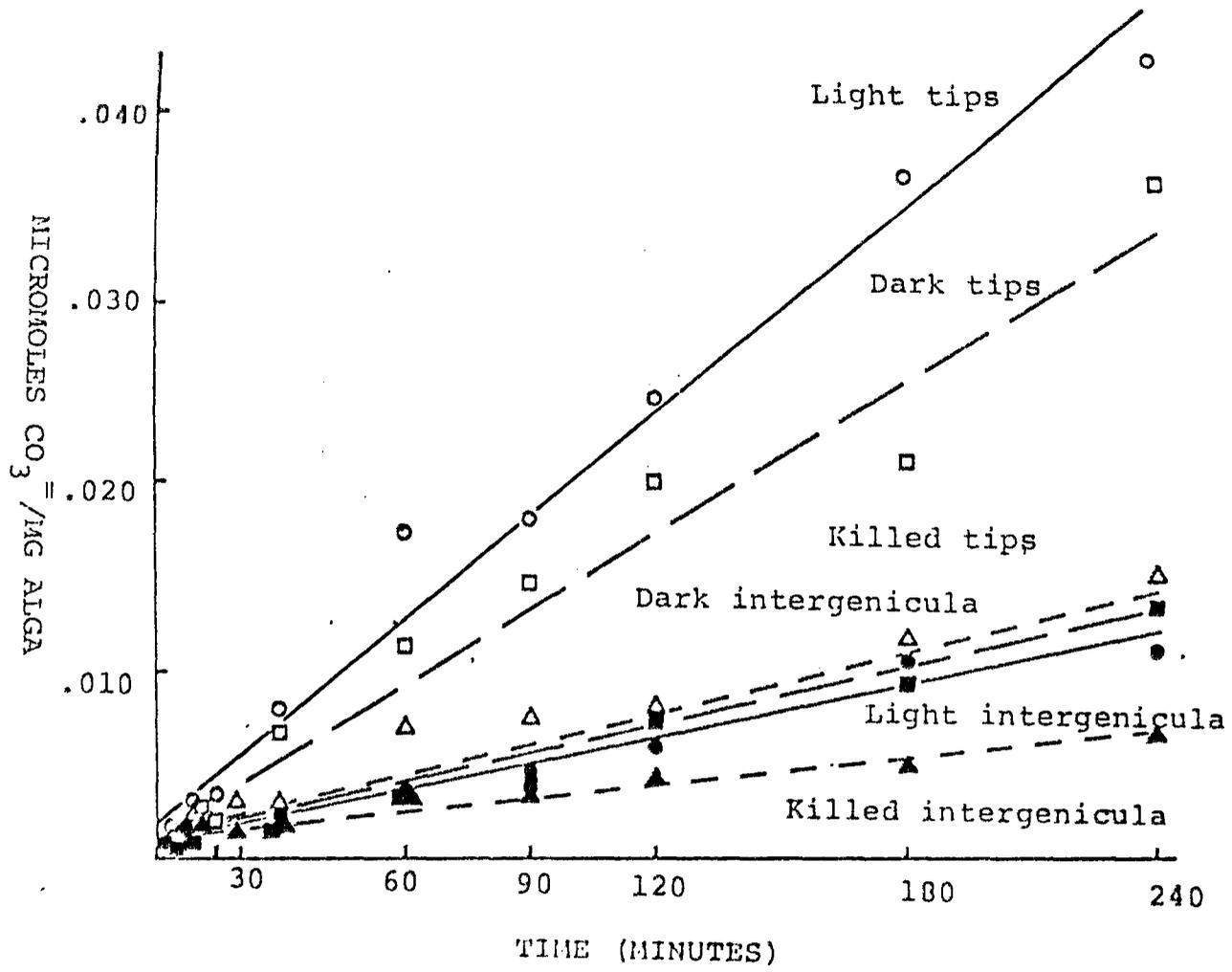


Figure 3-8.

III. Discussion

The chlorophyll:protein ratio for the tip samples (Figure 3-2) appears high considering the white unpigmented appearance of these segments. However, this value is probably exaggerated since: (1) it was impossible to avoid the inclusion of pigmented thallus when separating the tips from adjacent intergenicula and (2) pigmented cells are confined to the outermost cell layers in the Corallinaceae (Lind, 1970) and thus chlorophyll is a function of surface area rather than protein content. Correcting for the latter factor (assuming a spherical tip and a cylindrical intergeniculum) yields a tip chlorophyll:protein ratio about one third that of the first intergenicula. When one considers that most of the tip pigment is concentrated in the portions of pigmented thallus contaminating the tip samples, the chlorophyll content of the white tips becomes even smaller.

As previously supposed (Pearse, 1972), tips are relatively non-photosynthetic. However, no explanation for light-enhancement in these tips can be found in the data presented here. The remote effects of photosynthesis in the white tips are further examined in Chapter 5 of this dissertation.

The white tips contain less mineral calcium than do the intergenicula. Protein:dry weight ratios (Figure 3-2)

decrease between tip and intergenicula samples and percent mineralization increases (Figure 3-1). The density of the intergenicula, however, remains constant in increasingly older segments. Since the intergenicula grow slowly, calcification rates may be limited by the amount of organic growth. Indeed, Lind (1970) has shown that the cell walls in mature thallus are completely impregnated with calcite crystals. The low intergenicular rates are probably due to physical space limitations on crystal growth.

This slow calcification in the intergenicula is intuitively expected to be greatly influenced by exchange phenomena associated with the Ca-45 technique. This intuition is supported by the comparison of rates determined by isotopic and non-isotopic methods (Table 3-2).

For the intergenicula samples, Ca-45-based rates seriously underestimate the light-enhancement effect. Underestimation is the result of a two-fold overestimation of dark calcification rates. Even though the Ca-45-based rates were taken from regression lines designed to compensate for initial exchange and binding of label, a significant error remains in the data from the dark incubations. An explanation lies in an examination of Ca-45 uptake by the killed algae. The exchange of calcium is shown to be a slow process, continuing through the 12 hour incubation period. Since the killed algae are not accreting calcium, this extended exchange period is probably due to the slow

Table 3-2

Calcification rate comparison among the four measurement methods used in this study.

Treatment	Total Alkalinity	EDTA Titration	H ¹⁴ CO ₃ Incorporation	Calcium-45 Incorporation
LIGHT INCUBATIONS				
Tips	--	--	7.40+-0.9	18.60+-4.1
IG	1.35+-0.09	1.09+-0.16	1.96+-0.3	1.50+-0.25
DARK INCUBATIONS				
Tips	--	--	5.60+-0.9	6.80+-1.4
IG	0.60+-0.07	0.40+-0.023	2.13+-0.6	0.90+-0.13
KILLED INCUBATIONS				
Tips	--	--	2.14	2.58
IG	-0.40	-0.20	1.03	--
LIGHT/DARK PATIOS				
Tips	--	--	1.34	2.75+-0.242
IG	2.29+-0.222	2.72+-0.366	0.92	1.67+-0.137

Rates are expressed as ng of calcium deposited/mg alga/min. It was not possible to obtain values for tip calcium deposition using the total alkalinity and EDTA titration techniques. Intergenicula (IG) values using these two methods are actually rates determined by measurement of changes in seawater calcium caused by whole algal fronds. The uptake curve for calcium-45 by killed intergenicula was too non-linear for an estimation of calcium "deposition" rate by linear regression of the points between 1 and 8 hours of incubation.

diffusion of label into the interior of the thallus. A similar effect, on a smaller scale, would be expected in the live algae. If large enough, this exchange could bias uptake results.

It is significant that uptake of inorganic carbon-14 also produced low light:dark ratios. The method proposed by Borowitzka and Larkum (1976b) to replace the Ca-45 technique for calcification measurements must also suffer from exchange errors. The data lead to the conclusion that accurate light:dark ratios cannot be obtained by current isotopic techniques. Future investigators should include a non-isotopic method to control their use of radioisotopes.

The above results should not be considered to diminish the usefulness of radioisotopes in the investigation of calcification processes. Instead, they should serve to define limitations on isotopic techniques and so designate their proper use and facilitate data interpretation. In subsequent chapters the utility of the Ca-45 and C-14-bicarbonate methods is clearly shown.

To explain the differences between isotopic and non-isotopic measurements, the following hypothesis is suggested.

It is likely that Ca-45 will trace "gross" calcification. Gross here is defined as the sum of the uptakes due to exchange and to net accretion. Given a constant exchange

rate, the extent of exchange error in the data will depend on the magnitude of the net accretion rate. Where net accretion greatly exceeds exchange (e.g., in the tips), gross and net calcification should be comparable, especially after a long incubation. This becomes more evident when one considers that label recycled from the mineral phase by exchange is likely to be rapidly redeposited because of the high net accretion rate. In addition, label will be "buried" in the crystals as layers of CaCO_3 are deposited over the crystal surfaces. This "burial" will effectively remove the label from the exchangeable phase.

Where calcification rates are low and of the same order as the exchange rates, a significant bias would be introduced. It is suggested that net accretion rates of the intergenacula are of this order. Rates measured in the light are probably exaggerated by exchange when Ca-45 is used but only to a small extent. In the light, differences between isotopic and non-isotopic methods are insignificant. However, in the dark, Ca-45 uptake produces a two-fold overestimation of net accretion. The error in the dark accounts for the low light:dark ratios (L/D ratios) for calcification rates reported by previous workers using Ca-45 (i.e., Goreau, 1963; Pearse, 1972).

The underestimation of L/D has obscured an important characteristic of the light-enhancement phenomena. In red alga, light-enhancement processes influence calcification

rates of the intergenicula to the same extent as the growing tips. The report by Pearse (1972) that light-enhancement becomes undetectable in older intergenicula is erroneous and due to the bias of exchange on the low calcification rates of mature segments.

The L/D ratios also clearly show that algae are calcifying in the dark. Contrary to the suggestion by Borowitzka and Larkum (1976b, 1976c) on Halimeda, dark calcification cannot be accounted for by exchange and binding alone. One must explain the basic calcification mechanism as well as the light-enhancement of this mechanism. Stated in these terms, dark calcification and light-enhancement are separated into two interrelated processes. The most obvious and simple explanation for light-enhancement of any basic calcification process is the precipitation hypothesis (Blinks, 1951; see Chapter 1, Introduction). This hypothesis is pleasing since it implies that the alga is able to use the ionic changes naturally induced by photosynthesis to augment the calcification of its cell walls. The finding that light-enhancement occurs throughout the algal thallus supports this hypothesis. In the next chapter support for simple precipitation is extended in experiments on the washout of Ca-45 from the algal thallus.

A major argument against the precipitation hypothesis is the remote effect of photosynthesis on calcification

in the non-photosynthetic tips. This problem is further addressed in Chapter 5.

CHAPTER 4

CALCIUM METABOLISM II. WASHOUT, FLUXES AND
COMPARTMENTALIZATION OF CALCIUM

Calcium-45 taken up by the thallus of the green calcareous alga, Halimeda, is retained to a greater extent during a wash incubation in the light than in the dark (Stark et al., 1969). These authors interpreted the differential washout of Ca-45 as indicative of a two-step calcification process whereby calcium is first bound or otherwise sequestered in the alga and subsequently deposited, in a light-enhanced step, as CaCO_3 . Similar washout phenomena have been observed in the coralline alga, Bossiella orbigniana (Pearse, 1972). Although the data are consistent with a two-step calcification mechanism, the latter author suggests that alternative processes are equally likely. For example, "The organic and mineral phases may continue to exchange calcium until, in the light, photosynthetically produced specific organic substances are laid down in the walls, inhibiting exchange and/or binding additional calcium" (Pearse, 1972, page 95). Other alternatives can be imagined but information on which to base the choice for

the actual process is not available.

In Chapter 3 it was shown that exchange and binding of calcium-45 produce a distorted view of calcification unless net accretion rates measured by non-isotopic methods are employed. In view of this finding, it is recognized that washout data, such as those described above, may require alternative explanation in terms of exchange and binding of calcium label.

In this chapter the washout of calcium-45 from algal thallus is reexamined. The research was designed to answer two questions. First, the study sought to test the relationships between calcium uptake and net accretion proposed in Chapter 3; are these relationships supported by data on calcium fluxes, calcium pools and label retention? Second, the study examined the hypotheses of Stark et al. (1969) and Pearse (1972); are there specific calcium pools which play a fundamental role in the calcification process or can washout of calcium be explained in terms of exchange at the crystal surfaces?

I. Materials and Methods

A. Calcium Labelling

Algae were collected and maintained in aquaria as described in Chapter 3. For labelling with Ca-45, algae were incubated in small glass aquaria or 2000 ml beakers.

Up to 20 grams (dry weight) of algae were incubated for 18-20 hours in 1500 ml of seawater containing 0.01 to 0.05 microcuries/ml. Aquaria were aerated with an air stone and covered to retard evaporation. At the end of the labeling period seawater and algal samples were taken and counted as described in Chapter 2. The remaining alga was rinsed quickly 5 times (45 seconds total rinse time) with cold (10° C) unlabelled seawater. Algae were placed in large (350 ml) wash vessels in either the light or dark. Seawater and algal samples were taken at intervals for scintillation counting. All incubations were carried out at 11° C in either a cold box or cold room. Light was supplied by banks of cool white fluorescent tubes supplying about 1000 foot-candles to the surfaces of the incubation media.

B. Curve Analysis

To analyze the data from washout experiments, curves were fitted to exponential expressions using the computer program, DISCRETE, written and supplied by Dr. S. W. Provencher (for references see Provencher, 1976a, 1976b). This program provides an extremely powerful alternative to traditional graphical and iterative methods for analyzing the sums of exponential expressions. The advantages of the approach are two-fold.

First and most importantly, no initial estimates for pool sizes, rate constants, or the number of components are needed. Such estimates are almost universally arrived at "by eye" (see Branson, 1961) and may be unintentionally biased by the previously established expectations of the investigator. In this way, it is possible to "choose" to some extent the number of components and arrive at values which appear to fit the data but actually misrepresent the true situation. This human bias is likely to be extremely misleading in the case of exponentials since the severe nonorthogonality of these expressions can allow a grossly incorrect solution to reproduce the data well enough to be accepted (Laiken and Printz, 1970; Lanczos, 1956; Isenberg et al., 1973). Since only the raw data are input for DISCRETE, no personal bias may enter the calculations and the danger of accepting an incorrect solution is greatly reduced.

The second important feature of DISCRETE is that it uses a non-linear least squares approximation to fit the curves. Thus, standard statistical parameters associated with least squares analysis may be modified to document the "goodness of fit" of the curves to the data. DISCRETE, then, not only fits the data to curves with from 1 to 9 exponential components, but also generates statistical parameters on which to base the choice of the expression which best fits the data.

A detailed description of the methods employed in the curve fitting and estimations of goodness of fit are provided in the papers of Provencher. However, a little should be said about the choice of the number of exponential components. DISCRETE selects the best solution by computing a value $P_{NG}(K:J)$. This is defined as the probability that the solution where $NLAM = J$ is better than the solution where $NLAM = K$. The test for P_{NG} is defined so that the burden is placed on the solution with larger $NLAM$ to pass an F test that has been corrected for non-linearity at that solution using Beale's non-linearity parameter, NPFI (Beale, 1960. The best solution is that for which P_{NG} is > 0.5 when tested against all other solutions. Normal criteria for acceptance of a solution are applied so that for any value of $P_{NG}(K:J) < 0.95$ the solution with $NLAM = K$ is considered a significant possibility.

II. Results and Discussion

A. Stability of Calcium Labelling

Label washed out of the thallus during incubation in unlabelled seawater is "unstable." The term, stability, is defined as the percent retention of the initial Ca-45 activity in the alga (Stark et al., 1969; Pearse, 1972; Borowitzka and Larkum, 1976b). By the above expression calcium is most stable in the light (66% retention), less

Figure 4-1. Washout of Ca-45 from C. tuberculosum. Values are presented as the log of the specific activity of the alga (normalized by expression as micrograms calcium/mg alga) vs. time. The open circles are the light values, the closed circles the dark values and the squares the killed values. Note the different scales for the light data and for the dark and killed data. Each point is the mean from 8-12 determinations in 3 experimental series normalized for differences in initial specific activities. Percent standard errors ranged from 3-14 with the highest variability associated with measurement early in the incubation.

