PHYSIOLOGICAL AND BIOCHEMICAL STUDIES OF *LAURENCIA SPECTABILIS* AND ITS SYMBIONT *JANCZEWSKIA GARDNERI* (CERAMIALES, RHODOPHYCEAE)

by

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ABSTRACT

This thesis is a two part study on the biochemistry and physiology of the red algae Janczewskia gardneri Setchell & Guernsey and Laurencia spectabilis Postels & Ruprecht (Ceramiales, Rhodophyceae). The first part consists of photosynthesis, translocatory, microscopic, and cultural studies of the symbiotic association between the supposed parasite J. gardneri and its host L. spectabilis. Mature plants of J. gardneri were pigmented, contained typical red algal chloroplasts when viewed by electron microscopy, and were capable of photosynthesis. Both J. gardneri and L. spectabilis incorporated ^{14}C-label from $NaH^{14}CO_3$ into similar compounds, including sugars (floridoside, isofloridoside, galactose, glucose) and amino acids (alanine, aspartic acid, glutamic acid, glycine, serine). Translocation of ^{14}C-labeled photosynthetic products between mature J. gardneri and its host did not occur. Released spores of J. gardneri were pigmented, contained mitochondria, proplastids and floridean starch reserves, and germinated in the absence of the host; however, the germlings died within two weeks. This research suggested that mature individuals of J. gardneri were obligate epiphytes.

The second part of this thesis reports on the isolation and partial characterization of a proteoglycan from L. spectabilis. The proteoglycan was isolated by extraction in a dilute buffer-NaCl solution followed by gel and ion exchange chromatography, using cellulose acetate strip electrophoresis for monitoring purification. The non-sulfated proteoglycan contained 92% carbohydrate and 8% protein. Galactose (85%) was the major neutral sugar detected. Uronic acids, glucose, xylose and a trace of arabinose were also present. A small quantity of hydroxyproline was present in the molecule.

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FRONTISPIECE

JANCZEWSKIA GARDNERI (ARROW) UPON LAURENCIA SPECTABILIS

P A R T I

STUDIES OF *JANCZEWSKIA GARDNERI* AND ITS SYMBIOTIC RELATIONSHIP WITH *LAURENCIA SPECTABILIS*

INTRODUCTION

Red algae involved in symbiotic relationships have been the subjects of recent research (e.g., Harlin 1971b; Kugrens 1971; Evans, Callow and Callow 1973; Goff 1975; Harlin and Craigie 1975; Turner and Evans 1977). These associations are symbiotic on the basis of De Bary's original definition that symbiosis is the living together of dissimilar organisms in a constant, intimate association (Henry 1966). This broad definition encompasses many kinds of associations including epiphytism, endophytism, and parasitism. The partners of the red algae in these symbioses include bacteria (Bland and Brock 1973), fungi (Kazama and Fuller 1970; Kohlmeyer 1975), sea grasses (Harlin 1971b; McRoy and Goering 1974), green algae (Wilson 1977), brown algae (Markham 1969; Rawlence and Taylor 1970; Hawkes 1977), and other red algae (Kugrens 1971; Evans, et al. 1973; Goff 1975). The symbiosis between the red algae Janczewskia gardneri Setchell & Guernsey and Laurencia spectabilis Postels & Ruprecht, both members of the order Ceramiales, was the subject of this research. This relationship has been known since J. gardneri was first reported (Setchell 1914) as a parasite of L. spectabilis. Chapman and Chapman (1973) considered species of the genus Janczewskia to be either hemi- or holoparasitic, but the exact nature of this relationship was uncertain.

Characteristics of Red Algal Symbiotic Relationships

The symbiotic relationships of the algae, especially the Rhodophyta, exhibit a broad spectrum of association from epiphytes to obligate

parasites (Setchell 1918; Fritsch 1935, 1945; Scott 1969; Joubert and Rijkenberg 1971; Evans, et al. 1973; Goff 1975; Harlin and Craigie 1975). A number of hypotheses to explain the evolution of the various associations between red algae have been presented (Setchell 1918; Sturch 1926; Feldmann and Feldmann 1958; Fan 1961). Setchell (1918) suggested that mutated spores of an alga may germinate on its parent plant and initiate parasitism. On the other hand, Sturch (1926) and Fan (1961) suggested that in most instances of red algal parasitism the parasites originated from epiphytes which had sunk ramifications into their hosts to gain better attachment. Later these attachment organs may have become adapted to absorb nourishment from the host. If the food material was suitable, the 'intruder' may have become irreversibly dependent by loss of its photosynthetic system and/or become reduced in size. If changes occurred with the transition from epiphyte to parasite, they expected to and did find a progression from independence to interdependence between the algae which had entered into the symbiotic relationships.

In other discussion, Setchell (1918) identified three criteria which may be indicative of parasitism in the red algae: penetration, reduction of thallus size, and loss of color. He considered these criteria to apply to epiphytes, endophytes and parasites, and concluded that as morphological features they provided only preliminary indications of a possible parasitic relationship. He recognized the need to demonstrate metabolite transfer from the host to the parasite and a dependence of the parasite upon the host to establish the existence of a parasitic relationship. Setchell also pointed out that many of the parasitic red algae are found

on closely related hosts with host and parasite usually in the same family. In the approximately 40 genera of suspected red algal parasites, about 90% belong to the same family or order as their host (Feldmann and Feldmann 1958; Dawson 1966). Parasites which exhibit such close systematic relationships with their hosts were called adelphoparasites, whereas those lacking a close relationship were termed alloparasites (Feldmann and Feldmann 1958).

Janczewskia gardneri

J. gardneri was first described as occurring "on Laurencia pinnatifida, particularly that broad, coarse form called L. spectabilis by Postels and Ruprecht" (Setchell 1914). The range of J. gardneri extends along the eastern Pacific coast from Vancouver Island, British Columbia to Baja California (Abbott and Hollenberg 1976). L. spectabilis is the predominant host species throughout the range of J. gardneri except for rare reports of L. splendens as the host species from central California and Baja California. J. gardneri is an adelphoparasite on the basis of the terminology of Feldmann and Feldmann (1958), since it and its hosts are classified in the subdivision Laurencieae of the family Rhodomelaceae in the Ceramiales (Hommersand 1963).