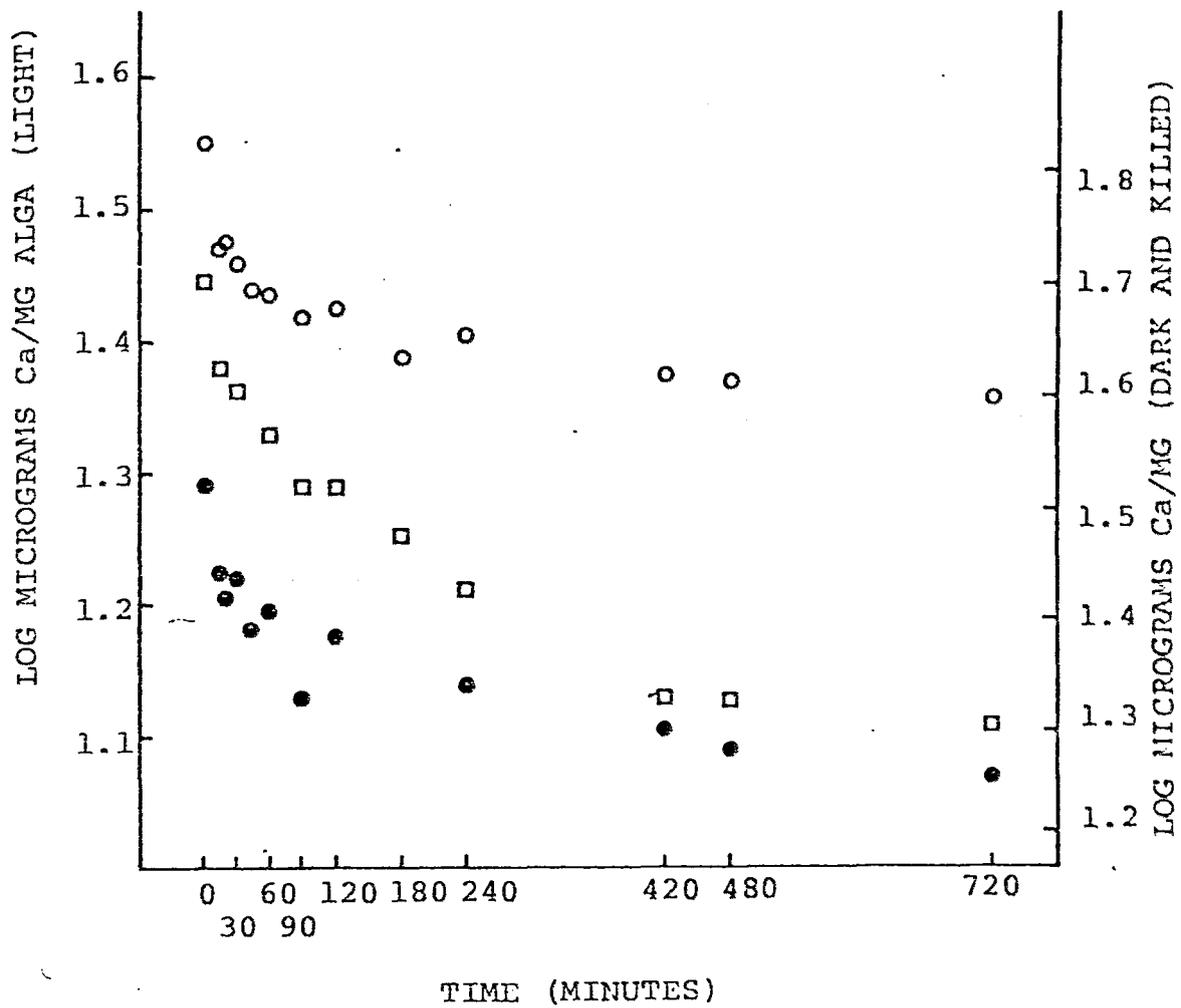


Figure 4-1.

stable in the dark (55%) and least stable in the killed controls (39%) after a 12 hour wash in "cold" seawater (Figure 4-1).

These data provide no new information on algal calcification, but serve to illustrate a key problem limiting the interpretation of washout data. It is not possible, in the experiments above, to determine the origin of the unstable calcium-45. If calcium is being bound and/or sequestered prior to light-stimulated deposition (Stark et al., 1969), then the unstable calcium will not originate from the exchangeable mineral phase. On the other hand, if specific photosynthetic compounds are inhibiting exchange (Pearse, 1972), then exchange at the mineral surfaces would be a primary source of unstable label. A third hypothesis, that Ca-45 exchange can be explained by recycling and burial of label (Chapter 3) suggests no particular source for unstable calcium.

To determine the origin of the unstable Ca-45 activity, the amount of label removed during a typical incubation in "cold" seawater was compared with that removed by chelation with 2% EDTA at pH8 (Figure 4-2). The amount of label lost to the EDTA solution compares to that released to seawater in the light (Table 4-1), but is much greater than that removed by the water washing.

Chelation of calcium by EDTA removes only the label at or near the thallus surface. It is unlikely that the

Figure 4-2. The experimental flow chart for the EDTA washing of alga during the washout of Ca-45. The experiment is described in the text. Separate study showed that the seawater washing between the loading and wash incubations removed the same amount of label, within 15%, as the H₂O washing in (1).

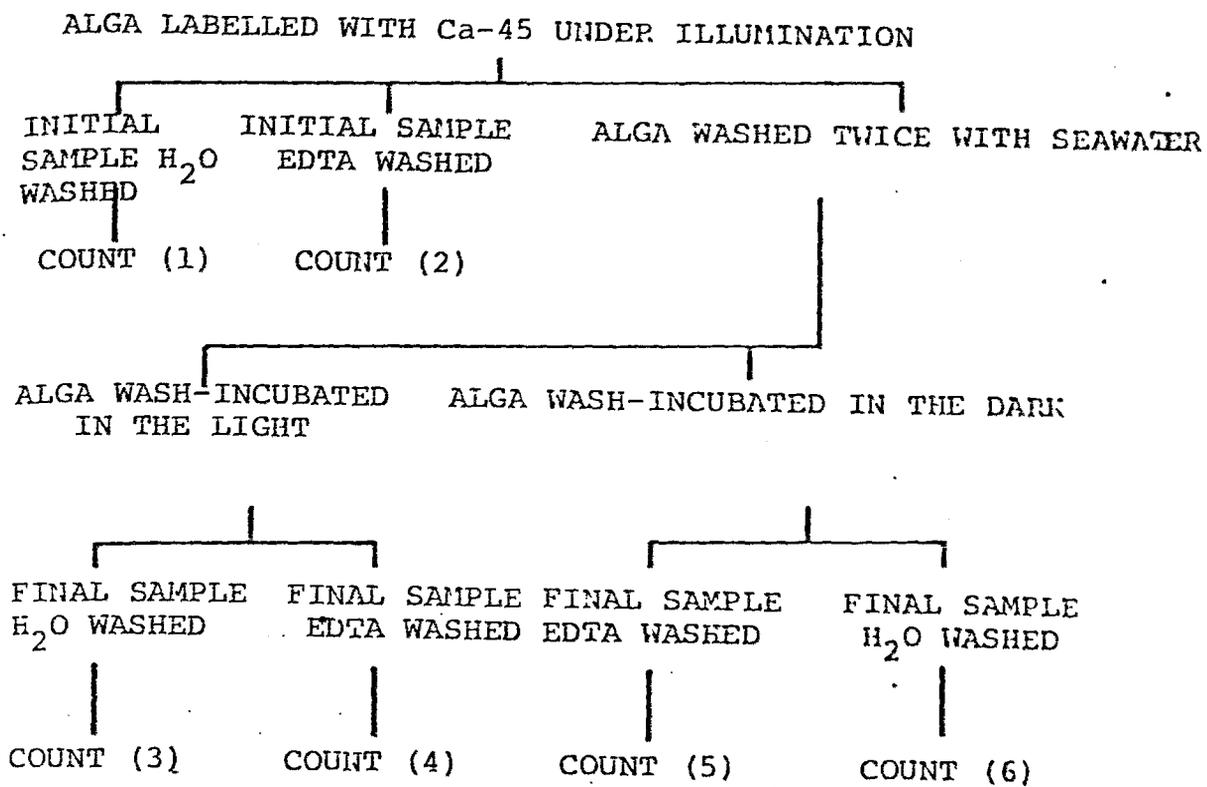


Figure 4-2.

Table 4-1

The effects of EDTA chelation on the washout of calcium-45.

S.#	Sample	CPM/mg alga	% ret.
INTERGENICULAR SAMPLES			
1	EDTA washed, initial activity	333+-63	--
2	H ₂ O washed, initial activity	1079+-78	--
3	EDTA washed, light incub.	304+- 9	91
4	H ₂ O washed, light incub.	376+-59	35
5	EDTA washed, dark incub.	262+-17	77
6	H ₂ O washed, dark incub.	297+-11	27.5
TIP SAMPLES			
1	EDTA washed, initial activity	773+-184	--
2	H ₂ O washed, initial activity	2358+-998	--
3	EDTA washed, light incub.	790+-280	102
4	H ₂ O washed, light incub.	969+-294	41
5	EDTA washed, dark incub.	542+- 26	70
6	H ₂ O washed, dark incub.	628+- 37	27
KILLED CONTROL SAMPLES			
1	EDTA washed, IG initial activity	25.1+-2.7	--
2	H ₂ O washed, IG initial activity	37.8+-2.1	--
1	EDTA washed, tip initial activity	66.6+-11.7	--
2	H ₂ O washed, tip initial activity	89.1+-16.1	--
3	EDTA washed, IG final activity	5.0+-0.3	20
4	H ₂ O washed, IG final activity	5.0+-0.1	17.8
3	EDTA washed, tip final activity	9.9+-0.8	14.8
4	H ₂ O washed, tip final activity	8.7+-3.0	9.8

Samples are identified by the number assigned in Figure 4-3 and by the treatment used to remove label (i.e., whether washed with water or an EDTA solution). Initial samples refer to those taken immediately following the loading incubation (incubation with Ca-45 in the light). Final samples are those taken after the wash incubation (killed controls). For live algae, the condition of the wash incubation (i.e., whether in the light or the dark) is indicated. Specific activities for live algae are the means of 8 samples from 2 experimental series. For the killed controls, activities are the means of 8 samples from a duplicate series in one experiment. Activities are reported plus or minus the standard deviation. IG refers to intergenicular samples.

EDTA solution could penetrate deeply into the thallus in 30 seconds. Furthermore, subsequent 30 second washes remove much less label than the first (Figure 4-3). Much of the unstable Ca-45 is probably superficial and bound or adsorbed on or near the thallus surface. These data do not support the hypothesis that specific photosynthetic compounds inhibit mineral exchange (Pearse, 1972); the main source of unstable calcium is not the calcite crystals. Calcite crystals are not found at the thallus surface (Bailey and Bisalputra, 1970; Lind, 1970). One should also note, in connection with this hypothesis, that many organic compounds, especially phosphates, effectively coat CaCO_3 crystals. The production of specific compounds during photosynthesis uniquely suited for this purpose seems unlikely. More importantly, organic compounds which coat crystal surfaces act as crystal poisons, inhibiting not only exchange but also deposition of mineral. Such inhibition conflicts with photosynthetic enhancement of calcification rates. (A discussion of crystal poisons is found in Simkiss, 1964.)

The removal of calcium by EDTA chelation does not eliminate the differential washout of calcium-45 from algae in the light and dark (compare numbers 3 and 5 and 4 and 6, Table 4-1). Since the EDTA solution has removed a great deal of non-mineral label, it can be assumed that some major fraction of the remaining Ca-45 is in the form of CaCO_3 .

Figure 4-3. Percent of total label removed by consecutive 30 second washes in 2% EDTA, pH 8.0. Line represents the cumulative removal of label. Each value is the mean of 8 samples of 10 to 20 segments (intergenicula). Vertical bars are one standard deviation of the mean.

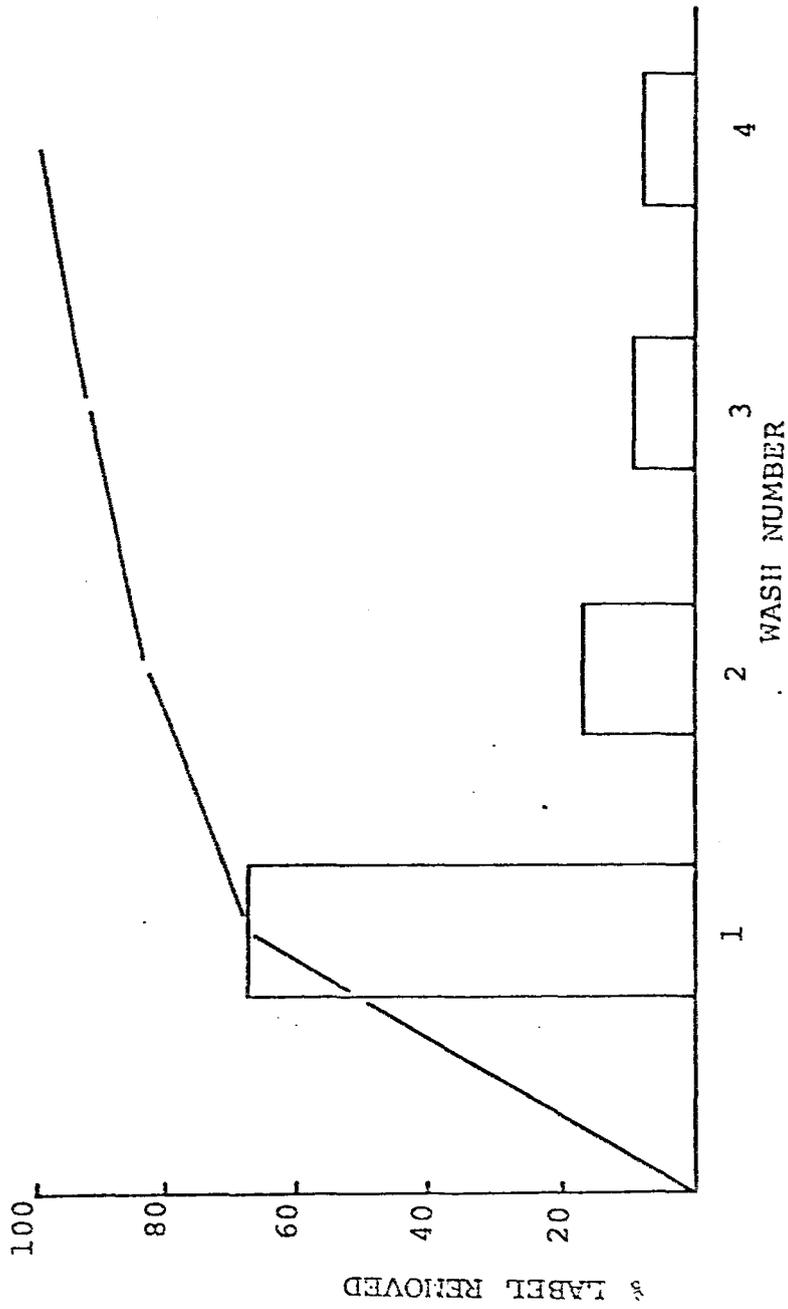


Figure 4-3.

This suggests that recycling and burial of label (Chapter 3) might be used to explain differential washout phenomena. The instability of the system may be the result of a constant exchange rate of soluble calcium-45 (= bound and free calcium ions) and a differential exchange of mineral calcium-45. In the light, mineral exchange of Ca-45 will be slow due to the rapid recycling and burial of label resulting from the faster calcification rate. In the dark, recycling and burial proceed more slowly allowing more label to diffuse into the seawater. Support for the suggestions that live algae are burying and, hence, "stabilizing" label is seen in the data from the killed algae (Table 4-1). These algae are not accreting CaCO_3 (Chapter 3). Hence, label should not be buried. The high instability of the label in the killed alga shows that nearly all of the calcium label remains in the exchangeable pool.

The above hypothesis suggests that "unstable" Ca-45 activity may originate from both the mineral and soluble calcium phases. The data are consistent with this view, but such exchange makes it difficult to tell if specific pooling of Ca occurs. Though it seems unlikely that the calcium bound on or near the thallus surface is being specifically sequestered, a small pool of calcium sequestered prior to deposition could be masked by the washout of calcium from larger pools (i.e., the mineral, the superficially bound calcium, etc.). In order to examine

more critically the pooling of calcium in the algal system, a compartmental analysis was run on the uptake and washout data.

B. Compartmental Analysis

The DISCRETE analyses of the data from uptake experiments (Figure 4-4) show that the decreases in seawater calcium are logarithmic and can be expressed by single exponential terms (Table 4-2). The rate constants (LAMBDA's) for the uptake expressions increase from algae incubated in the light to those incubated in the dark to the killed controls. Recalculated as half-lives (Table 4-2), these rate constants demonstrate that the live algae continue to take up calcium after the killed algae reach equilibrium. This can be partially explained by the high influx rate for the killed algae as compared to the live plants. However, rate constants must also be influenced by net accretion of calcium in both light and dark-incubated algae (Chapter 3). The influence of net CaCO_3 deposition on rate constants and pool sizes has not been discussed in the literature. Clearly, interpretations of kinetic data cannot follow the example set by Ca-45 studies of vertebrate bone, which is in equilibrium with the body fluids (Branson, 1961; Atkins, 1969). Instead, pool sizes and rate constants must be considered the total effect of both an exchange equilibrium and a constant removal of calcium label.

Figure 4-4. Removal of calcium-45 activity from seawater by C. tuberculosum during calcium uptake experiments. Values are presented as the log of the specific activity of seawater (normalized by expression as micrograms calcium/mg alga/ml of seawater). The open circles are the light values, the closed circles the dark values and the squares the killed values. Each point is the mean from 10 to 14 determinations in 3 experimental series normalized for differences in initial specific activities and amounts of alga. Error in the data ranged from 3 to 15% (standard error) with the most variable data being found in the values from early in the incubation.

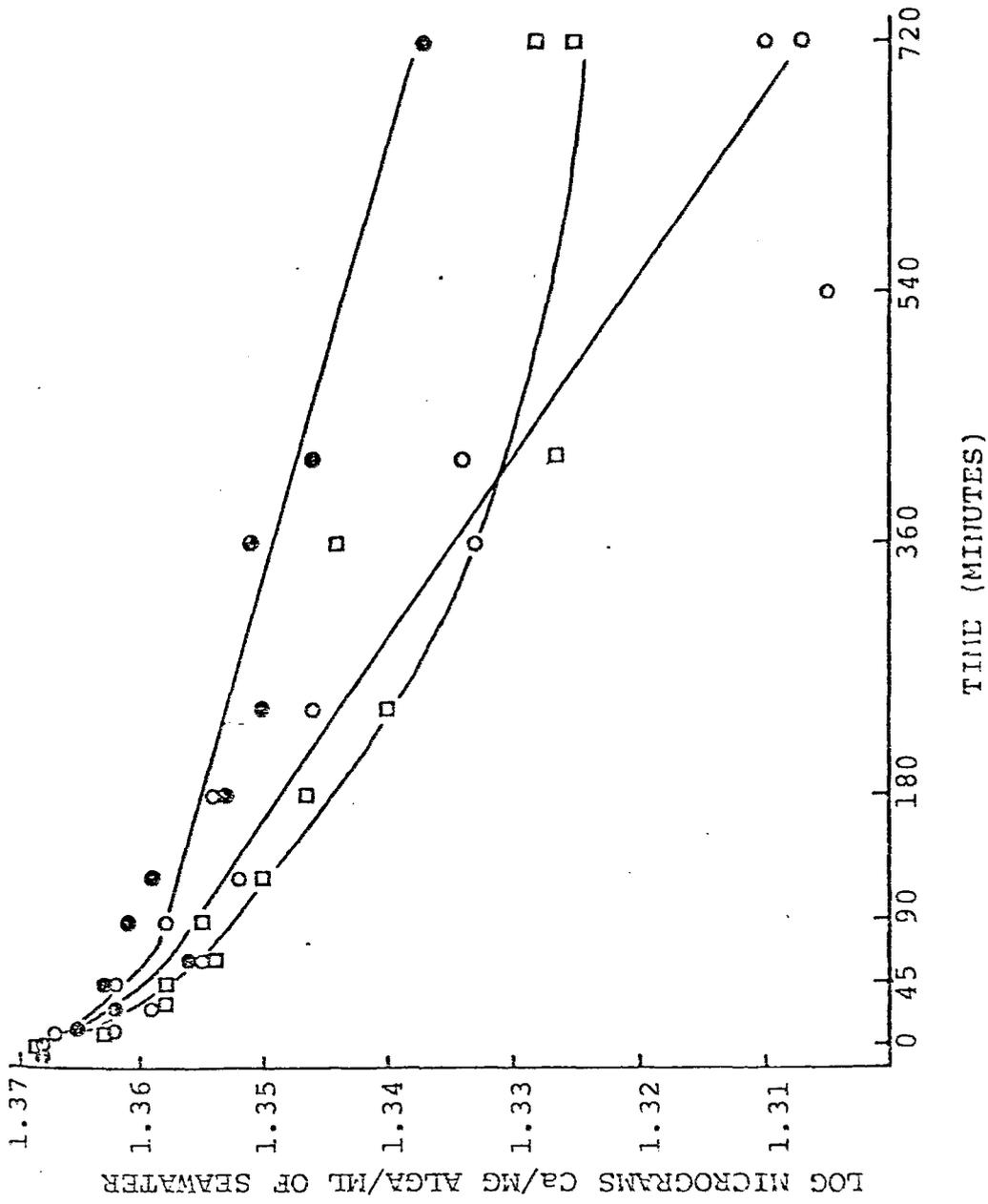


Figure 4-4.

Uptake data are inadequate for in-depth analyses since binding and recycling of tracer bias results. In addition, uptake is poorly documented due to limitations of technique. Data taken between 0 and 60 minutes of incubation are not accurate enough to allow a rapidly exchanging calcium pool to be detected. Fortunately, washout data (Figure 4-1) are not subject either to recycling (since the seawater calcium pool is large compared to the algal pool) or high variability. Such data are expected to provide a more detailed description of calcium exchange in the algal system.

Two-component expressions were returned by DISCRETE for all three washout data sets (Table 4-2). The results thus suggest two exchanging calcium pools within the algal system. It is difficult, however, to assign potential exchangeable pools in the alga to the kinetically distinguished compartments. A long-lived component in Halimeda might be due to slow exchange of calcium bound to the cell walls (Borowitzka and Larkum, 1976b). Yet, in the Corallinaceae, the cell wall serves to nucleate and orient growing calcite crystals (Lind, 1970). The cell wall fibers are thus buried in the mineral and presumably cannot participate in exchange reactions. An alternative explanation for the "slow" component is the gradual diffusion and release of calcium from the thallus interior. Prolonged calcium uptake in killed algae appears to be the result of

Table 4-2

Analyses of calcium-45 time-series data by the computer program DISCRETE.

Treatment	Alpha ₁	Lambda ₁	Alpha ₂	Lambda ₂	Half-time ₁ (min)	Half-time ₂ (min)	Influx	Efflux	P _{NG}	Constant Term
Light uptake	4.527 (3.2)	.000789 (.00029)			878		.0265 (.0122)		0.969	12.85 (3.2)
Dark uptake	.9386 (.16)	.003052 (.0013)			227		.0198 (.009)		1.000	15.93 (.17)
Killed uptake	1.845 (.20)	.005762 (.0017)			120		.0621 (.014)		0.907	21.38 (.18)
Light washout	.02233 (.0021)	.004915 (.0015)	.02342 (.0028)	.1302 (.050)	141	5.3		.0122 (.003)	1.000	.08438 (.0018)
Dark washout	.04027 (.130)	.000799 (.0037)	.07005 (.0076)	.04455 (.009)	867	15.6		.01835 (.0049)	0.977	.04469 (.130)
Killed washout	.06255 (.0043)	.003341 (.00079)	.04046 (.0053)	.1206 (.046)	207	5.8		.01575 (.0027)	1.000	.07169 (.0055)

Alpha's are pool sizes and Lambda's are the rate constants. The P_{NG} given is the probability that the solution reported is really better than the second best solution. Influx and efflux data were not calculated by the computer program, but were extrapolated from log-log plots of calcium-45 uptake or washout vs. time (see text). Half-times for components were calculated from the expression, half-time = .693147/Alpha. Numbers in parentheses are standard errors.

diffusion of calcium deep into the plant (Chapter 3). The reverse phenomenon would be expected in washout experiments. Other possible calcium pools include: (1) calcium bound on the thallus surface, (2) cytoplasmic calcium, (3) calcium in organelles, (4) calcium bound to anionic molecules such as mucopolysaccharides (Bohm and Goreau, 1973), and (5) calcium in the exchangeable phase of the calcite. At present these possibilities cannot be separated.

Whatever the identities of the kinetic components, certain trends in their pool sizes and rate constants are evident. The calcium pools of algae incubated in the light appear smaller and more rapidly exchanging than are those of the dark-incubated algae. Since both light- and dark-incubated plants were loaded with Ca-45 in the same vessel, exchangeable Ca-45 pools in both plants were initially the same. Since there is net uptake of calcium from seawater in both the light and the dark (Chapter 3), it is unlikely that the sizes of the Ca-45 pools increased during the washout period. In addition, since flux rates for calcium in light- and dark-incubated algae are not significantly different (Table 4-2), changes in permeability and/or mediated transport of calcium cannot be used to explain the differences in rate constants.

An explanation again lies in the concept of recycling and burial of label. Pool size, as measured in washout experiments, depends upon label released to the seawater.

Label which is removed from the exchangeable phase by other means (e.g., recycling and burial in the CaCO_3 crystals) cannot be detected. Because algae incubated in the light are removing Ca-45 rapidly and undetectably by recycling and burial, pool sizes in the light should appear to be smaller and to equilibrate more quickly. In the dark, net accretion removes label from the exchangeable phase more slowly and smaller rate constants result. Furthermore, exchangeable calcium label remains "unstabilized" (i.e., not buried in the mineral) longer, increasing the likelihood that it will be exchanged to seawater. Thus pool sizes in the dark appear larger than those in the light.

Since the washout of calcium-45 from the algal thallus is accounted for on the basis of the above physical-chemical exchange phenomena, it is not necessary to suggest the two-step calcification mechanism (Stark et al., 1969). Rather, both kinetically distinguishable pools are influenced by net accretion of CaCO_3 (Table 4-2). Instead of a pool of calcium sequestered specifically for a light-enhanced calcification reaction, calcium is apparently supplied to the CaCO_3 from both kinetic pools. This view is consistent with the data on calcium flux rates (Table 4-2). When compared with net accretion rates (Chapter 3), intergenicular flux rates are very large, the flux-accretion ratios being 25 and 40 in the light and dark respectively. This suggests that calcium diffusion is not limiting to

CaCO_3 deposition. Thus, the supply of calcium at seawater concentration is sufficient and no specially sequestered pool of easily available calcium is necessary. However, it is possible that calcium is being actively transported and that this transport produces a calcium pool at high concentration. Such a mechanism could be associated with the vesicles which have been shown to fuse with plasmalemma and open to the cell wall in Corallina (Bailey and Bisalputra, 1970). If these vesicles contained high calcium concentrations, their emptying into the cell wall space could enhance calcification by decreasing the solubility product of CaCO_3 at the sites of mineral deposition. If the fusion of vesicles with the plasmalemma or active calcium transport was light-stimulated, the result would be a mechanism such as that proposed by Stark et al. (1969). The data here do not allow the elimination of the above possibility. The problem is investigated in Chapter 5.

It must be noted that there are problems with the compartmental analyses. These are primarily in connection with the data from algae incubated in the dark. The DISCRETE program found no exact fit to the transforms (see Provencher, 1976). Thus the dark washout analysis is statistically poorly defined. This fact hampers quantitative evaluation of the data. Another problem is the unexpectedly low value for calcium efflux from the killed alga. This low figure is in conflict with the previous findings that

the killed algae release more label to seawater than do live algae over a comparable period of time. Very precise analyses are extremely difficult to obtain with current techniques, but such analyses are required before the kinetics of calcium uptake and washout in the coralline algae are fully interpreted.

A final problem is that the interpretation of calcium exchange itself is complicated by at least two major factors: (1) changes in pool sizes and rate constants caused by net accretion of calcium, and (2) the large number of potential calcium pools within the algal system. These problems have not always been addressed by recent investigators. The work of Bohm and Goreau (1973) and Borowitzka and Larkum (1976b) should be reexamined at least to the degree that some statistical information should be supplied to support the kinetic expressions reported.

III. Summary

Much of the calcium-45 activity lost to seawater during a typical washout experiment with labelled algae can be attributed to Ca-45 present at or near the thallus surface. Yet, non-surface label continues to show differential washout in the light and dark. A comparison of calcium fluxes shows that calcium is equally mobile across the algal thallus regardless of the light conditions. This suggests that differential washout is due to differences in

the exchange of mineral calcium. These differences can be accounted for by recycling and burial of label.

Refined analyses of calcium washout show that calcium exchange in C. tuberculosum involves at least two kinetically distinct calcium pools. The apparent size and turnover rates of these pools vary among light-incubated, dark-incubated and killed algae. These data are consistent with the recycling and burial of label from both of the exchangeable calcium pools. Alternative hypotheses (Stark et al., 1969; Pearse, 1972) for the mechanisms of calcium-45 washout are not supported. However, because active processes are not eliminated by the data presented, a "two-step" calcification mechanism cannot be totally excluded. Active processes are further investigated in Chapter 5.

CHAPTER 5

TRANSLOCATION OF CALCIUM, BICARBONATE
AND ORGANIC COMPOUNDS AND THE ENERGY
REQUIREMENTS OF CALCIFICATION

Previous study of the articulated coralline algae has shown that light-enhancement of calcification rates occurs in the apical tip segments (Pearse, 1972; Chapter 3). These tips are white and contain little photosynthetic pigment (Chapter 3). This lack of pigment together with the demonstration of light-enhancement indicates that photosynthesis influences calcification at sites remote from the chloroplasts. This fact has been used as an argument against the precipitation hypothesis. This hypothesis (Blinks, 1951) suggests that the ionic changes surrounding photosynthesis are responsible for the light-enhancement effect. Since the tips are non-photosynthetic, the precipitation model predicts that light-enhancement should not be observed.

To explain light-enhancement in the apical tips, it has been suggested that materials are translocated from the pigmented thallus to the tips (Pease, 1972). In one speculative mechanism high energy phosphates or cell wall/matrix precursors are translocated. In the light the abundant supply of these materials stimulates calcification. In the

dark a smaller supply limits CaCO_3 deposition (Pearse, 1972). This type of mechanism implies that calcification is directly dependent upon energy input. Such energy input to the calcification process, in the form of active calcium transport, has been reported in Acropora acuminata (Chalker, 1975). Thus, there is a precedent for an energy requiring calcification process in the hermatypic corals. (For a discussion of the parallels between coral and coralline algal calcification processes see Chapter 1.)

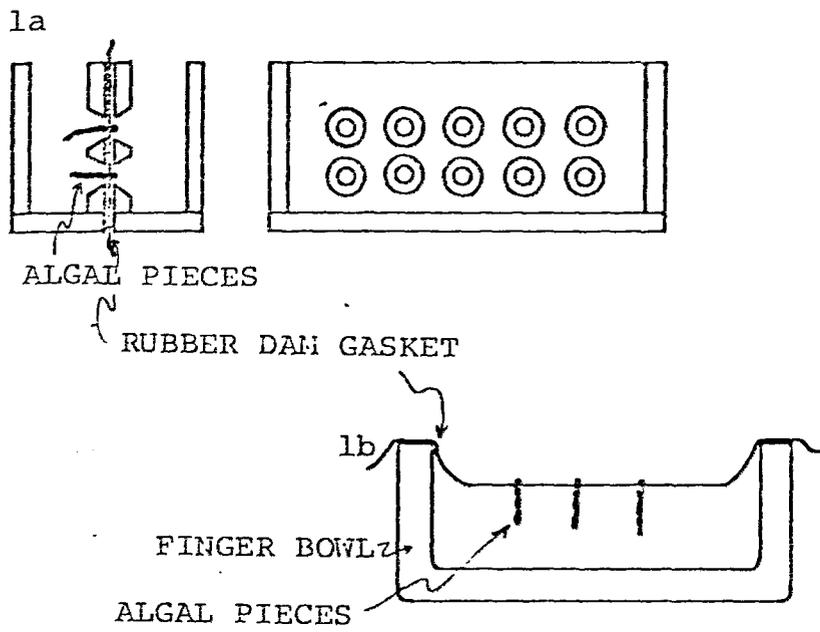
In this chapter, translocation phenomena are investigated. The research sought to determine: (1) if translocation of organic or inorganic materials occurs, (2) what effect translocated materials have on calcification, and (3) what the energy requirements, if any, for translocation, light-enhancement and calcification are.

I. Materials and Methods

A. Translocation Studies

A method for determining the direction and amount of translocation of radioisotopic tracers was designed using rubber dental dam as a gasket to separate tips and their adjacent intergeniculae. Illustrations of two designs used are shown in Figure 5-1a and Figure 5-1b. The "translocation chamber" in Figure 5-1a was used for experiments requiring one or the other of the tips and intergenicula

Figures 5-1a and 5-1b. Two types of translocation "chambers" used in the analysis of transport of materials between intergenicula and tips in C. tuberculosum. Figure 5-1a is made from 1/3" plexiglas. For aeration of Figure 5-1b a syringe needle was slipped under the rubber gasket at one edge of the finger bowl.



Figures 5-1a and 5-1b.

to be incubated in the dark. Pieces of algae were cut from algal fronds to contain a white growing tip and 3 or more intergenicula, and were selected for uniform cylindrical terminal segments and the absence of mature genicula at the growing tips. The algal pieces were pushed through pinholes in the rubber gasket so that only the white tip portion protruded on one side. The rubber dam gasket was then placed between the halves of the translocation chamber and the halves were bolted together. Seawater solutions were then added to each half of the chamber. During this entire operation, algal pieces were kept submerged in cold seawater to avoid desiccation damage. After a 30 minute preincubation, label was added to only one side of the chamber and incubation continued for 2 to 4 hours. Agitation and pH control were provided by aeration of the seawater by compressed air forced through a syringe needle. At the end of the incubation, the algal pieces were removed from the apparatus and the tips separated from the rest of the alga with a razor blade. The algal samples were then treated as described in Chapter 2 for scintillation counting. Seawater samples were taken of both sides of the chamber both at the start and end of the incubation to determine initial activities and to check for leakage of label between the two sides. All incubations were carried out at 11° C in a cold room. Illumination was provided by a bank of 4 cool white fluorescent tubes

supplying about 1000 foot-candles to the surfaces of the incubation vessels. For dark incubations, one half of the translocation chamber was darkened with black tape and aluminum foil. To avoid heating effects from the light fixtures, a fan was used to circulate cool air over the incubation vessels.

B. Use of Metabolic Inhibitors and Artificial Seawater

In experiments in which seawater containing various calcium concentrations was needed, artificial seawater (Lyman and Fleming, 1940) supplemented with a trace metals solution (Provasoli et al., 1957) was used. Concentrated stocks of the major ions were used to make up fresh seawater before each experiment. Concentrations of calcium used ranged from 20 to 200% of normal seawater calcium. The minor changes in the salinity produced by raising or lowering the calcium concentration were ignored.

Metabolic inhibitors used in this study were made up in concentrated stocks and diluted to the appropriate concentrations just before use. For DCMU (dichloromethyl-urea) and DNP (2,4-dinitrophenol) stocks were made up in absolute ethanol. Controls were run with algae without inhibitor but with the small amount of ethanol (1000:1, vol:vol) to check the effects of the added alcohol. For KCN, the stock solution was made up in seawater; the pH of

a 0.001M KCN solution was 8.3.

For incubations involving metabolic inhibitors or artificial seawater, algal fronds were always preincubated for 30 minutes before the addition of label. The standard incubation vessels were 50 ml beakers containing 40 ml of medium and covered with watch glasses or sealed with rubber stoppers. Incubation conditions were the same as previously described for the translocation studies.

C. Bicarbonate Labelling

Algae were labelled by incubation in seawater containing $\text{H}^{14}\text{CO}_3^-$. Methods were as described in Chapter 3.

II. Results

A. Translocation

A surprisingly large amount of organic C-14 label is incorporated into the white tip samples during incubation with $\text{H}^{14}\text{CO}_3^-$ (Figure 5-2). As previously noted (Chapter 3), photosynthesis occurs only at the algal surface. Expression of photosynthetic rates based on dry weights gives a distorted view of the actual relative incorporation of tips and intergenicula. Recalculation of the rates as $\text{CPM mm}^2 \text{ algal thallus}^{-1} \text{ min}^{-1}$ results in rates for CO_2 fixation of 167 and 254 for tips and intergenicula respectively. Even this latter rate for the tips is high and

Figure 5-2. Uptake of $H^{14}CO_3$ (into organic fraction) by C. tuberculosis. Circles represent the data from tips (open) and intergenicula (closed) and the triangles are the killed control tips (open) and intergenicula (closed). Vertical bars are the standard deviations of the means of 8 samples from duplicate vessels in two separate experiments.