The thallus of J. gardneri is reduced and tubercle-like, forming pinkish cushions of irregular size (see Frontispiece). Setchell (1914) reported that from the thallus "slender hyphal branches" penetrated to the host's elongated central cells and that no connections between the two plants were present. On the basis of these morphological and

anatomical characters J. gardneri was regarded as parasitic by Setchell (1914), Fritsch (1945), Chapman and Chapman (1969), Smith (1969), and Abbott and Hollenberg (1976). Kugrens (1971) examined the ultrastructure of vegetative and reproductive structures of ten genera of parasitic red algae, including J. gardneri. He reported that J. gardneri possessed "typical red algal chloroplasts with respect to thylakoidal structure and presence of phycobilisomes" (He detected chlorophyll a and r-phycoerythrin, but not r-phycocyanin.). Due to intermingling of tissues, Kugrens did not identify any pit connections between J. gardneri and L. spectabilis, nor could he see the distinct rhizoidal filaments mentioned in Setchell's original description. Kugrens also tried to determine if ^{14}C-labeled photosynthetic products of the host were translocated to the parasite or vice versa, but he obtained no conclusive answers. Nevertheless, he did obtain evidence that J. gardneri was capable of photosynthetically assimilating the label, although at a much lower rate than its host. He germinated tetraspores of J. gardneri in culture, but they died within two weeks even in the presence of host plants. His study did not resolve the exact nature of the association between J. gardneri and L. spectabilis.

Other Red Algal Symbioses

Two red algal epiphytes have been the subjects of recent research. Smithora naiadum is an obligate epiphyte found normally on the sea grasses Phyllospadix scouleri or Zostera marina. Radioactive tracer studies (Harlin 1971a, b) with ^{32}P and ^{14}C showed translocation of those elements to proceed in both directions between host and epiphyte. Later work by

McRoy and Goering (1974) also indicated a direct transfer of carbon and nitrogen from Z. marina to its leaf epiphytes, including S. naiadum. Although this exchange occurred, field studies by Harlin (1971b, c, 1973a) using an artificial substrate (fibrous polypropylene strips) resulted in the epiphytes attaching and growing into irregularly-shaped plants. Harlin 1973a) suggested that "this supposedly obligate epiphyte does not require a chemical factor from its normal host plant for (1) substrate selection and adhesion, (2) growth of basal cushions and young blades or (3) maturation of thick cushions", and "that the primary role of the host is to provide a physical environment for an opportunistic species, while it secondarily may provide biochemical factors for normal development of an epiphyte and subsequent completion of its life history".

Polysiphonia lanosa occurs upon the brown alga Ascophyllum nodosum or occasionally upon species of Fucus (Rawlence and Taylor 1970). P. lanosa differs from S. naiadum in that it has host-penetrating rhizoids. Subsequent studies supplied evidence that the penetration was due more to chemical than mechanical means (Rawlence and Taylor 1972; Rawlence 1972). Degeneration and almost complete disappearance of host tissue occurred in advance of and surrounding the rhizoid. Ultrastructural changes indicated increased cell activity for P. lanosa, but no tests for enzymes were performed. The physiological aspect of possible metabolite transfer between P. lanosa and A. nodosum was examined with ^{14}C-labeled glutamic acid by Citharel (1972a, b) and with inorganic radioisotopes (^{32}P, ^{86}Rb, ^{99}Mo, ^{24}Na) by Penot (1974). These studies showed that there was some transfer of radioactive substances from the brown alga to the red alga. Later

studies (Harlin and Craigie 1975; Turner and Evans 1977) determined that P. lanosa could assimilate ^{14}C-bicarbonate photosynthetically and that it did not appear to receive nor to depend upon ^{14}C-labeled products of photoassimilation from A. nodosum. Harlin and Craigie (1975) suggested that the association of the two algae was based on the need for growth factors from the host for germination or cell division in an early stage of the life history of the epiphyte or on surface characteristics of the host critical for the attachment of P. lanosa spores. Turner and Evans (1977) suggested that carbon metabolism need not be the sole basis for such a relationship between any two algae and that some aspect of epiphyte spore settlement and attachment may be of greater importance. Rawlence (1972) also suggested that "any nutrition dependence on the host is incidental to attachment".

The red alga Gonimophyllum skottsbergii, an adelphoparasite, has a small, leafy, pinkish thallus and is associated with the red alga Botryoglossum ruprechtiana (Harlin 1971b). In Kugrens (1971) ultrastructural study of ten genera of 'parasitic' red algae, he reported that G. skottsbergii had 'typical' red algal chloroplasts and could photosynthesize. Harlin (1971b, 1973b) also found that it assimilated ^{14}C-label photosynthetically. In addition, she reported that the 'parasite' translocated the assimilated ^{14}C-labeled materials to its 'host' as well as receiving translocates from its host.

Evans, Callow and Callow (1973) examined "the parasitic, chloroplast-free, red alga Holmsella pachyderma", an alloparasite, and its red algal host Gracilaria verrucosa. Their studies indicated that metabolite

movement occurred from host to parasite only as the carbohydrate floridoside, the main photosynthetic product of G. verrucosa, was translocated to the parasite. A significant amount of the detected ^{14}C-label in H. pachyderma was found in mannitol, a compound which was not identified in the host, so H. pachyderma was capable of metabolizing substances translocated from its host. Since H. pachyderma was shown to be chloroplast-free by electron microscopy and therefore heterotrophic, the transfer of the floridoside established that H. pachyderma was indeed parasitic on G. verrucosa. It was suggested that the movement of the carbohydrate was most likely through the endophytic filaments which arise from the base of the parasite and penetrate between the cells of the host. The large number of mitochondria present in the endophytic filament cells was suggested to be important in providing energy for the movement of substances between host and parasite.