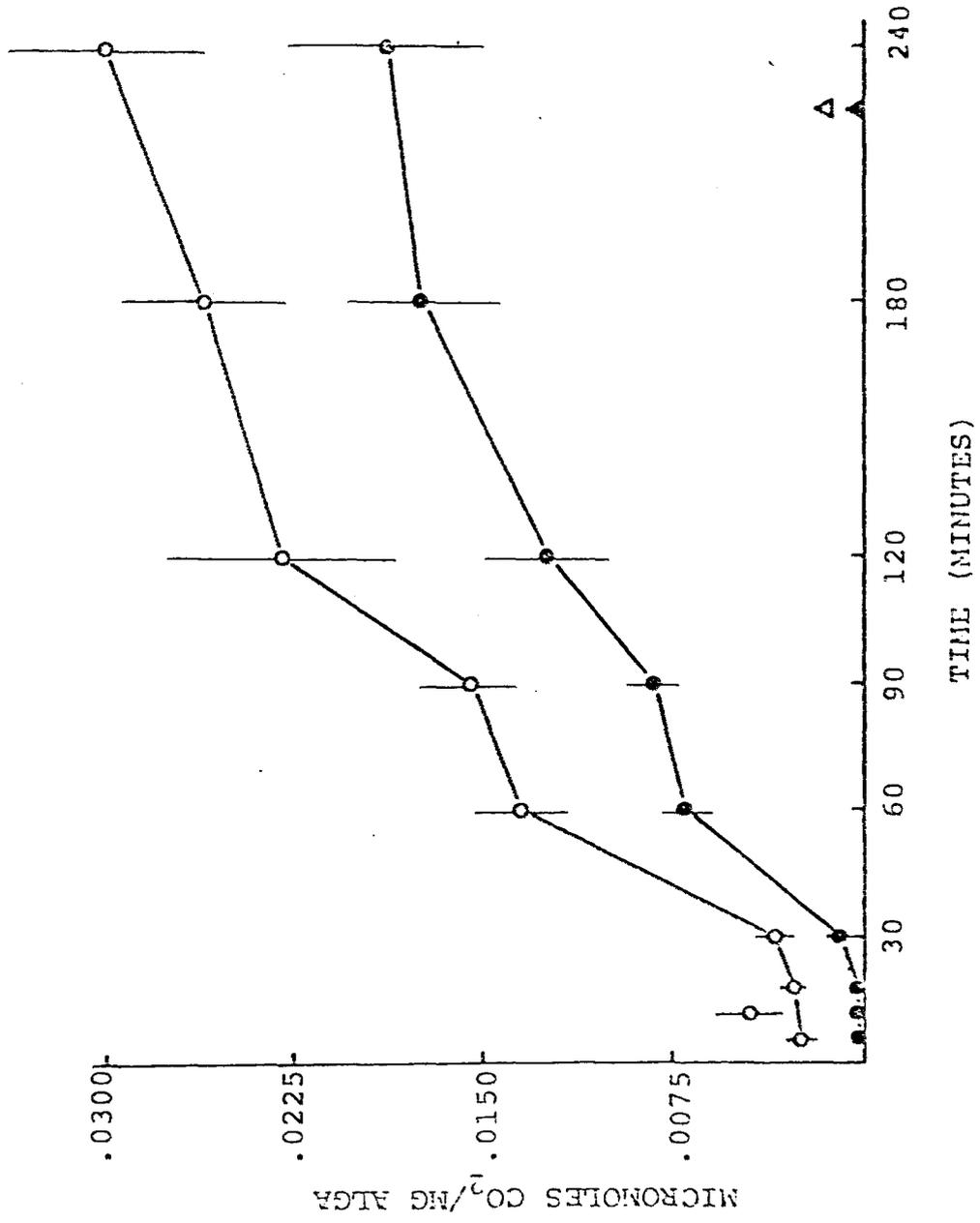


Figure 5-2.

obviously a significant amount of fixed carbon is present in the tips after a short incubation. The large amounts of label in the tips support the contention that translocation of organic material from the adjacent pigmented thallus is occurring. However, due to sampling errors (Chapter 3), some pigmented thallus was included in the "white tip" samples. It is, then, not possible to tell how much, if any, organic material is being translocated. Hence, organic translocation phenomena were investigated in the translocation chamber.

Calcium-45 is translocated from the intergenicula to the tips in C. tuberculosum (Table 5-1). These results are based on experiments using the translocation chamber described in Figure 5-1a. Both sides of the chamber were filled with filtered seawater and Ca-45 added only to the side into which the intergenicula protruded. The live alga, incubated in these experiments in the light, showed a significantly greater translocation of Ca-45 than did the killed controls. Tip:intergenicula incorporation ratios were 0.300 ± 0.103 and 0.039 ± 0.045 for live and killed algae respectively (ratios based on CPM [Ca-45] mg alga⁻¹ min⁻¹). In all data reported the amount of label in the final activity samples from the "cold" seawater side of the chamber was not significantly different from background. In several experiments the backflow of calcium-45, the flow from tips to ingernicula, was measured by addition of label to

Table 5-1

Translocation in live alga and killed controls.

Tip incorporation	Intergenicula incorporation	Tip: Intergenicula ratio
LIGHT-INCUBATED ALGA		
.1166	.3217	.3624
.1140	.2772	.4112
.1416	.4934	.2870
.1743	.4950	.3521
.0506	.4262	.1187
.1154	.4222	.2733
Means .1188+- .0407	.4060+- .0894	.3008+0.1027
KILLED ALGA		
.0036	.1185	.0304
.0003	.1254	.0024
.0000	.1488	.0000
.0182	.1666	.1092
.0069	.1332	.0518
Means .0058+- .0075	.1335+- .0193	.0338+- .0448

Data are for the translocation of calcium-45. Values are expressed as micrograms calcium per milligram algae. Each value is the result of the determination of activity from a sample containing 10 to 20 segments. Means are provided plus or minus one standard deviation.

the "tip" side of the chamber. Back translocation was undetectable in the live algae. In the killed controls the intergenicula:tip ratio was 0.080 ± 0.036 . There is, in the light, a unidirectional flow of calcium from the intergenicula to the tips.

For an additional translocation experiment, the translocation chamber in Figure 5-1b was utilized. The calcium label was added to the tip side (top solution) once again and artificial seawater containing various concentrations of Ca^{++} was used to fill the bottom of the chambers. It was predicted that, if large amounts of calcium were being translocated, the calcium ions from the intergenicula would "dilute" the calcium-45 taken up by the tips from seawater. Since it could be expected that higher calcium concentrations in the intergenicula would produce greater translocation in absolute terms, label incorporated into the tips would be increasingly diluted as calcium concentrations in the intergenicula increased. The data support the prediction; label incorporation decreases as a function of the calcium concentration surrounding the intergenicula (Figure 5-3). No such effect is seen in the killed algae and none is expected because of the small translocation rate in these formalin-fixed controls.

When $\text{H}^{14}\text{CO}_3^-$ is used in place of Ca-45 in translocation experiments both organic and inorganic labels move from intergenicula to the tip (Table 5-2). For these experiments,

Figure 5-3. Uptake of Ca-45 into tips isolated by a rubber membrane from the adjacent intergenicula as a function of the calcium concentration surrounding the bases. Circles are the light-incubated alga, squares are the killed controls. Vertical bars are one standard deviation of the mean. Each point is the mean of 8 determinations on duplicate samples from two experimental series.

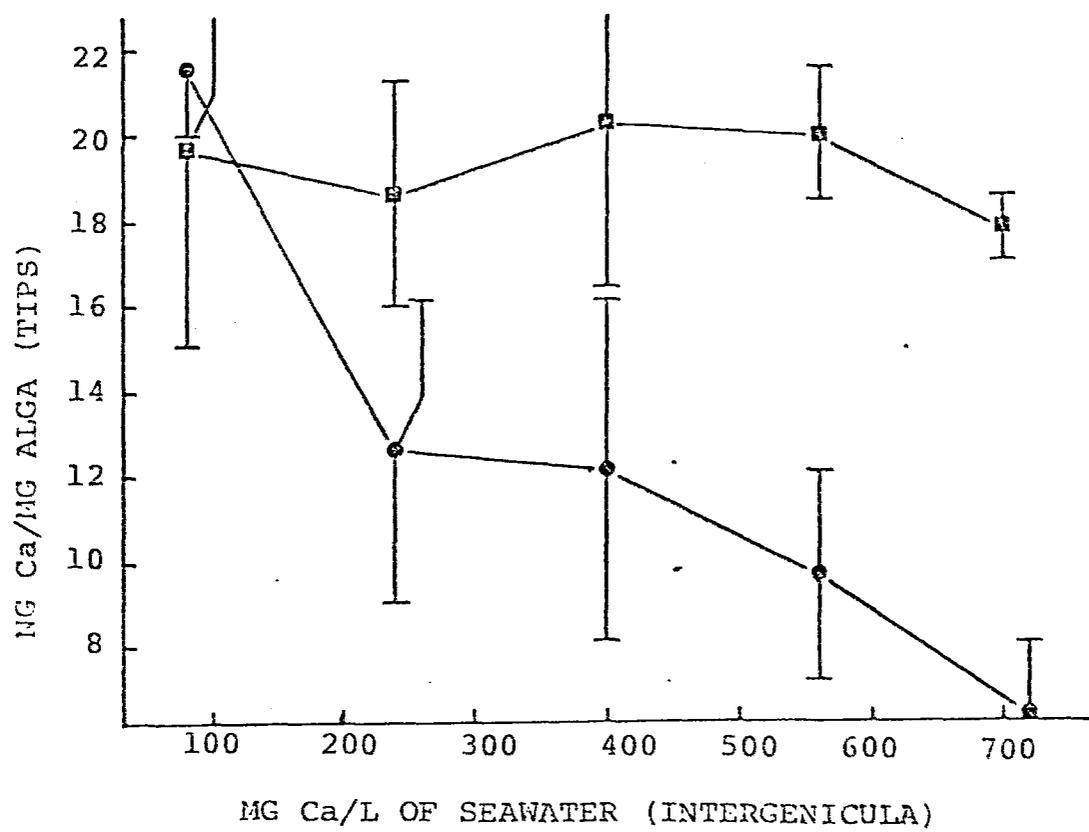


Figure 5-3.

Table 5-2

Translocation of organic and inorganic carbon-14 activity in live alga and killed controls.

Tip incorporation		Intergenicula Inc.		Tip: Intergenicula ratios	
Organic	Inorganic	Organic	Inorganic	Organic	Inorganic
LIGHT-INCUBATED ALGA					
5.6	2.0	16.3	7.1	.3436	.2817
9.3	3.6	16.6	8.9	.5602	.4045
4.8	2.2	17.4	9.0	.2759	.2444
4.6	2.2	17.2	8.9	.2674	.1798
5.6	1.6	17.0	8.9	.3294	.1798
2.0	3.5	15.7	7.6	.1274	.4605
MEANS					
5.3+-2.3	2.5+-0.8	16.7+-0.5	8.4+-0.8	.3173+- .1415	.3030+- .1070
KILLED ALGA					
	0.6		6.7		.0896
	0.4		7.4		.0540
	0.6		7.6		.0789
	0.2		7.4		.0270
MEANS					
	0.4+-0.2		7.2+-0.4		.0624+- .0279

Data are from experiments using $H^{14}CO_3$. Values are expressed in nanomoles CO_2 per milligram alga plus or minus one standard deviation. Each value is the result of the determination of activities in a sample of 10 to 20 segments. Incubation time was 2 hours.

the half of the translocation chamber into which the tips protruded was darkened before the preincubation. At the beginning of the incubation label was added to the intergenicula side of the chamber.

When the intergenicula are incubated in the light, the use of $\text{H}^{14}\text{CO}_3^-$ allows the detection of translocation of both inorganic carbon and organic compounds containing label fixed during photosynthesis (Borowitzka and Larkum, 1976b). Significant amounts of both organic and inorganic carbon move from the intergenicula to the tips. Tip:intergenicula ratios are 0.317 ± 0.142 and 0.303 ± 0.107 respectively. These ratios are remarkably similar to those obtained with Ca-45 translocation. For killed controls, the translocation ratio for inorganic carbon is 0.062 ± 0.028 . As before, backflow of label in the live algae was not detectable by the methods used.

The effect of translocation on calcification was investigated using the chamber in Figure 5-1a. Calcium-45 label was added to the "tip side." Three experimental series were run, one with both sides of the chamber in the light, one with only the tip side darkened and one with only the intergenicula side darkened.

The amount of Ca-45 incorporated into the tip is directly correlated with photosynthesis in the intergenicula (Table 5-3). Tips whose adjacent intergenicula were incubated in the light took up 2.1X as much label as did

Table 5-3

Calcium-45 incorporation rates in the tips of algal pieces in the translocation chamber.

Light Rates	Dark Rates
16.1	5.4
12.9	5.5
11.1	3.3
9.5	1.9
21.7	8.9
14.4	8.9
12.1	10.1
17.5	10.7
	MEANS
14.4+-3.9	6.8+-3.3

Light/Dark ratio 2.12

t-test for difference of means $t = 4.14$ significant
at .001 level

For the light rates, label was added to the tip side and the intergenicula incubated in the light. For the dark rates, label was added to the tip side and the intergenicula incubated in the dark. Rates are expressed as $\text{ng Ca mg}^{-1} \text{ alga min}^{-1}$. Each value is the result of the determination of activity on a sample of 10 to 15 segments. Means are provided plus or minus one standard deviation.

the tips whose intergenacula were kept in the dark. As a control, if the tips were kept in the dark, but the intergenacula were illuminated, calcium-45 incorporation rates remained high ($12.7 \pm 2.6 \text{ ng Ca mg}^{-1} \text{ alga min}^{-1}$). The results clearly show that light-enhanced calcification in the tips is dependent in some way upon the adjacent intergenacula.

B. Effects of External Calcium, Photosynthesis and Respiration on Calcification

The dependence of Ca-45 uptake on the external calcium concentration can be expressed as a simple function. The data from both light-incubation (Figure 5-4) and killed algae (Figure 5-5) are linear over the range of 10 to 210% of seawater calcium (80 to 680 mg Ca per liter). Within experimental error, saturation kinetics are not seen. Mediated calcium transport on a large scale must be excluded. This result supports the data from Chapter 4 which showed that calcium fluxes greatly exceed net accretion rates. However, because of this large disparity, the actual deposition process could be mediated, but masked by the exchange of the calcium label. To examine this possibility an experimental series identical to the one above was run and calcification rates were measured by EDTA titration (Chapter 3). Definite saturation kinetics are shown (Figure 5-6). An Eadie-Hofstee replot of the data

Figure 5-4 . The uptake of calcium-45 by live algae as a function of external calcium concentration. Vertical bars are one standard deviation of the mean. The line is the least squares regression of the data.

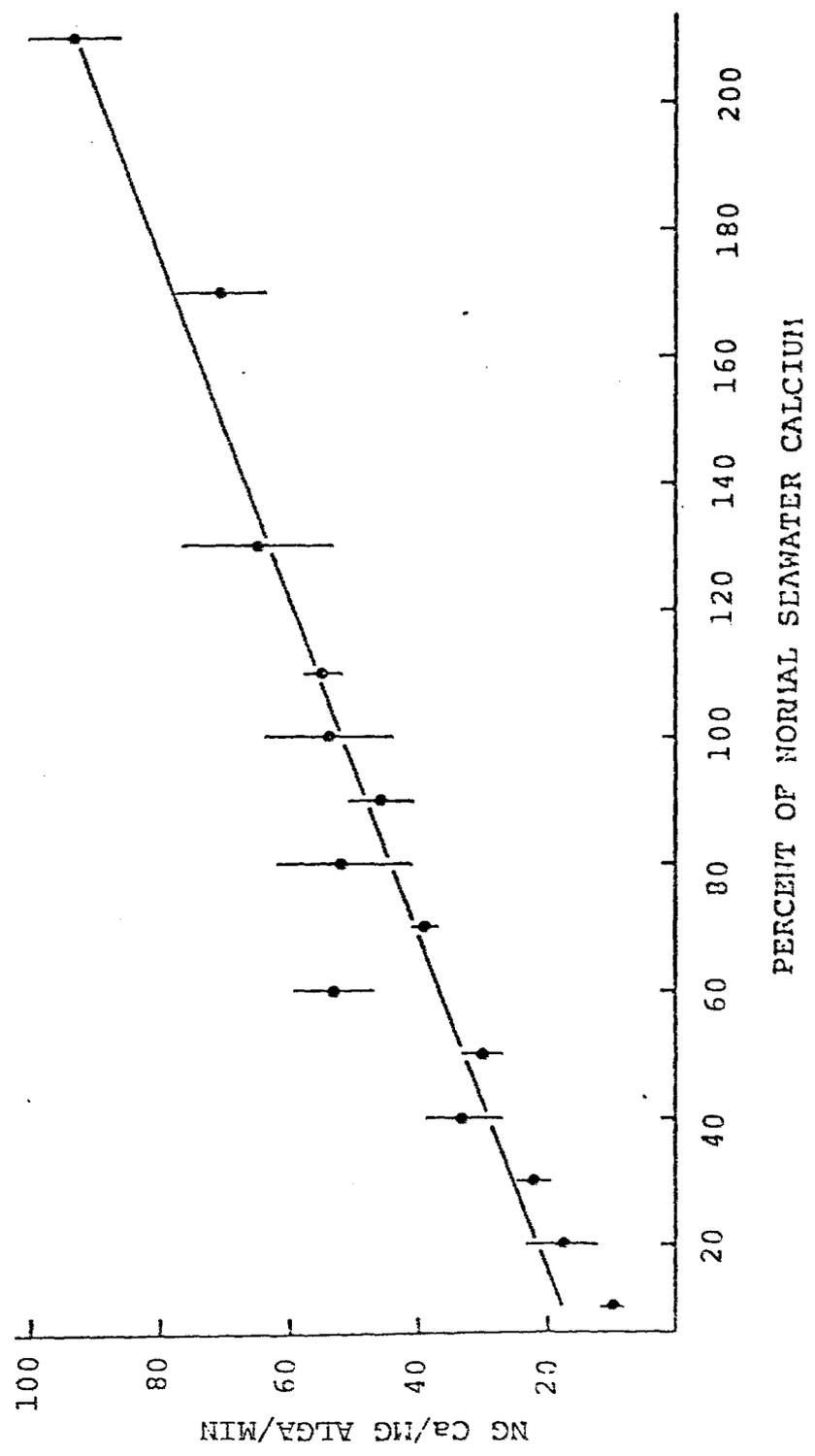


Figure 5-4.