Goff (1975) investigated morphological and physiological aspects of another red algal alloparasite, Harveyella mirabilis, with its red algal hosts Odonthalia floccosa (the primary host in the northeast Pacific), O. washingtoniensis, and Rhodomela larix. Field and laboratory studies of the development and reproduction of H. mirabilis showed that the completion of its reproductive life history was dependent on the presence of a suitable host for germination and that the development and reproduction were affected by seasonal changes in seawater temperature and photoperiod. H. mirabilis was found to be physiologically dependent upon the host O. floccosa since the parasite lacked 'normal' red algal chloroplasts and did not photoassimilate ^{14}C-bicarbonate, but it did receive

^{14}C-labeled photoassimilated products from O. floccosa. Light microscopic radioautography showed the primary flow of label to occur through the rhizoidal cells of the parasite. A secondary source of label was from isolated host cells dispersed in the H. mirabilis pustule. Carbohydrates (which could not be specifically identified) were apparently the main radioactive substances translocated from host to parasite, although the presence of proteins and lipids as well as carbohydrates was associated with a vacuole/vesicle transport system detected in the rhizoidal cells of H. mirabilis.

From this survey of work on red algal associations, it can be seen that the relationships between the attached organism and its host are often quite complex. There is evidence for a strong degree of host specificity which may be related to physical attachment or a biochemical factor supplied by the host. Any of these relationships has to involve the attachment and germination of a spore, which may involve the production of enzymes for degrading the host's cells. Additional substrate specificity can involve the availability of 'suitable' metabolites of the host for the symbiont. The ability of a parasite to obtain metabolites and to convert them into other utilizable compounds has been established for two alloparasites, Holmsella pachyderma (Evans, et al. 1973) and Harveyella mirabilis (Goff 1975). In addition, evidence from both studies suggests that the endophytic attachment organs of such algae can be specialized for actively transporting translocates from their hosts.

Objectives For This Study

I decided to investigate a red algal symbiotic relationship involving an adelphoparasite because it was of interest to increase the limited information about this large group of red algal parasites. I chose the supposedly parasitic Janczewskia gardneri because of its specific association with the red alga Laurencia spectabilis, its unclear physiological relationship with its host, and its abundance almost year-round in the local flora.

The general purpose of this study was to determine the exact relationship of an adelphoparasite (J. gardneri) with its host (L. spectabilis), including the extent of independence and interaction of the parasite with its host, and the possible nature of substrate selection by the parasite. The specific objectives were:

(1) to determine photosynthetic rates for the host and parasite when separate and when associated.

(2) to identify the photosynthetic products of host and parasite.

(3) to determine if translocation between host and parasite can occur in either direction, and if so, what substances are translocated.

(4) to determine if there is a preferential selection of a host life history stage for invasion by the parasite.

(5) to determine if the reproductive spores of the parasite have the capacity (e.g., chloroplasts, starch reserves, mitochondria) to grow independently from the host.

(6) to try to grow the parasite from spores in culture.

MATERIALS AND METHODS

CHEMICALS AND SOLVENTS

Chemicals and solvents, reagent grade ACS or better, were obtained from the suppliers as indicated: glycerol (Allied Chemical Canada, Ltd., Pointe Claire, Quebec); acetic anhydride, uranyl acetate (J.T. Baker Chemical Co., Phillipsburg, New Jersey); Beckman Amino Acid Calibration Mixture Type 1 (Beckman Instruments, Inc., Spinco Division, Palo Alto, California); D-galactose, D-galactosamine HCl, pyridine AnalaR ACS (used for GLC preparations), sodium borohydride (British Drug Houses Ltd., Poole, England); all amino acids (Calbiochem, Los Angeles, California); caprylic acid, trifluoroacetic acid (Eastman Kodak Co., Rochester, New York); lead citrate, most reagents for Spurr's resin (Electron Microscopy Sciences, Fort Washington, Pennsylvania); germanium dioxide, D-mannose, D-mannitol, "Scinti-Verse" scintillation cocktail (Fisher Scientific Co., Fair Lawn , New Jersey); sodium glucuronate (Koch-Light Laboratories Ltd., Colnbrook, England); L-fucose, D-galacturonic acid (monohydrate), D-glucose, D-xylose (Nutritional Biochemicals Corp., Cleveland, Ohio); methyl cellosolve, ninhydrin (Pierce Chemical Co., Rockford, Illinois); glutaraldehyde, OsO_4 (Polysciences, Inc., Warrington, Pennsylvania); D-glucosamine HCl, myo-inositol (Sigma Chemical Co., St. Louis, Missouri). Floridoside and isofloridoside were provided by Dr. J.S. Craigie, Atlantic Regional Laboratory, Halifax, Nova Scotia. D-galactitol, D-glucitol, D-mannitol and their acetates, L-fucitol pentaacetate, and D-xylitol pentaacetate were provided by Dr. J.N.C. Whyte, Fisheries and Marine Service, Vancouver, British Columbia. All other chemicals and solvents were obtained locally and were of reagent grade ACS quality or better.

SEAWATER

Seawater from the collection site was used to transport fresh plant material to the laboratory. All other seawater used in this study was obtained from Botany Beach near Port Renfrew, British Columbia and before use was membrane-filtered (0.45 µm membrane; Millipore Corp., Bedford, Massachusetts).

COLLECTION SITES AND PROCEDURES

Laurencia spectabilis with and without attached *Janczewskia gardneri* was obtained from four locations on Vancouver Island, British Columbia (see Fig. 1A) between December 1973 and 1976. The main collection area was Botany Beach (the north end) approximately 1 km SE of San Juan Point near Port Renfrew, B.C. The other three sites were Cable Beach on Barkley Sound near Bamfield, B.C.; Whiffen Spit at the harbor entrance, Sooke, B.C.; and the harbor entrance breakwater, Victoria, B.C. Additional collections were made at least once between November 1972 and December 1976 at Saturnina Island, B.C. (Fig. 1A), two sites in Oregon (Fig. 1B), and three sites in California (Fig. 1C). Herbarium specimens of *L. spectabilis* and *J. gardneri* were examined at The University of British Columbia, Vancouver, B.C.; University of California, Berkeley, CA.; and California Polytechnic State University, San Luis Obispo, CA. These specimens had been obtained along the entire known range of *J. gardneri* on the eastern Pacific coast from Vancouver Island, British Columbia to Baja California.