Figure 5-5. The uptake of calcium-45 by formalin fixed algae as a function of the external calcium concentration. Vertical bars are one standard deviation of the mean. The line is the least squares regression of the data.

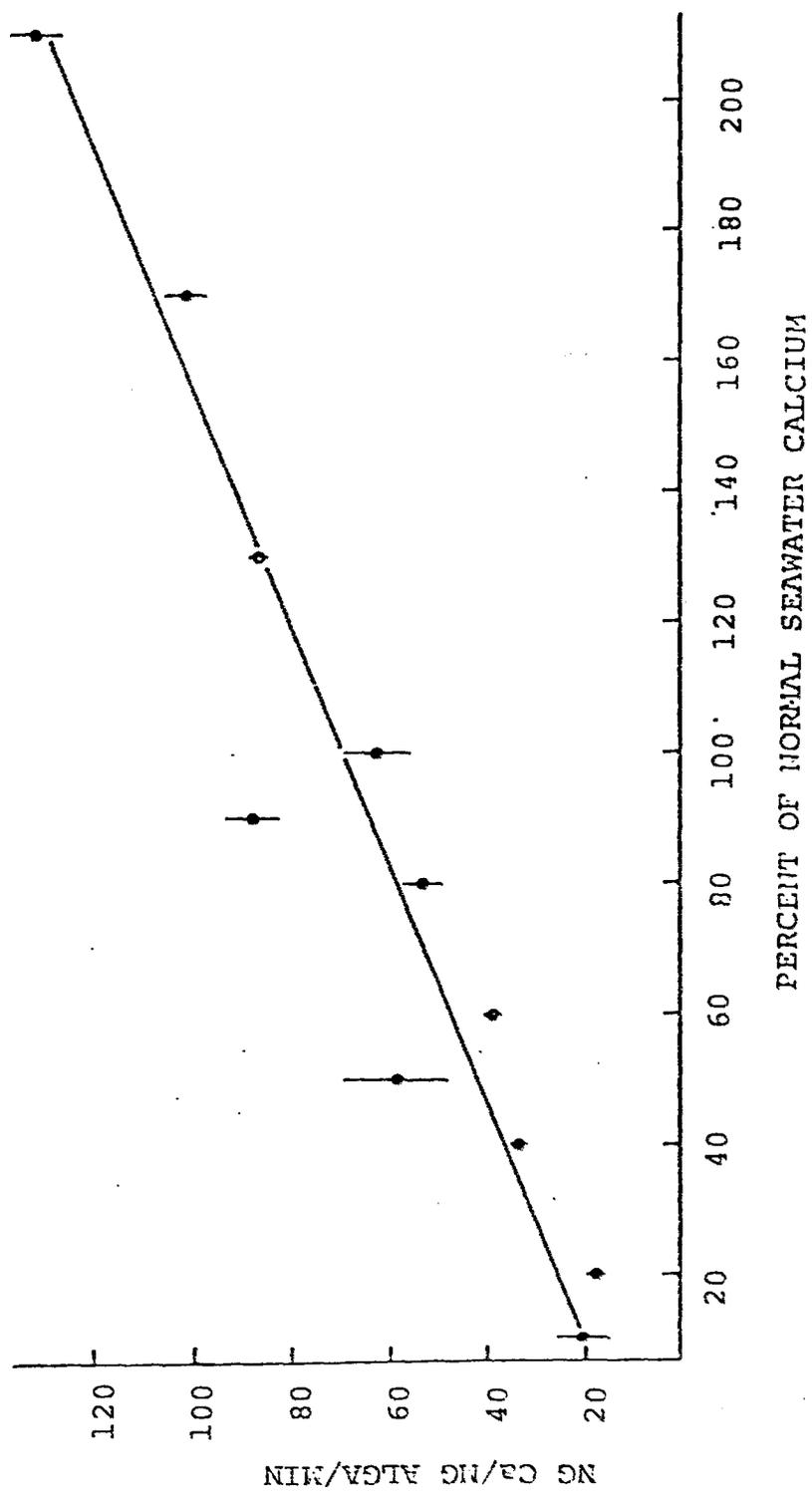


Figure 5-5.

Figure 5-6. Net accretion of calcium by EDTA titration as a function of the external calcium concentration. Each point is the mean of 6 titrations on duplicate samples. Vertical bars are one standard deviation of the mean. Curve is estimated best fit.

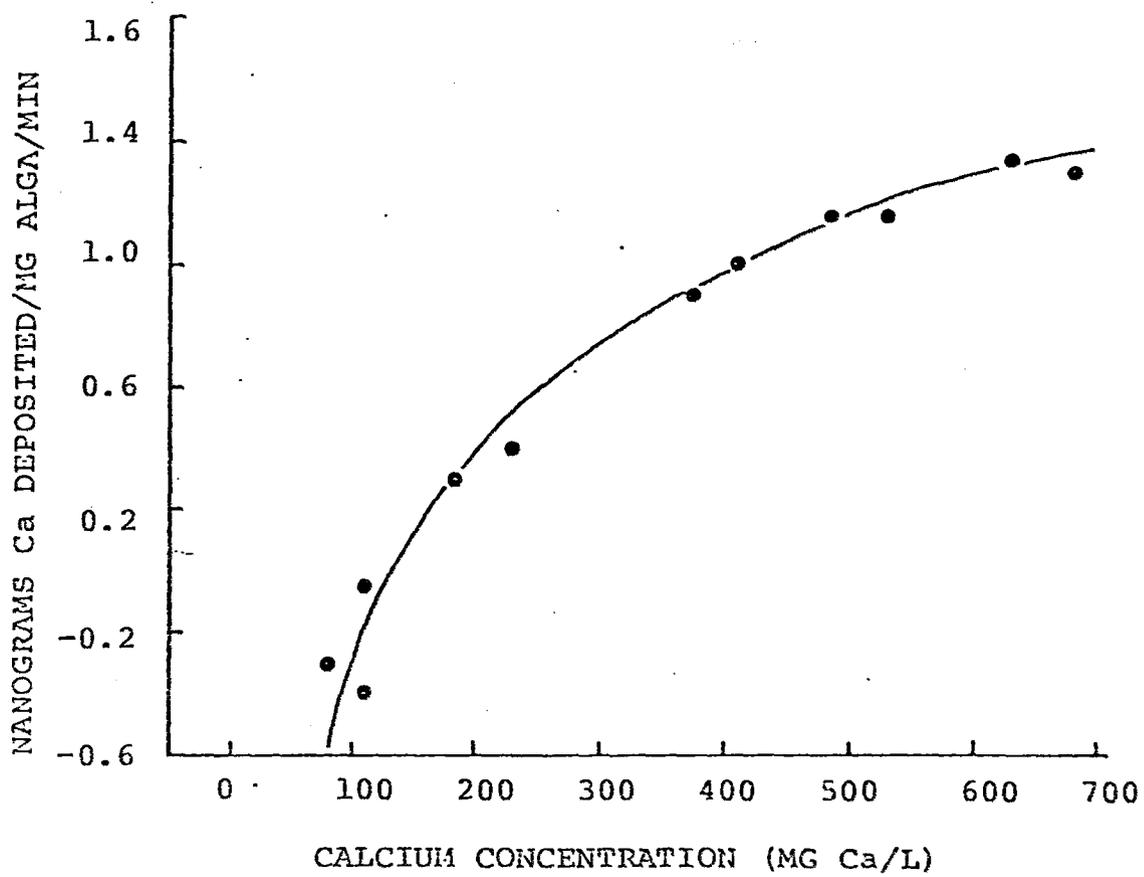


Figure 5-6.

(Figure 5-7) produces a V_{\max} of $2.5 \text{ ng Ca mg}^{-1} \text{ alga min}^{-1}$ and a K_m of 7.6 nM l^{-1} . Accretion of CaCO_3 (but not calcium uptake) may be an enzyme mediated process. Yet, aside from the active transport of calcium, no enzyme reaction substrate dependent upon calcium is known. The possibility of a small amount of calcium transport intimately associated with CaCO_3 deposition is considered in the discussion (see also Chapter 4).

To investigate the direct effects of photosynthesis on light-enhancement, the inhibitor, DCMU, was used. DCMU is thought to block electron transfer in photosystem II and also the Hill reaction (Izawa et al., 1969). Respiration should not be directly affected by this compound. When calcification and photosynthesis were measured concurrently in the presence of DCMU (by H^{14}CO_3 uptake) photosynthesis was completely inhibited at a DCMU concentration of 10 micromolar. At the same concentration calcification rates were reduced to dark levels (Figure 5-8). This is strong evidence that light-enhancement is a function of photosynthesis and not some other photobiological process.

DNP and CN^- were chosen to inhibit oxidative and photophosphorylation. DNP acts as an uncoupler of electron transport and phosphorylation. Even at inhibitor concentrations of 0.1 mM, DNP has no effect on either photosynthesis or calcification (Figure 5-9). If DNP were

Figure 5-7. Eadie-Hofstee replot of the data in Figure 5-6. The y-intercept is V_{\max} and the slope of the line is $-K_M$. Line is best least squares fit.

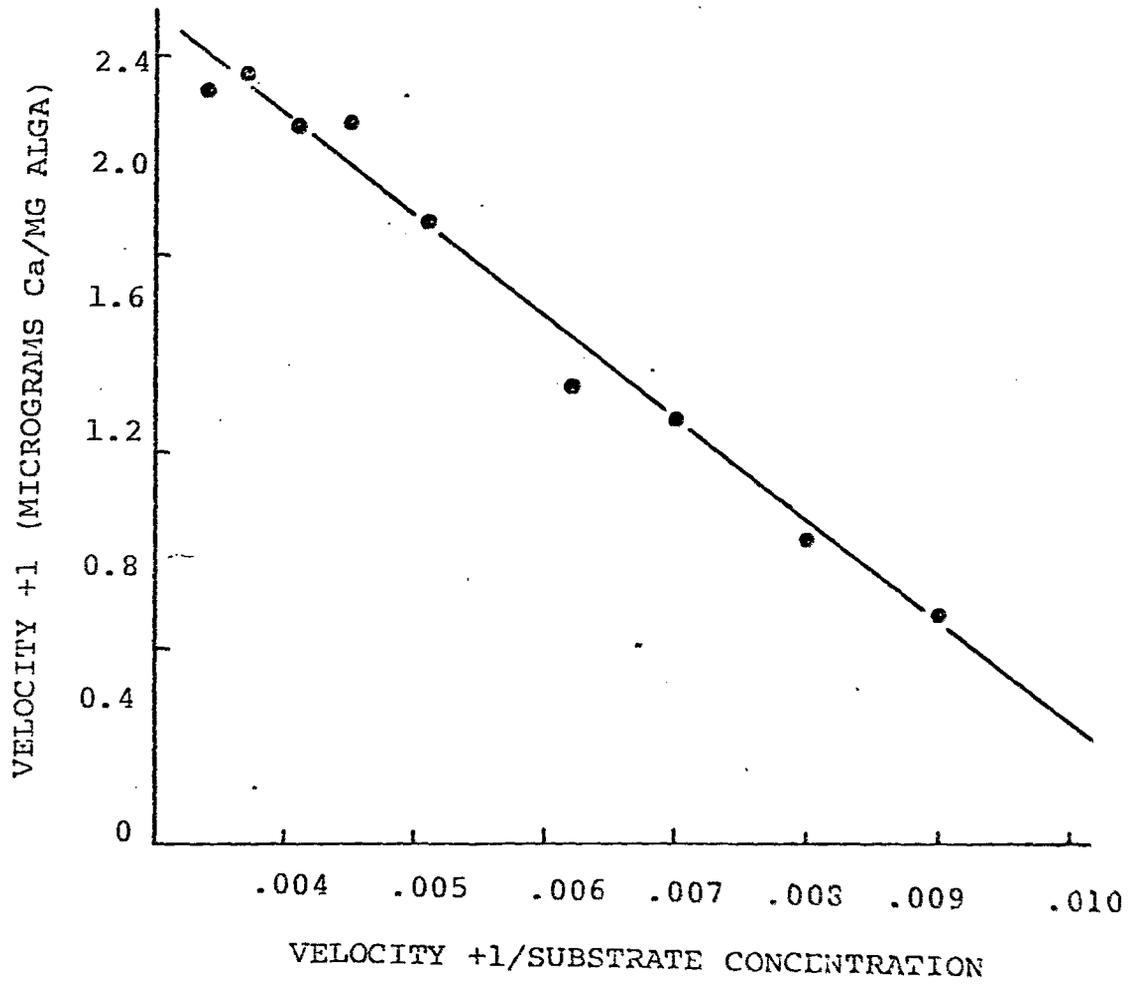


Figure 5-7.

Figure 5-8. Uptake of H^{14}CO_3 by intergenicula in the presence of DCMU. Open circles are inorganic carbon, closed circles organic carbon, open squares dark control inorganic uptake and closed squares dark control organic uptake. Vertical bars are standard deviations. Each point is the mean of 4 samples of 10 or more segments from duplicate vessels in two separate experiments.

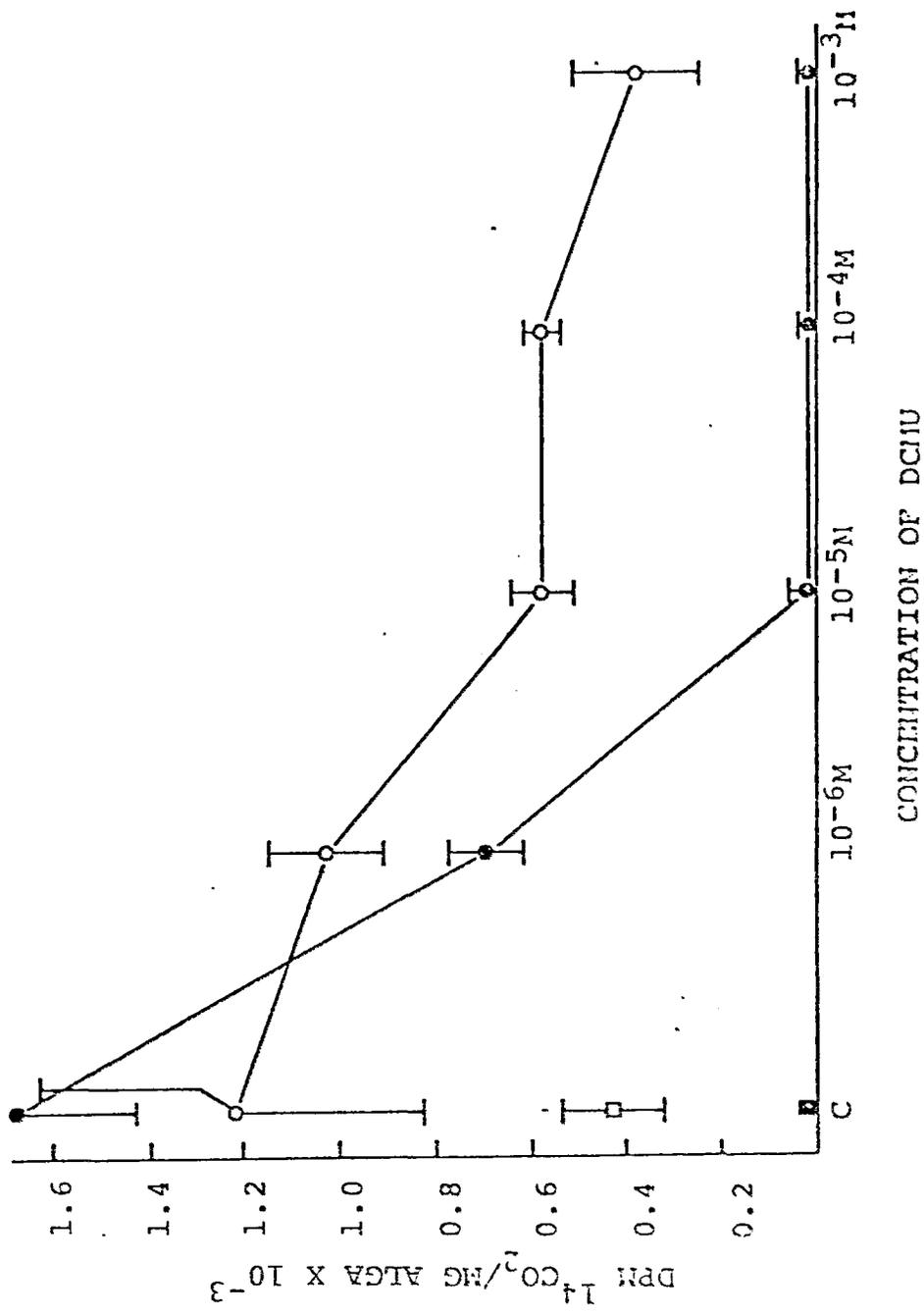


Figure 5-8.

uncoupling phosphorylation, photosynthesis should be inhibited. The data show that DNP was not effective in the algal system.

Cyanide is a classic inhibitor of oxidative and photophosphorylation. At 1 mM, CN^- nearly completely inhibits CO_2 fixation (Figures 5-10 and 5-11). This concentration also inhibits calcification rates to near dark levels. Calcification rates measured in the dark are unaffected by CN^- .

III. Discussion

Cyanide, if it has any effects outside the inhibition of photosynthesis (Figures 5-10 and 5-11), should also block respiration. If true, then dark calcification may not be directly energy dependent. Theoretically, energy should not be needed for the precipitation of CaCO_3 since seawater is supersaturated with respect to this mineral. It may be that the nucleation of crystals is required before CaCO_3 deposition can occur (Lind, 1970; Chapter 6).

If dark CaCO_3 deposition is not directly energy dependent, the possibility of active calcium transport as part of the algal calcification mechanism would be eliminated. Furthermore, the suggestion that light-enhancement is due to the increased supply of ATP generated by photosynthesis (Pearse, 1972) would be invalid. In support of this interpretation, active transport on a large scale is

Figure 5-9. Uptake of H^{14}CO_3 by tips and intergenacula as a function of DNP concentration. Open symbols represent inorganic uptake and closed symbols organic uptake. Circles are tip values; triangles are intergenicular values. The square is the dark control. Means are 8 samples from 2 experiments. Vertical bars are one standard deviation of the mean.