Freshly collected plants were placed in plastic bags containing seawater and kept on ice during transport to the laboratory. They were

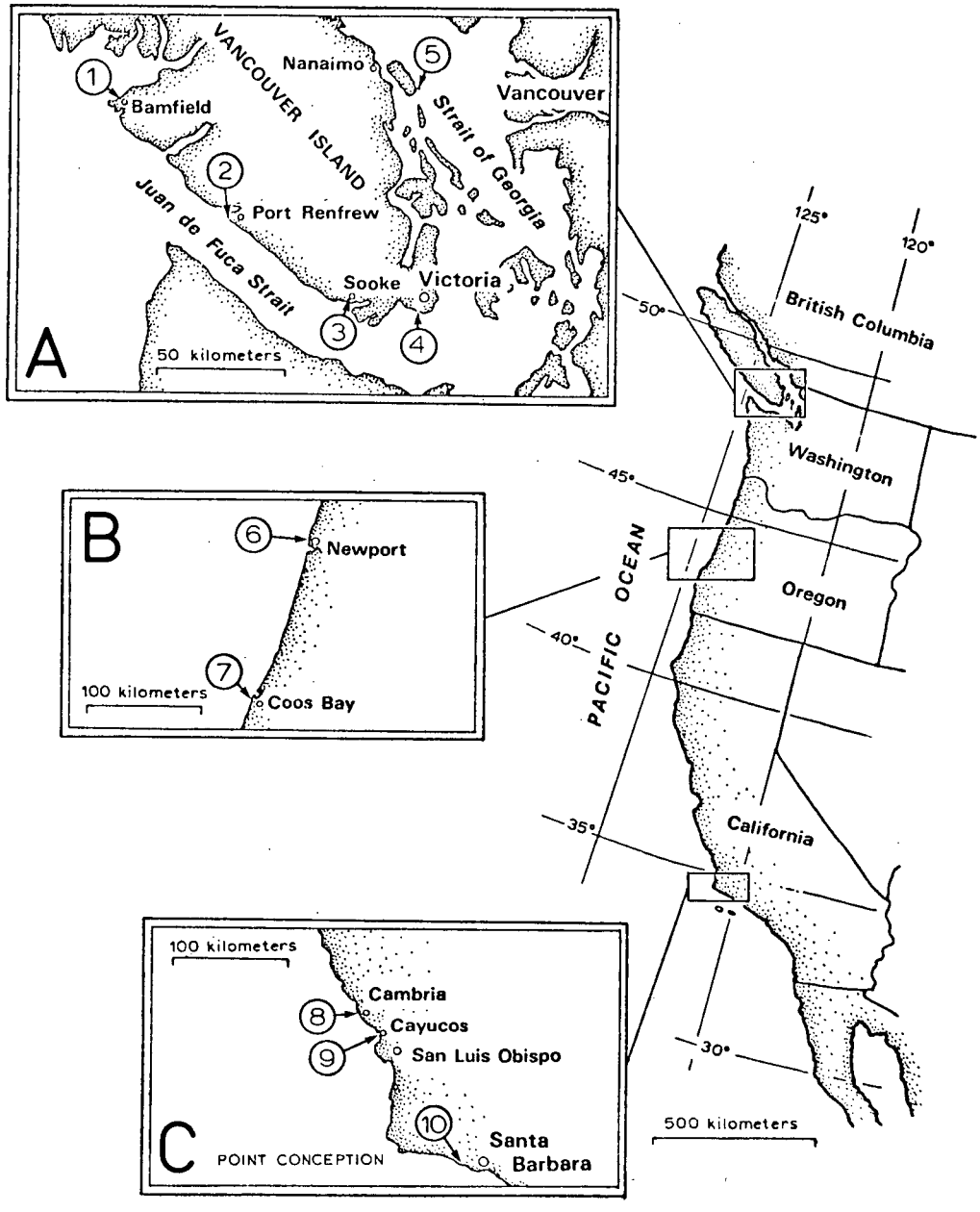
Fig. 1. Collecting sites in British Columbia (A), Oregon (B), and California (C).

(A) Sites in British Columbia were (1) Cable Beach, (2) Botany Beach, (3) Whiffen Spit, (4) Victoria breakwater, and (5) Saturnina Island, one of the Flat Top Islands off the eastern tip of Gabriola Island.

(B) Sites in Oregon were (6) Yaquina Head, north of Newport, and (7) South Cove, Cape Arago, south of Coos Bay.

(C) Sites in California were (8) Cambria, (9) Cayucos, and (10) Coal Oil Point, north of the University of California, Santa Barbara campus.

FIGURE 1


A
VANCOUVER ISLAND
Nanaimo
Bamfield
Port Renfrew
Juan de Fuca Strait
Strait of Georgia
Vancouver
Sooke
Victoria
50 kilometers
B
Newport
Coos Bay
100 kilometers
C
100 kilometers
Cambria
Cayucos
San Luis Obispo
Santa Barbara
POINT CONCEPTION
125°
120°
50°
45°
40°
35°
30°
British Columbia
Washington
Oregon
California
PACIFIC OCEAN
500 kilometers

maintained in open containers with aeration in a 10°C growth chamber and provided with a 12 hr photoperiod (5.6 x 10^3 ergs/cm^2/sec light; YSI-Kettering Model 65 radiometer).

Specimens were preserved by fixation with either 30% ethanol or Karpechenko's solution (Papenfuss 1946). The material was dehydrated in a graded ethanol series and stored in 70% ethanol.

LIGHT MICROSCOPY

Observations were made primarily on fresh material. Reproductive structures were usually examined from squashed plant preparations. The attachment region between the two plants was studied in tissue sections cut with a freezing microtome and were examined unstained or stained with toluidine blue.