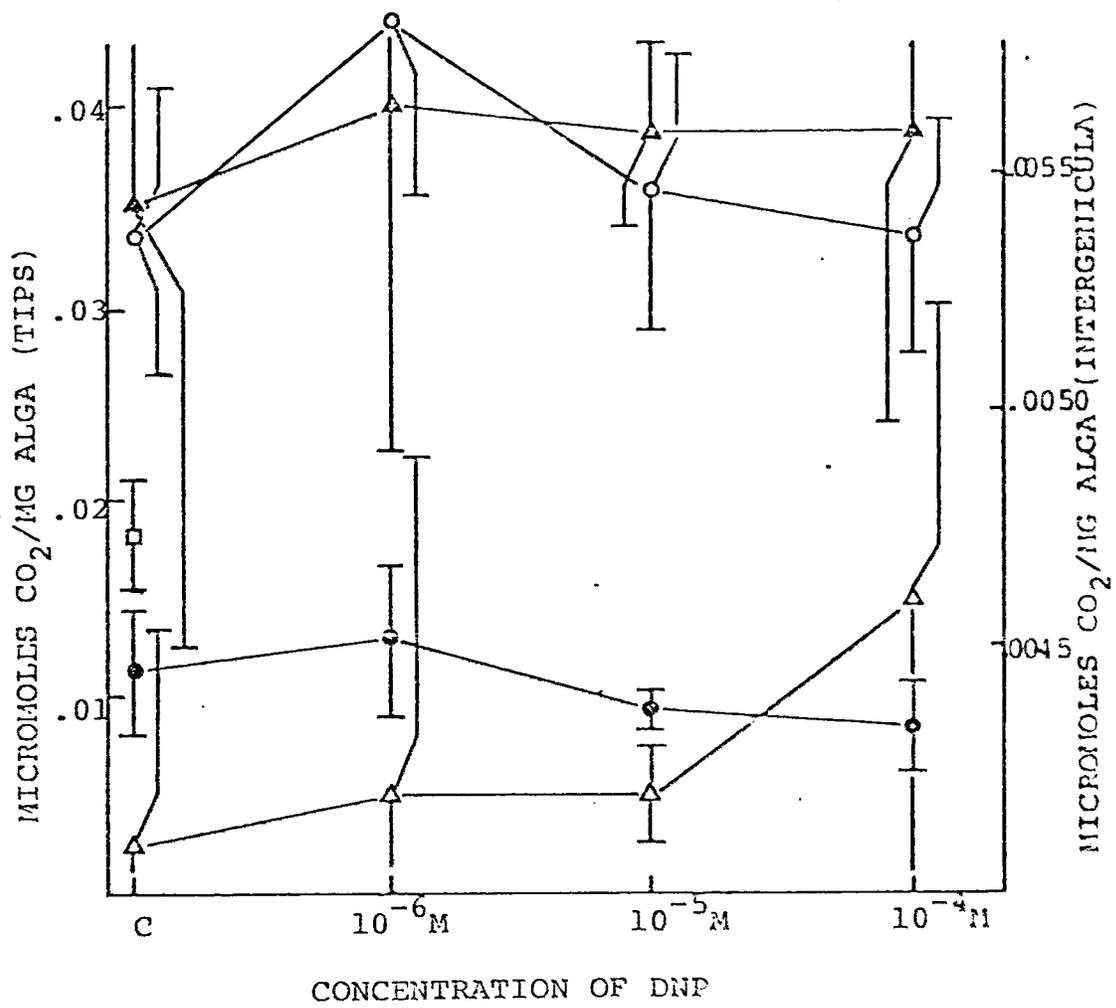


Figure 5-9.

Figure 5-10. Uptake of H^{14}CO_3 by tips in the presence of cyanide. Open circles are inorganic carbon, closed circles organic carbon and squares the dark controls. Vertical bars are one standard deviation of the mean. Means of 8 samples from 2 experiments.

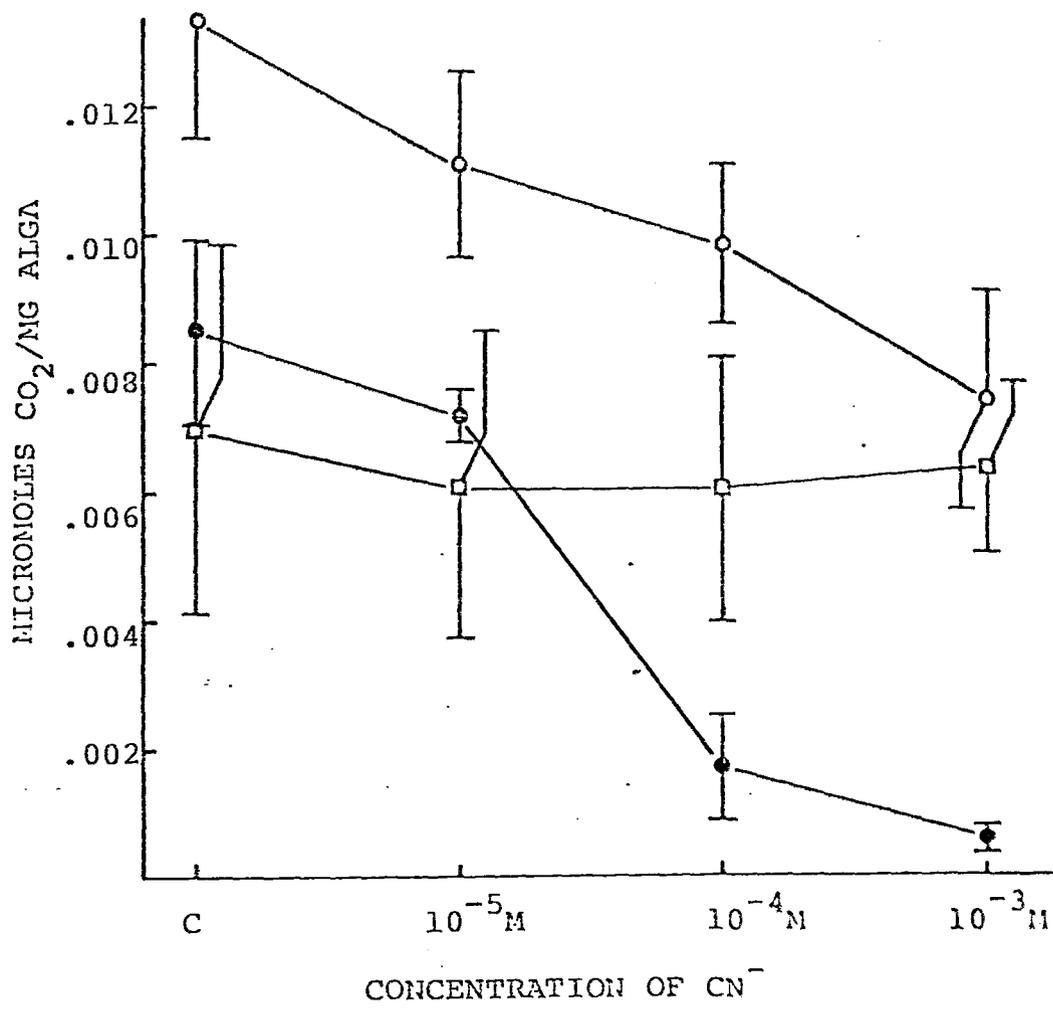


Figure 5-10.

Figure 5-11. Uptake of H^{14}CO_3 by intergenicula in the presence of cyanide. Open circles are inorganic carbon, closed circles organic carbon and squares the dark controls. Mean of 8 samples from 2 experiments. Vertical bars are one standard deviation of the mean.

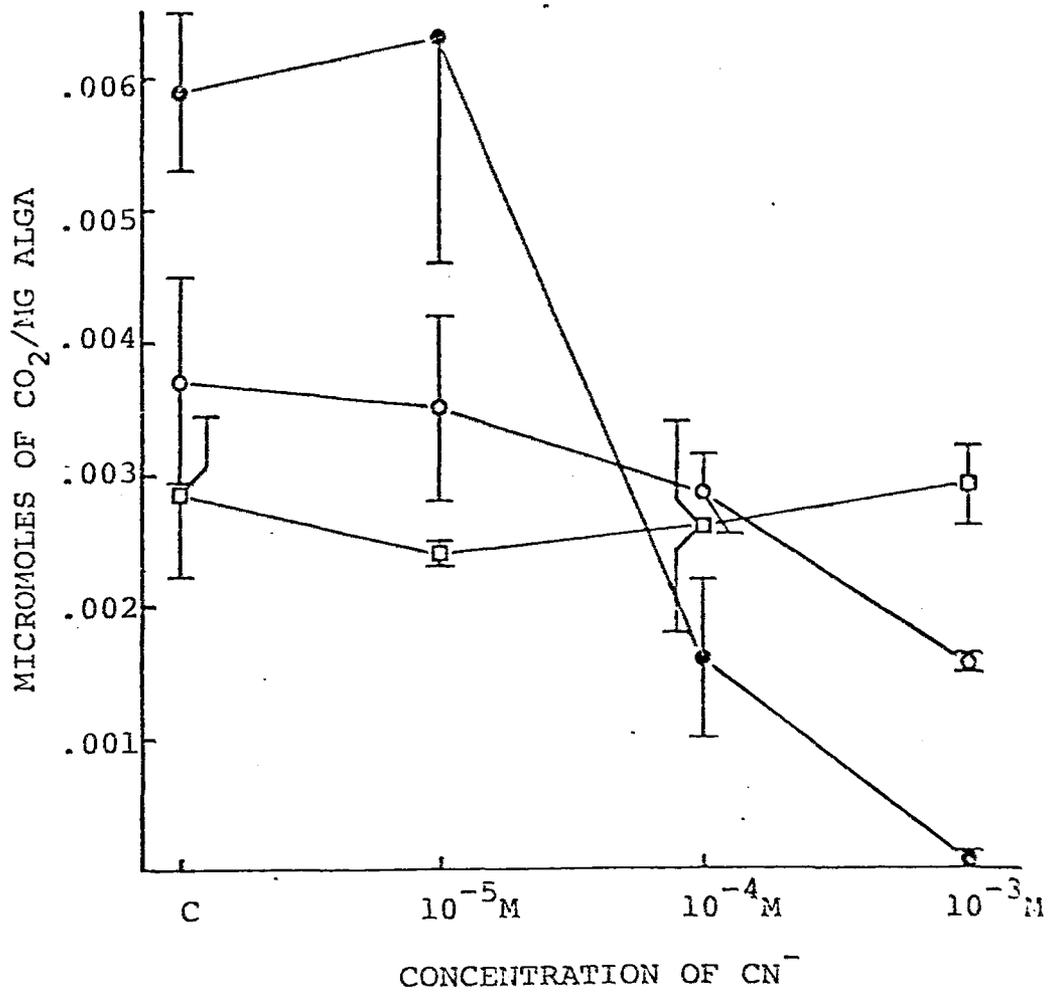


Figure 5-11.

not found in Calliarthron (Figure 5-4). The possibility of active transport on a small scale is unlikely since the calcite crystals form outside the cell plasmalemma (Lind, 1970; Bailey and Bisalputra, 1970). Calcium transport to the sites of mineral deposition, then, would involve the entire cell surfaces and should have a major effect on Ca uptake. Nevertheless, net accretion data show saturation kinetics (Figure 5-6). In the absence of light-enhanced active calcium transport the only obvious explanation of these data is the following:

Increasing either of the calcium or carbonate concentrations will cause the product of these concentrations to more greatly exceed the solubility product of calcium carbonate. In the light, photosynthesis raises the pH and, hence, the $\text{CO}_3^{=}$ concentration, to a point limited by factors governing CO_2 fixation. Thus, the data represent the total effect on the product of calcium and carbonate concentrations of photosynthesis and external calcium. Since photosynthesis (and, hence, $\text{CO}_3^{=}$) were constant, the saturation kinetics are due to the limitations on calcification rate imposed by the concentration of $\text{CO}_3^{=}$. This interpretation is supported by the behavior of the process at low calcium concentrations. Below a concentration of about 150 mg Ca l^{-1} , the algal thallus loses calcium, indicating that the reduced calcium concentration has increased the solubility product to the point where deposition can no

longer occur.

The translocation assay was run over a time period which allowed the possible influences of calcium pool sizes on the translocation of label to be ignored. When the data in Figure 5-6 are used to calculate the total exchangeable calcium (Heany, 1963), the results are about 1%, or $0.01 \text{ mg Ca mg}^{-1}$ alga. Thus, during the incubations in the translocation chamber the amount of calcium transported to the tips is less than 5% of the total pool. Thus the ratios in Table 5-1 should be proportional to the rates of translocation. There is no direct evidence that the same is true for inorganic and organic carbon. This was assumed, however, since the total amount of carbon transported is small compared to the expected pool size of CO_2 (250 nM mg^{-1} alga) based on the concentration of CO_2 in seawater and assuming that the exchangeable phase of the mineral calcium and the mineral CO_3 are the same size.

Rates of translocation of calcium, bicarbonate and organic compounds are remarkably similar (Tables 5-1 and 5-2). This suggests that all are present in the solution being transported between intergeniculum and tip. Instead of the translocation of a specific compound it appears that there is a general flow of materials. The flow is shown to be unidirectional, i.e., materials are moving only from the intergenicula to the tips. In addition, the data (for the first time) directly show that the light-enhancement

effect in the tips is dependent upon photosynthesis in the adjacent intergenicula (Table 5-3). The only obvious link between the tips and the adjacent thallus is translocation. Thus, it is likely that translocation is in some way responsible for light-enhancement.

The previous arguments for the lack of a direct chemical energy requirement in algal calcification are consistent with the contention that translocation does not specifically involve high-energy phosphates. (Though translocation itself probably requires energy.) However, it must be considered that the tips are dependent for their supply of fixed carbon on the adjacent thallus. Calcification will, of course, depend ultimately on the energy supply for organic growth. Thus the speculation that light-enhanced calcification in the tips is due to the translocation of matrix precursors (Pearse, 1972) apparently remains a possibility. Yet, an examination of crystal formation in the Corallinaceae shows that the matrix is probably necessary only for the nucleation of oriented seed crystals and not for subsequent growth (Lind, 1970). The bulk of the CaCO_3 is deposited independently and the effects of reduced availability of matrix precursors should be long-term and indirect.

The simplest explanation of the data is that the solution translocated to the tips is sufficiently well buffered to maintain the pH environment in the intergenicula. That

is, the ionic changes induced by photosynthesis are translocated. At high pH the 2 mM CO_2 system and the borate system in seawater provide the major buffering capacity. The finding that inorganic carbon is translocated suggests that the translocated solution is buffered by the CO_2 system. This hypothesis would require, in essence, the translocation of some hydroxide. The hydroxyl ions would be consumed in the precipitation of CaCO_3 . Since calcium is also being translocated, after CaCO_3 deposition there need be no cation excess. Whether or not hydroxyl ions are being translocated is not demonstrated.

A potentially interesting side-light to this hypothesis is the finding of very high concentrations of organic acids in the corallinaceae as compared with non-calcareous algae (Furuya, 1965). If these acids were translocated, they might supply substrates for anabolic metabolism and respiration, thus meeting the tips' demand for fixed carbon. In addition, many organic acids weakly chelate divalent cations so that their translocation might partially explain the translocation of calcium. Finally, organic acids might marginally assist in the buffering of the translocated solution. The nature of the translocated compounds is further considered in Chapter 6.

The argument that "remote" light-enhancement effects rule out the precipitation hypothesis is not valid. The findings here suggest that these effects may be satis-

factorily explained in terms of the ionic changes surrounding photosynthesis and their translocation to the growing tips. The hypothesis is consistent with the demonstration that calcium-45 uptake (Chapter 3) and washout (Chapter 4) can be satisfactorily explained by physical-chemical processes. No direct metabolic intervention seems necessary. The precipitation hypothesis is suggested as the most likely mechanism of light-enhancement in the Corallinaceae.

CHAPTER 6

CHEMICAL AND MORPHOLOGICAL CONSIDERATIONS

It has been suggested (Chapter 3) that organic acids, found in high concentration in the coralline algae (Furuya, 1965), might be translocated from the intergeniculum to the tip. The data reported in the first half of this chapter are the results of a preliminary identification of translocated organic compounds. The data are presented as evidence that organic acids are indeed translocated and that there is a general flow of organic materials from the intergenacula to the growing tips.

In the second half of the chapter, the results of calcium-binding experiments with soluble and insoluble polysaccharide components are presented. These studies were prompted by: 1) investigations which have shown that soluble (Bohm and Goreau, 1973) and insoluble (Lind, 1970) polysaccharide fractions bind substantial amounts of calcium and might be important to the calcification process, and 2) the demonstration (Chapter 3) that calcium exchange and/or binding significantly biases Ca-45-based calcification rate measurements.

Bohm and Goreau thought that a calcium-binding muc-

polysaccharide from Halimeda might sequester calcium for subsequent deposition. Lind suggested that the cell wall/matrix of Lithothamnion provides nucleation sites for the growth of calcite crystals. The fact that there is significant binding of calcium by C. tuberculosum indicates that polysaccharide binding in this system should be investigated. The results presented in this chapter constitute an attempt to identify and locate within the algal system, calcium-binding organic fractions analogous to those reported by Bohm and Goreau and Lind.

I. Materials and Methods

A. Fractionation of Algal Homogenates and Binding Studies

Algae labelled in the light with $\text{H}^{14}\text{CO}_3^-$ or unlabelled algae were fractionated according to the outline in Table 6-1 into lipid, ethanol soluble, alkali soluble and insoluble fractions. Lipid fractions were taken by homogenizing 10 grams net weight of algae in absolute methanol, centrifuging and repeatedly extracting the pellet in 1:1 methanol: ether until the extract was clear. The lipid extracts were pooled, reduced in volume in a stream of air, made up to a known volume and, on labelled samples, aliquots taken for activity determinations.

The residue from the lipid extractions was further

Table 6-1

Distribution of C-14 label in various fractions of extracts from C. tuberculosum tips.

Sample	CPM/ml of extract	
1. Lipid fraction	908	956
2. EtOH soluble fraction	13121	12097
3. Alkali soluble fraction	13163	12700
4. Insoluble fraction	26950	25328

Each value is one determination on a 2 ml sample of extract. Volumes were kept constant to hold counts comparable.

fractionated with two volumes of 80% ethanol at 90 degrees C for thirty minutes. The ethanol extracts were combined, evaporated in a stream of air and made to a known volume. For labelled samples aliquots were counted. This fraction was also analyzed by high voltage electrophoresis and descending paper chromatography (see Section B).

The residue from the ethanol extractions was re-extracted, this time with 0.0001N NaOH under reflux for thirty minutes (suggested by Bohm, 1973). Two such extractions were combined, freeze-dried and taken up in a known volume of 0.001M NH_4HCO_3 buffer, pH 7.8. This fraction was separated on Sephadex columns as described in Section B. For labelled samples aliquots of both the extract and the washed and resuspended residue were taken for counting.

B. Electrophoresis and Chromatography

Labelled compounds in ethanol extracts prepared as in Section A above were separated by high voltage electrophoresis using a Shandon model L24 set at 4000V. Runs were commonly made for forty-five to sixty minutes with a current of 80 to 135 milliamps. The buffer used was 8% formic acid, pH 2.0. Labelled compounds were located on a strip scanner and by ninhydrin staining. Descending paper chromatography was carried out using the methods of Bassham and Calvin (1957).

Compounds in the alkali soluble fraction prepared as in Section A were separated on 50cm by 1.6cm columns of Sephadex G-100, G-25 and G-10. One to 3 ml fractions were analyzed for protein by absorbance at 280nm and for polysaccharide using a quantitative adaptation of the Molische test described by Bohm (1973) except that thymol was used instead of carbazole and color was read at 507nm. Carbon-14 activity was determined by counting aliquots of the fractions collected from the columns in toluene:methanol fluor (Chapter 2). This fraction was also prepared from non-labelled algae and the binding of calcium determined using Ca-45 by the method of Bohm (1973).

C. Binding Studies

Cell wall/matrix material was prepared by slow decalcification of the algal thallus in cold, neutral 4% EDTA. Decalcification was continued for three months with weekly changes of the EDTA solution. Bacterial and fungal growth was discouraged by the addition of a few crystals of thymol. For binding studies cell wall/matrix material was homogenized in distilled H₂O, washed five times by centrifugation and resuspension in distilled H₂O and placed in a bag of dialysis tubing. The cell wall/matrix suspension was then dialyzed against seawater containing Ca-45 for twenty-four hours. After the experiment, aliquots of the whole matrix

suspension, the supernate solution after centrifugation and the seawater were taken for liquid scintillation counting. Also one ml aliquots of the suspension were pipetted onto planchets, dried and weighed. A control dialysis with distilled H₂O and no matrix was also run.

D. Microscopy

Mature thallus for light microscopy was fixed and decalcified in Susa fixative (Johansen, 1969), dehydrated, embedded in paraffin and sectioned by conventional methods. Sections were stained with Harris hematoxylin and eosin. Sporelings (settled on slides in the aquarium) were fixed in 1% glutaraldehyde in 1M sodium cacodylate buffer, pH 7.8, dehydrated, embedded in Epon 812 and sectioned 1 micron. The sections were stained with Richardson's blue.

E. Autoradiography

Sporelings for use in autoradiography were decalcified in 4% neutral EDTA for forty-eight hours. Complete decalcification was checked by calcium staining (Kashiwa, 1966) using glyoxal bis-2-hydroxanil. Decalcified sporelings (still on the glass slides) were incubated in seawater containing 0.1 microcuries/ml of calcium-45 for one hour. The sporelings were then rinsed in distilled water for two minutes, quickly dehydrated through an ethanol series and embedded in Epon 812. One micron sections were cut, stained

and coated with Kodak NT emulsion. After two weeks the slides were developed and photographed.

II. Results

A. Translocated Organic Compounds

The relative incorporation of C-14 label into the organic fractions isolated from the algal tips is provided in Table 6-1. The lipid fraction is seen to contain relatively little activity. Since lipid would not be expected to be translocated and relatively small amounts of carbon were funneled into the lipids, this fraction was not further analyzed.

The two soluble extracts were found to contain large amounts of label. Of these two extracts, the EtOH soluble material is expected to contain the small molecules likely to move between intergeniculum and tip. To identify the labelled compounds in this extract, the solution was used for separation and analysis described below.

Organic compounds translocated to the white algal tips (see Chapter 5) were investigated by high voltage electrophoresis. Ethanol extracts were prepared as described in the methods from tips which had been isolated in the translocation chamber diagrammed in Figure 5-1a. Several runs were made using this apparatus with $\text{H}^{14}\text{CO}_3^-$ label added only to the side into which the intergenicula were extended.

After one hour incubations, tips were isolated from the rest of the algal pieces and frozen until ready for use.

Figure 6-1 shows the pattern of labelled compounds produced by strip scanning after sixty minutes of electrophoresis. A number of labelled compounds are present. The mobilities of the compounds are in accord with those expected of acidic compounds, but are less than those of the acidic amino acids. Since organic acids have been found in great abundance in the coralline algae (Furuya, 1965), it is tempting to guess that these compounds are TCA cycle intermediates such as malate, citrate and succinate. However, Majak, Craigie and McLachlan (1966) found that sugars (floridoside, mannitol and glucose) were the most common carbon accumulation products found in Corallina officinalis after incubation in the light in the presence of $\text{H}^{14}\text{CO}_3^-$. Also, Bidwell (1958) found large amounts of labelled glyceric acid in C. officinalis in similar experiments. Sugar compounds and glyceric acid would also be rather non-mobile in the electrophoresis conditions used and, thus, it is not certain that the labelled compounds are the organic acids mentioned.

Figure 6-2 shows the pattern of labelled compounds from the same extract as in Figure 6-1, but separated by two dimensional descending paper chromatography. The labelled compounds show a low mobility in the phenol:water solvent, but a much greater movement with the butanol:propionic

Figure 6-1. High voltage electrophoresis of EtOH soluble fraction from C-14 labelled algae. Strip scan of EtOH soluble fraction after paper high-voltage electrophoresis. Strip figure above the scan trace shows ninhydrin positive areas of the paper after electrophoresis in relation to the radioactive compounds. Ninhydrin positive are the cross-hatched areas on the strip. Only the first two-thirds of the scan trace are shown since no additional labelled compounds were found nearer the anode.

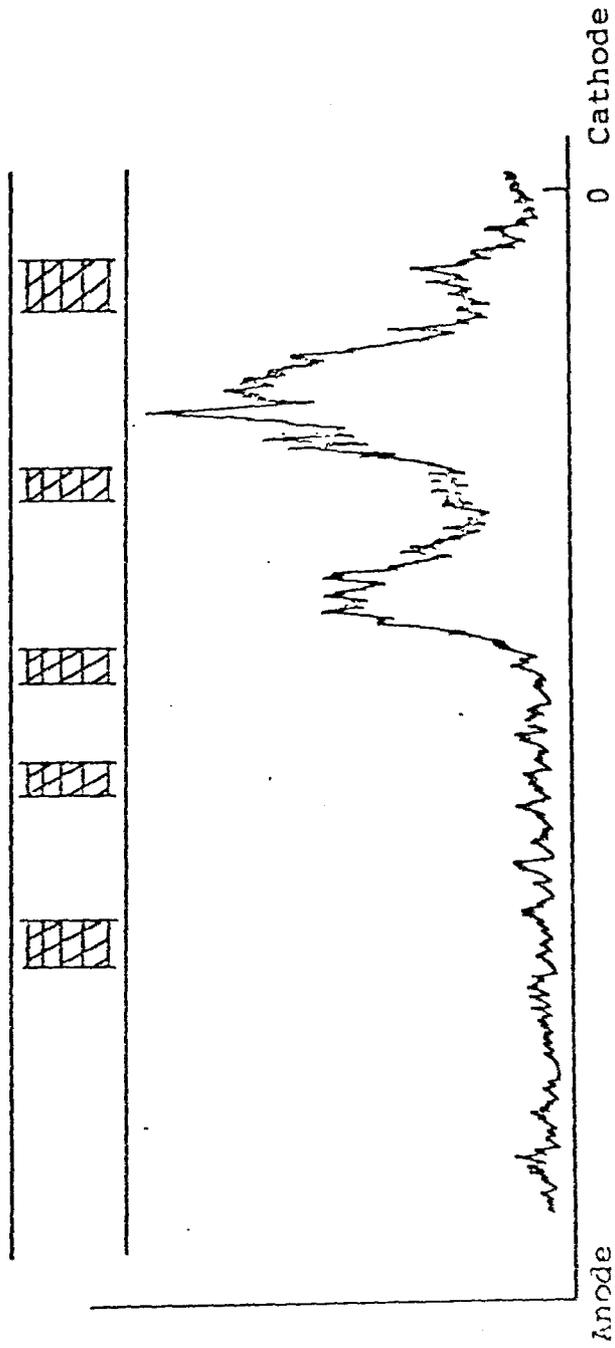


Figure 6-1.

Figure 6-2. Radioactive spots separated by two dimensional paper chromatography. The spots outlined by the solid lines represent radioactive compounds. The spot outlined by the dotted line is the major ninhydrin positive area. The origin is at the spot in the lower right corner. Radioactivity at this position indicates that some of the label did not move in either solvent system. Since the EtOH extract was not completely clear after reconstitution in H₂O after freeze drying, this label probably represents insoluble material. The crosses are alignment marks.

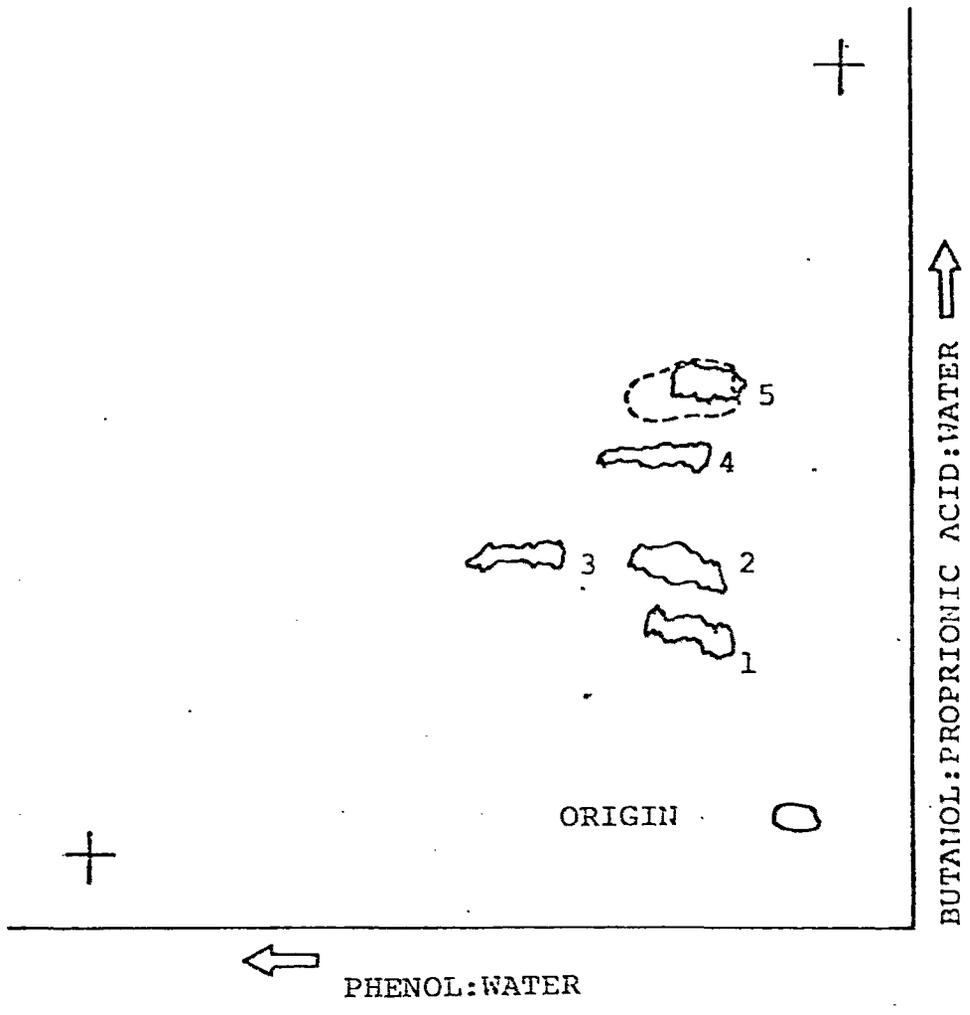


Figure 6-2.

acid:water solvent. These mobilities are characteristic of organic TCA-cycle acids. A comparison of the location of radioactive spots on the chromatogram in Figure 6-2 with the maps produced by Bassham and Calvin suggest that spots 2, 4, and 5 have migrated in fashions similar to those expected for aspartate, citrate and malate, respectively. However, spot 2 shows no ninhydrin color expected from an amino acid and spot 5 may be associated with ninhydrin color not expected of an organic acid. Spot 3 has migrated far enough in the phenol:water solvent that it may be a sugar, possibly glucose or trehalose, both of which have been reported in C-14 labelled photosynthate from Corallina officinalis (Majak et al., 1966; Craigie et al., 1968). It is difficult to guess at the identity of spot 1. It has migrated to a position which might indicate a disaccharide, a sugar phosphate or even a nucleotide. Co-chromatography of labelled compounds with suspected authentic compounds was inconclusive due to the failure of locating agents. However, the data indicate that acidic compounds are found in the extract and a possibility that organic acids are translocated in the coralline system.

B. Polysaccharides

A search was made, using the methods of Bohm (1973), for a calcium binding mucilage fraction. The alkali soluble fraction prepared from non-labelled alga was fraction-

ated further using the Sephadex columns. In each case Ca-45 was added to the extract before the run and in each case the activity of the calcium label traveled with the salt peak (Figures 6-3, 6-4, and 6-5). There was no evidence for a calcium binding polysaccharide fraction in the alga. The polysaccharides which were present eluted earlier than the calcium activity at positions of 10,000 and 1000 molecular weight. (Molecular weights are approximations based on the elution of cytochrome c from the G-100 column.) The soluble mucilage fraction from C. tuberculosum does not appear to contain a calcium-binding species such as the one implicated by Bohm and Goreau (1973) in calcification in Halimeda. Soluble mucilages capable of sequestering calcium are not of importance to coralline algal calcification. Recent work by Borowitzka and Larkum (1976b) suggests that the calcium-binding polysaccharides in Halimeda do not participate in the deposition mechanism.

In contrast to the soluble polysaccharides, the insoluble cell wall/matrix of Lithothamnion has been found by Lind (1970) to bind appreciable amounts of calcium. This same phenomenon was examined in C. tuberculosum. Cell wall/matrix material was prepared by slow decalcification of non-labelled material as described in the methods. Dialysis-type binding studies indicated that the cell wall/matrix suspension would bind 9.6 micrograms of calcium per milligram (based on dry weight). Details of these

Figure 6-3. The elution of the alkali soluble extract from *C. tuberculosis* from a Sephadex G-100 column. Circles are absorbance at 507 nm for the Molische test for polysaccharide; squares are the activity of calcium-45 in CPM per 100 microliter aliquot; triangles are absorbance at 260 nm for protein. Arrows from left to right are the position of elution of blue dextran, Cytochrome C and K ferriicyanide. Two ml fractions were collected.

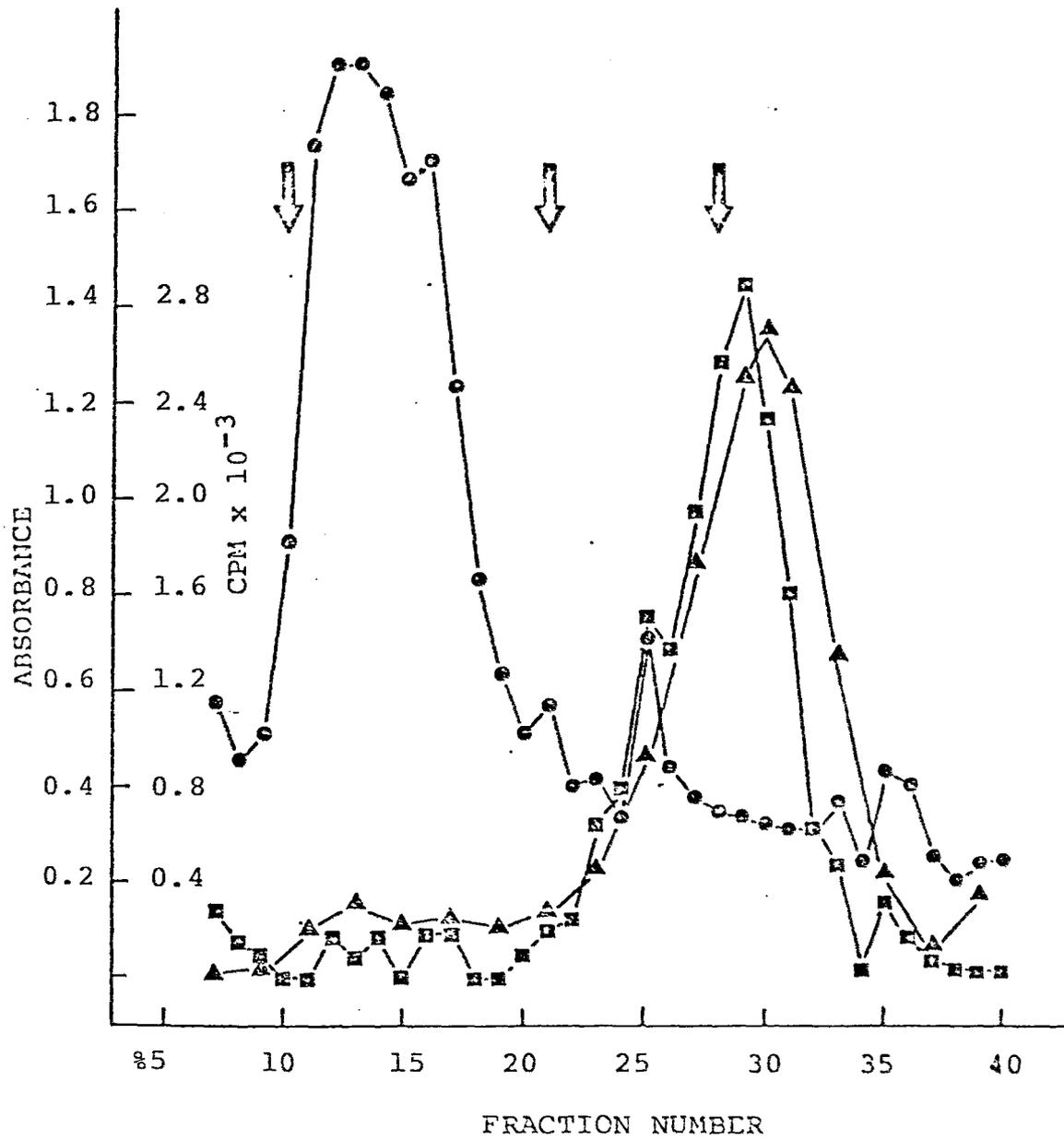


Figure 6-3.