ELECTRON MICROSCOPY

Fresh plant material for electron microscopy was fixed on ice in the field or in the laboratory. J. gardneri or pieces of L. spectabilis with attached J. gardneri were fixed for 1 hr in either (a) 50% glutaraldehyde: 0.07 M sodium phosphate buffer (pH 7.2): seawater (1:4:4 v/v) (adapted from McBride and Cole 1969) or (b) 5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0). Both methods of fixation were followed by post-fixation for 2 hr at 5°C in a solution containing equal volumes of 2% (w/v) OsO_4 and the phosphate buffer used for the fixation. The material was washed in the buffer, dehydrated in a graded ethanol series, and embedded in Spurr's resin (Spurr 1969). Sections were cut using glass

knives on a Reichert OM U3 ultramicrotome and were post-stained for 30 min with saturated uranyl acetate followed by 8 min with lead citrate (Reynolds 1963). Sections were viewed in a Zeiss EM 9S transmission electron microscope.

PHOTOSYNTHESIS AND TRANSLOCATION EXPERIMENTS

A. Preparation of Plant Material. Fresh plant material stored overnight in the 10°C growth chamber was used for the experiments. Plants for photosynthesis experiments #1, 2 and 3 were collected at Botany Beach on 18 August 1974, 24 February 1975, and 11 July 1976. Plants for translocation experiments #1 and 3 were from Botany Beach on 24 February 1975 and 11 July 1976, and for experiment #2 plants were from Whiffen Spit on 31 March 1975.

J. gardneri and pieces of L. spectabilis with or without attached J. gardneri were selected and cleaned of macroscopic epiphytes at least 14 hr before any uptake of ^{14}C-label was allowed. The clean material was put in petri plates (100 x 15 mm) containing seawater. The plants were maintained at ca. 10°C and were kept in the dark for at least 8 hr followed by a minimal pretreatment of 6 hr light or dark. The pretreatment condition (light or dark) in the photosynthesis experiments was the same condition under which the plants were allowed to take up the radioactive label. The pretreatment conditions in translocation experiments #1 and 2 were the same conditions as during the experimental translocation period, but in experiment #3 all plants were pretreated with light.

B. Radioactive Label. ^{14}C-sodium bicarbonate (sp. act. 56-60 mCi/mM) was used for the experiments. Seawater was acidified to pH 2.0 with HCl and stirred to remove CO_2. The pH was readjusted to pH 7.9 with NaOH. The radioisotope was then added to give a final concentration of 2 μCi $NaH^{14}CO_3$/ml.

C. Photosynthesis. The algae used in the three photosynthesis

experiments were allowed to take up the label for between 10 sec and 30 min in the light (2.6-2.8 x 10^5 ergs/cm^2/sec) or dark from 25 ml of radioactive seawater in 100 x 15 mm petri plates. In the three experiments there were three replicates for each light and dark (control) treatment used. After the uptake period, the plant material was removed immediately, rinsed thoroughly in seawater, killed by submersion in liquid N_2, and stored at ca. -70°C.

D. Translocation. Table I summarizes conditions of light and dark pretreatments, labeling periods, and light intensities used in the translocation experiments. In the three translocation experiments, pieces of L. spectabilis with J. gardneri were allowed to assimilate the label in the light and then were removed from the radioactive seawater (25 ml) and rinsed thoroughly with seawater. Three samples were taken, killed by submersion in liquid N_2 and stored frozen (ca. -70°C), while the other radioactive plant material was placed in petri plates containing 25 ml non-radioactive 'chase' seawater. These plants were allowed to translocate in the light or dark for periods of 2 to 12 hr. Three samples were removed at the end of each translocation period, killed by submersion in liquid N_2, and stored frozen. The chase seawater from translocation experiments #2 and 3 was kept for scintillation counting.

E. Measurement of Sample Radioactivity. Frozen radioactive plant material was thawed and any attached J. gardneri plants were dissected from their host material. The plants were blotted, weighed, and digested in scintillation vials using perchloric acid and hydrogen peroxide (Lobban 1974). The scintillation counting "cocktail", 10 ml of "Scinti-Verse", was added and mixed thoroughly with the digested material. The

TABLE I

SUMMARY OF CONDITIONS FOR THE TRANSLOCATION EXPERIMENTS AT $10^{o}C$

Experiment	Plant Material	Pretreatment Conditions ($ergs/cm^2/sec$)	Labeling Period (min)	Labeling Light Intensity ($ergs/cm^2/sec$)	Chase Conditions ($ergs/cm^2/sec$)
#1	Botany Beach 24 February 1975	Light 8×10^3	60	1.4×10^5	Light 8×10^3
#2a	Whiffen Spit 31 March 1975	Light 8×10^3	30	8.0×10^3	Light 8×10^3
#2b	Whiffen Spit 31 March 1975	Dark	30	8.0×10^3	Dark
#3a	Botany Beach 11 July 1976	Light 8×10^3	30	8.0×10^3	Light 8×10^3
#3b	Botany Beach 11 July 1976	Light 8×10^3	30	8.0×10^3	Dark

samples were counted using a Nuclear Chicago Unilux II-A scintillation counter and counts were corrected using the channel-ratio method (Wang and Willis 1965). Residual materials were also treated in this manner. Radioactivity in other samples was determined after addition of a suitable portion of the solution to 10 ml of "Scinti-Verse". Radioactivity of paper chromatograms was measured by placing portions of the paper in Bray's fluid (Bray 1960) for counting.

F. Extraction and Fractionation of Radioactive Compounds. Plant materials from photosynthesis experiments #1 and 2 were extracted separately and examined for radioactive compounds. J. gardneri plants and L. spectabilis portions which had been incubated separately in the radioactive seawater were used. These algae were exposed to the radioactive label in the light or dark for 30 min.

Each plant sample (0.1-0.9 gm wet wt) was extracted for 15 min with 100 ml boiling 80% ethanol, for 10 min with 100 ml boiling 50% ethanol, and again for 10 min with 100 ml of boiling 80% ethanol. The combined extracts were dried by rotary evaporation at 40°C and then dissolved in deionized water. The extracted residues were air-dried and later prepared for scintillation counting. Chloroform was added to each aqueous extract to partition lipids and pigments. The chloroform-phase was evaporated and counted. The volume of the water-phase was reduced to less than 5 ml by rotary evaporation at 40°C, then desalted and fractionated using a column of Rexyn 101(H) (3 x 25 cm; Fisher Scientific Co.) and next a column of Duolite A-4 (3.5 x 16 cm; Diamond Alkali Co., Redwood City, CA). The neutral fraction, which consisted of sugars, was collected after

passage of the extract through both columns. Amino acids were eluted from the Rexyn 101(H) cation exchange resin with 2 N NH_4OH, while organic acids were eluted from the Duolite A-4 anion exchange resin with 0.2 N formic acid. The volumes of these three fractions were reduced by rotary evaporation and samples were taken for scintillation counting.

The identification of sugars in the neutral fraction was facilitated by further fractionation using a column of acid-free charcoal (2 x 4.7 cm). The samples were applied in less than 5 ml of deionized water and eluted in water (100 ml) followed by a step-wise gradient (100 ml each) of 5, 10, 20, and 35% ethanol. These "fractionated-neutral" fractions were concentrated separately by rotary evaporation for paper chromatography.

G. Identification of Labeled Compounds.

1. Paper chromatography. The neutral, "fractionated-neutral", cationic and anionic fractions from each plant extract, and standard sugar mixtures were analyzed by one-dimensional paper chromatography on Whatman No. 1 paper using two solvent systems (Whyte and Southcott 1970): (a) ethyl acetate:pyridine:H_2O (10:4:3 v/v) and (b) ethyl acetate:acetic acid: formic acid:H_2O (18:3:1:4 v/v). Dried chromatograms were covered with thin plastic-wrap (e.g., "Handi-Wrap", Dow Chemical Co.) before radio-autography, otherwise the entire chromatogram became sensitive to the reagents used to detect sugars. The labeled compounds were detected by exposure to Kodak Blue Brand BB 14 medical X-ray film. Sugars were located on the chromatograms by modifications (J.N.C. Whyte, K. Mackie, pers. comm.) of the silver nitrate procedure of Trevelyan, Procter and Harrison (1950). Dried chromatograms were dipped in the silver nitrate

reagent, dried, and dipped in 0.5 n NaOH in 95% ethanol. Spot development was enhanced after the NaOH-ethanol dip by steaming the chromatograms for a few seconds, being careful not to overheat. The steamed chromatograms were placed in 5% $Na_2S_2O_3$ solution (25 gm sodium thiosulfate, 25 gm sodium acetate, 0.5 ml glacial acetic acid, plus 500 ml H_2O) for 10-15 min, then washed with tap water before drying.

2. Thin-layer chromatography. Organic acid fractions were separated on Avicel (American Vicose Co., Marcus Hook, PA.) thin-layers (ca. 0.65 mm) which were developed in one or two directions using the same solvent system of n-amyl alcohol:formic acid (97%):H_2O (20:20:1 v/v). Labeled compounds were detected by radioautography as described earlier. Acidic compounds were visualized by spraying the thin-layer with a solution of bromothymol blue (0.04 gm in 100 ml of 95% ethanol, adjusted with NaOH to pH 10.0).

3. High voltage paper electrophoresis. Neutral, cationic and anionic fractions, and mixtures of reference amino acids were separated by paper electrophoresis using a Michl-type liquid cooled apparatus (Michl 1951, 1959). The samples were applied to Whatman 3MM chromatography paper and electrophoresis was performed for 40 min at 3 kV (ca. 52.6 V/cm of paper length) in acetic acid:formic acid:H_2O (8:2:90 v/v, pH 2.0). Labeled compounds were detected by radioautography and amino acids were located on the electrophoregram by dipping in a cadmium acetate-ninhydrin solution (Heilmann, Barrollier and Watzke 1957).

4. Gas-liquid chromatography (GLC). Non-radioactive neutral fractions were obtained from fresh plant materials kept in conditions similar to those of the experimental plants. Alditol acetate derivatives

of the unknown and standard sugars were prepared as described by Albersheim, et al. (1967). Samples to be analyzed by mass spectrometry were derivatized by the method of Björndal, Lindberg and Svensson (1967). Samples were hydrolyzed for 1 hr at 110°C in 2 N trifluoroacetic acid and dried in a stream of air before derivatization. Varian Aerograph Model 1740 and Hewlett Packard Model 5710A dual column gas chromatographs were used for the analyses. The gas flow rates in both machines were 25 ml/min for N_2 and H_2, and 250 ml/min for air. Two analytical systems were used. (1) Stainless steel columns (6' x 1/8" o.d.) were packed with 5% (w/w) Silar 10C (Alltech Associates, Arlington Heights, IL.) on 100/120 mesh Gas Chrom Q (Applied Sciences Lab. Inc., State College, PA.). Samples were injected at 120°C followed by a programmed increase in temperature from 120° to 260°C at 2° or 4°/min. (2) Copper columns (6' x 1/8" o.d.) were packed with 3% (w/w) ECNSS-M on 100/120 mesh Gas Chrom Q (prepared by Applied Sciences Lab. Inc.). Samples were injected and held at 120°C for 10 min before an increase in temperature from 120° to 185°C was programmed at 2°/min. The samples used in both analytical systems were injected in various solvents: acetic anhydride, acetone, chloroform, ethanol, and ethyl acetate.

Samples for mass spectrometry were obtained using a preparative column (6' x 1/4" o.d. stainless steel) packed with 5% (w/w) Silar 10C on 100/120 mesh Gas Chrom Q in a F & M Model 720 gas chromatograph which was run isothermally at 250°C. Samples were collected in glass capillary tubes at the machine exit port.

5. Mass spectrometry and melting point determinations. The purity of samples collected from the preparative Silar 10C column was checked by analysis of a portion using the analytical Silar 10C columns. Mass spectrometry was performed in a Varian/MAT CH4-B mass spectrometer using the direct-insertion technique. Probe temperatures were 100° and 120°C, and the source temperature was ca. 150°C. The electron energy was 70 eV. Mass spectra were obtained for the unknown component(s) of the material resolved as a single peak by GLC and suspected to be floridoside (2-O-α-D-galactopyranosylglycerol), isofloridoside (1-O-α-D-galactopyranosylglycerol), or both. Reference samples of these two compounds were also analyzed. Melting points of all samples were determined, except for acetylated isofloridoside which was not obtained in solid form; acetylated floridoside has a value of 101°C (Putman and Hassid 1954).