Figure 6-4. The elution of the alkali soluble extract of C. tuberculosum from a Sephadex G-25 column. Symbols are described in Figure 6-3. Arrows indicate the exclusion limits of the column with blue dextran as the void volume indicator and K ferricyanide locating the salt peak. Three ml fractions were collected.

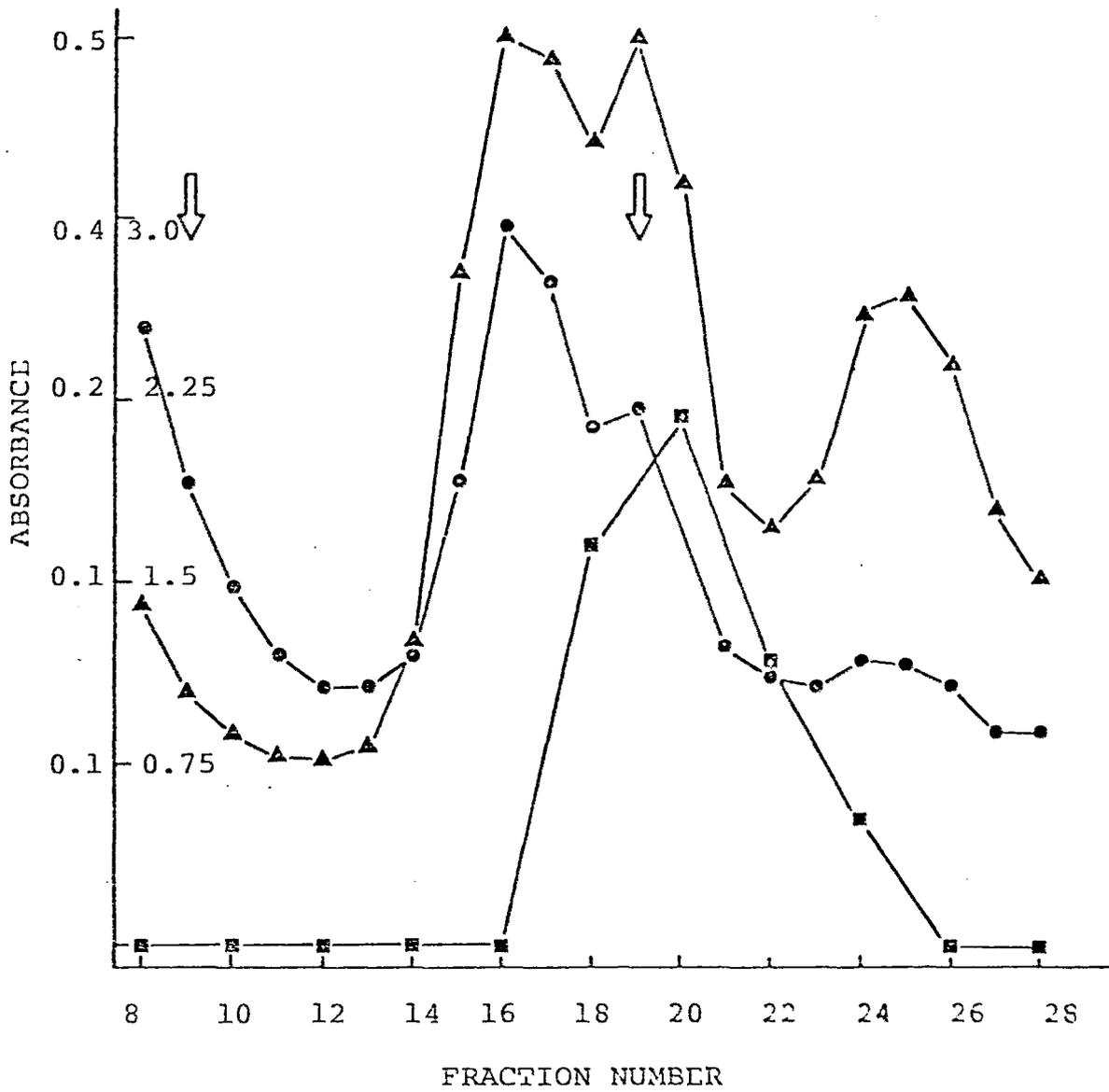


Figure 6-4.

Figure 6-5. The elution of the alkali soluble fractions from Figure 6-4 from a Sephadex G-10 column. Fractions 14 through 28 from Figure 6-4 were pooled, lypholized and rechromatographed. Symbols and arrows as in Figure 6-4. Three ml fractions were collected.

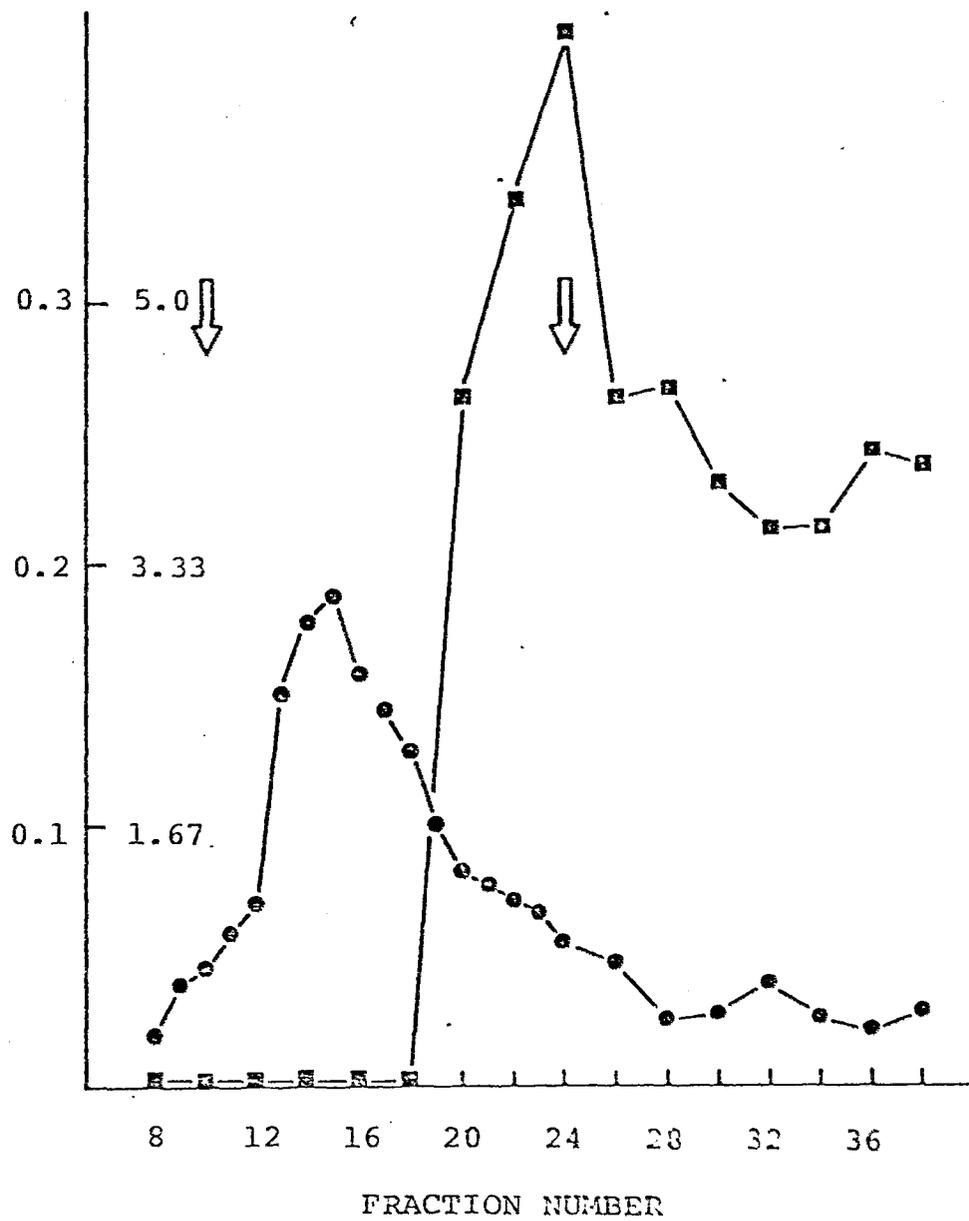


Figure 6-5.

data are provided in Table 6-2.

It was further shown (Figure 6-6) that this calcium binding activity of the cell wall/matrix is associated with the cell walls in developing sporelings. The figure is a photomicrograph of a 2 micron section of a sporeling which had been decalcified, labelled with calcium-45 and prepared for autoradiography. Developed grains are almost exclusively limited to areas over the cell walls. (This figure also shows intense labelling of the perimeter of the section, an indication of the binnding of calium at the surface of the thallus. This phenomenon was discussed in Chapter 4.)

The calcium binding activity of the extracted insoluble organics from C. tuberculosis is associated with the sites of mineral accretion in vivo. It is likely that the suggestions of Blinks (1963) and Lind (1970) are correct and that the cell wall/matrix material supplies nucleation sites for the growth of calcite crystals.

III. Conclusions and Summary

More study is needed to identify the translocated organic compounds moving toward the growing tips and the calcium-binding substances in the cell wall/matrix. Probably, organic acids are being translocated but it remains possible that other compounds may also be moving. In addition it is difficult to tell whether the similarity in the

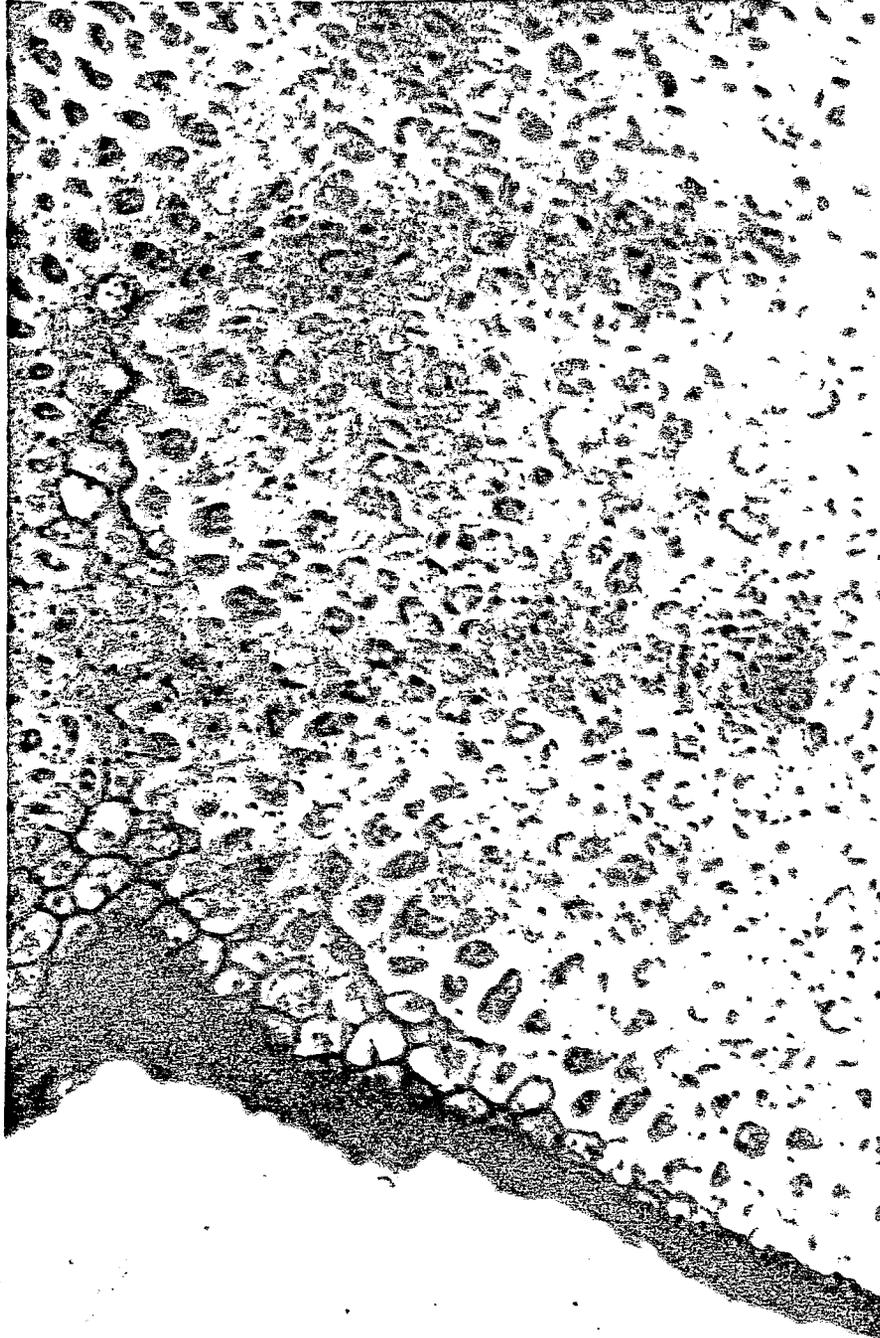
Table 6-2

The binding of calcium by the cell wall/matrix suspension estimated by dialysis against seawater containing Ca-45.

Sample	CPM/ml of solution	Micrograms Ca per milligram matrix
Initial seawater activity, matrix dialysis	78320	
Initial seawater activity, control	78129	
Final seawater activity, matrix dialysis	80527	
Final seawater activity, control	78241	
Final activity, matrix suspension	90281	116.65
Final activity, supernate of matrix suspension	82882	107.09
Final activity, control	78445	
Micrograms calcium bound per mg of matrix = 116.65 - 107.09 = 9.65		

The amount of bound calcium was calculated as the difference between the activity of the total suspension after dialysis and the activity of the supernate alone. The control was run with no matrix in the dialysis tubing and showed that the internal solution had reached equilibrium with respect to calcium during the incubation period.

Figure 6-6. Photomicrograph of C. tuberculosis sporeling used for autoradiography. Silver grains are small dark dots. For explanation see text.



patterns of translocated materials and that of compounds extracted from the intergenicula are due to a general flux of materials or the specific transport of a single compound which is subsequently metabolized into the variety of compounds observed. The data in Chapter 5 support the former alternative.

Calcium binding activity is associated with the insoluble cell wall constituents. As yet it is not possible to tell which organic compounds of the cell wall/matrix are responsible for calcium binding. It is likely that the calcium-binding material is a polysaccharide since coralline algal cell walls are composed primarily of various high molecular weight xylans, cellulose and other carbohydrate constituents (Turvey and Simpson, 1966). Yet, Lind (1970) shows that materials such as xylan and cellulose are rather inert, non-calcium binding and probably only of structural importance. Thus, calcium-binding activity is probably associated with mucopolysaccharides or sulphated forms.

CHAPTER 7

GENERAL SUMMARY

The use of calcium-45 for the estimation of calcification rates is shown to be significantly biased by exchange and binding, especially when calcification rates are low (Chapter 3). Because calcification rates in the intergenicular are low, past investigators have misinterpreted Ca-45 uptake data. Reports that light-enhancement disappears in progressively older segments are erroneous. Instead, light-enhancement can be observed, by non-isotopic techniques, to affect calcification to the same extent in both tips and intergenicula.

Differences between isotopic and non-isotopic calcification measures can be explained on the basis of physical-chemical exchange phenomena affecting the tracer techniques (Chapter 3). These same phenomena also are shown to influence the washout of Ca-45 label from the algal thallus (Chapter 4). The recycling and burial of tracer provide a complete explanation of Ca-45 kinetic data. The results are entirely consistent with the precipitation hypothesis as the mechanism of light-enhancement. Furthermore, the data do not support alternative hypotheses which suggest

direct metabolic involvement in the mineralization process.

A major argument against the precipitation hypothesis has been the observation of light-enhancement in the white tips of the articulated Corallinaceae. However, a mechanism is proposed (Chapter 5) whereby the ionic changes surrounding photosynthesis may affect calcification at a site remote from the chloroplasts. Translocation of both organic and inorganic species from intergenicula to the tips suggests a general flow of material. It is hypothesized that such a general flow would involve the transport of a buffered solution. This solution would preserve the pH (and, hence, $\text{CO}_3^{=}$) environment generated by photosynthesis and stimulate CaCO_3 deposition in the tips.

Recent physiological studies on the Corallinaceae suggest that metabolic processes participate directly in the deposition of CaCO_3 . This dissertation, however, argues against several possibilities, e.g., active transport of Ca^{++} , a two-step calcification process and increased supplies of high-energy phosphates and/or matrix precursor materials (Chapter 5). Further, this report shows that previous investigators have failed to control adequately their use of radioisotopes. The data indicate that the precipitation hypothesis is a valid explanation of light-enhancement and, in fact, must be considered the most likely mechanism for light-enhanced CaCO_3 deposition in the Corallinaceae.

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