6. Amino acid analysis. Non-radioactive cationic fractions were obtained from fresh plant material stored in the 10°C growth chamber. The ethanol-soluble free amino acids were analyzed by the method of Spackman, Stein and Moore (1958) using a Beckman Amino Acid Analyzer Model 120C. Basic amino acids were separated on a 15.5 x 0.9 cm column. The acidic and neutral amino acids were separated on a 58 x 0.9 cm column (Cameron 1972). The amino sugars glucosamine and galactosamine were resolved from each other and from other amino acids in both of these systems.

7. Computer analyses. Data from the photosynthesis and translocation experiments were analyzed with the aid of two computer programs used at the Institute of Animal Resource Ecology Data Centre, The University of British Columbia. Program SASTT was used to calculate the

means, variances and standard deviations of two sets of numbers, and to test for significant differences (α=0.05) between the two sets using a T-test. This program was used to analyze the photosynthesis data. Program ANVAR was used to analyze the data from the translocation experiments. The program performed a one-way analysis of variance (α=0.05) and an option was used to perform a Schefe's Test for multiple comparison among the levels of the one-way analysis of variance.

FREE SUGARS AND AMINO ACIDS

Fresh plant material of J. gardneri and L. spectabilis collected at Botany Beach (16 April and 14 May 1976), and L. spectabilis from Cable Beach (13 May 1976) was sorted according to detectable life history stages (male or female gametophyte, or tetrasporophyte). The plants were extracted for 30 min in boiling 80% ethanol. The extracts of ethanol-soluble free sugars and amino acids were prepared and analyzed by GLC and amino acid analyzer as described earlier, except that the extracts were not fractionated by ion exchange chromatography.

CULTURE OF SPORES

Material collected at the Victoria breakwater (28 June and 12 July 1976) and at Botany Beach (11 July 1976) was used. J. gardneri plants and portions of L. spectabilis, having either carposporangia or tetrasporangia, were cleaned and placed separately on glass cover slips in petri plates (60 x 15 mm) containing 10 ml of culture medium [1 l. membrane-filtered seawater, 20 ml enrichment solution (ES from Table 2-5,

McLachlan 1973), 5 ml germanium dioxide (250 mg/l. distilled water)]. The plants were maintained in a growth chamber (10°C) with a 12 hr photoperiod; the light was 3.4×10^3 ergs/cm^2/sec. Spores were usually released within 24 hr, and the source plant material was removed. Medium was changed every two to three days, and some spores were transferred on glass cover slips or by capillary pipettes to other petri plates. Culture conditions were also varied in an attempt to improve growth by agitating the cultures on a shaker table, or adding pieces of L. spectabilis to cultures of J. gardneri spores, or both.

OBSERVATIONS AND RESULTS

HOST LIFE HISTORY STAGES SELECTED BY SYMBIONT

Observations on fresh, preserved, and herbarium materials revealed that there was no preferential selection of host life history stages by the symbiont. Absence of preferential selection was confirmed during examination of fourteen collections, which consisted of over 500 plants of each species, from Botany Beach over a two year period (July 1974-76). The life history stages of the symbiotic partners in any given collection was apparently random; indeed, two or more life history stages of the symbiont were commonly observed on one host plant.

LIGHT MICROSCOPIC EXAMINATION OF THE ATTACHMENT REGION

Examination of sectioned material showed that J. gardneri had filaments which penetrated between cells of host tissue (Fig. 2), but there were no visible pit connections between the cells of the two plants. The region in which the tissues of the two plants intermingle was limited. Neither the larger host cells nor the smaller symbiont cells were spread throughout the other organism (Fig. 2). Thus photosynthesis and translocation experiments could be undertaken with minimal risk of interference from "contaminating" cells.

PIGMENTATION AND CHLOROPLASTS

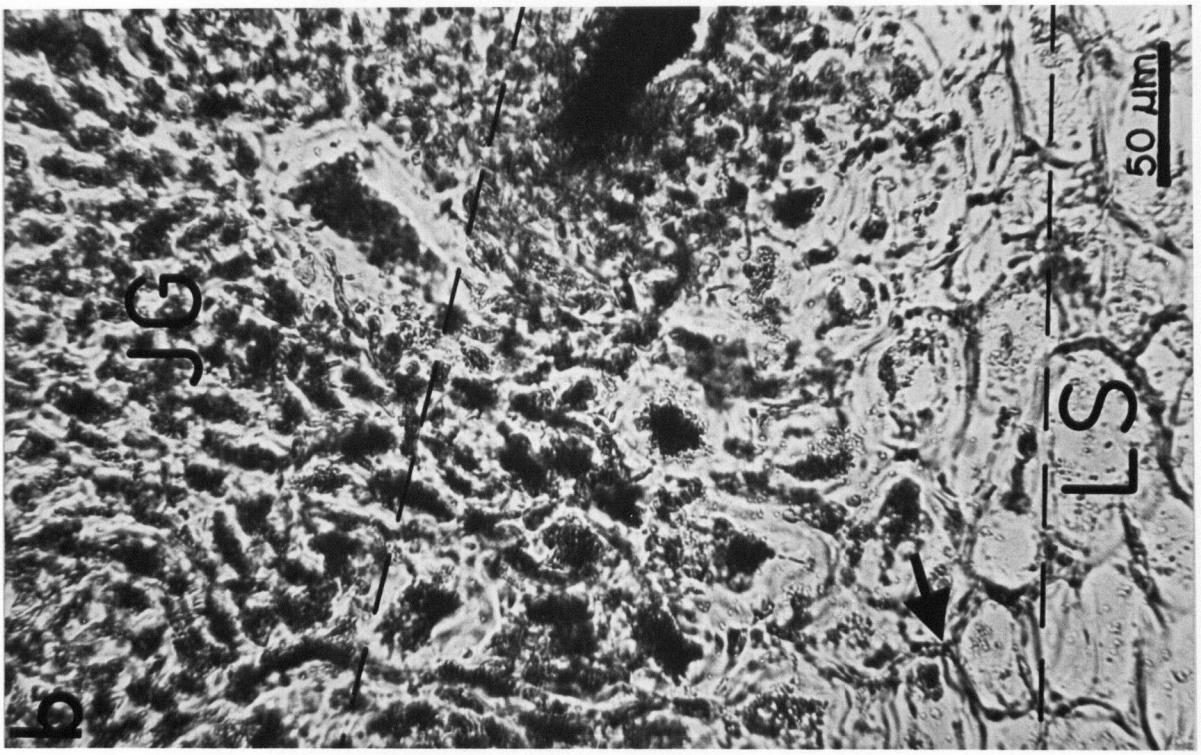
Chloroplasts of J. gardneri and L. spectabilis were observed by light microscopy in sections cut with a freezing microtome. Both whole plants

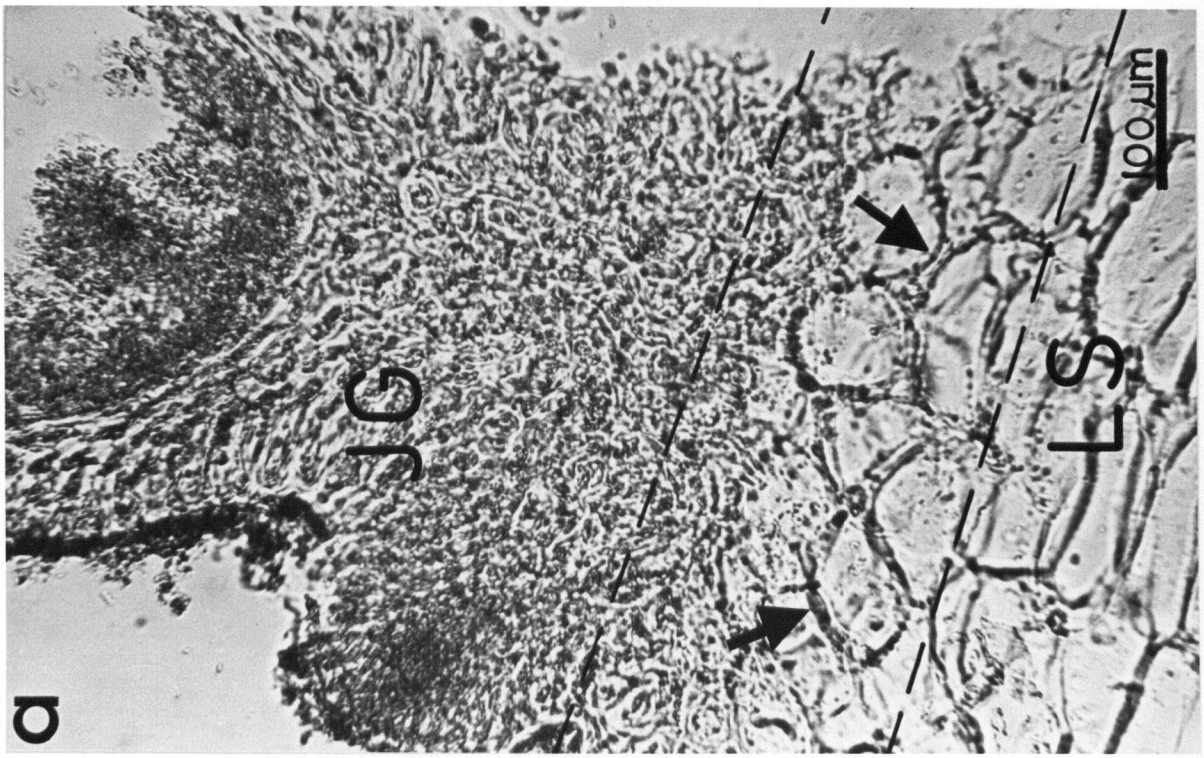
Fig. 2. Attachment region between J. gardneri and L. spectabilis.

The light micrographs are of unstained sections, cut with a freezing microtome, of J. gardneri (JG) attached to L. spectabilis (LS). Dotted lines indicate the boundaries of the attachment region between the two plants. Arrows in both micrographs point to slender filaments of J. gardneri cells penetrating between the large host cells.

(A) Attached J. gardneri plant is male, spermatangial branches visible in upper right-hand corner.

(B) Attached J. gardneri plant is vegetative.


b
JG
LS
50 µm


a
JG
LS
100 µm

(Frontispiece) and sections of J. gardneri had a reddish hue fainter than the red color of L. spectabilis plants and sectioned material. The carpospores and tetraspores of both plants were pigmented, but chloroplasts were not discernible by light microscopy due to other spore contents.

Electron microscopic investigation confirmed that J. gardneri had chloroplasts (Fig. 3). Some chloroplasts had thylakoids arranged stack-like, with an outer encircling thylakoid. Another common arrangement had only encircling thylakoids which appeared in cross-section as concentric rings. Observation of J. gardneri carpospores and tetraspores before their release from parent plants (Fig. 4) showed many proplastids as well as floridean starch, mitochondria, and dictyosomes present.

PHOTOSYNTHESIS EXPERIMENTS

A. Uptake of Radioactive Label. The results of photosynthesis experiment #1 are shown in Table II. Similar results were obtained in experiments #2 and 3. Both J. gardneri and L. spectabilis took up the radioactive label from the seawater containing $NaH^{14}CO_3$. This uptake by J. gardneri occurred whether or not it was attached to the host. Plants kept in the dark took up little of the label and from this I conclude that the net incorporation by both plants during the light treatment is a light-dependent phenomena. The quantity of label taken up by each alga (whether separate or attached to one another) did not in most instances differ significantly at the 5% level in the three experiments. One exception was in experiment #1 (Table II), when the incorporation by "separate" (non-attached) L. spectabilis in the light for 30 sec differed significantly from the uptake of "attached" plants. The other occurrence was in experi-

Fig. 3. Chloroplasts from a vegetative cell of J. gardneri.

The arrow points to a region where two chloroplasts (p) are dividing, also present are several mitochondria (m) and a nucleus (n). (Plant material was fixed for electron microscopy using method b in the Materials and Methods.).

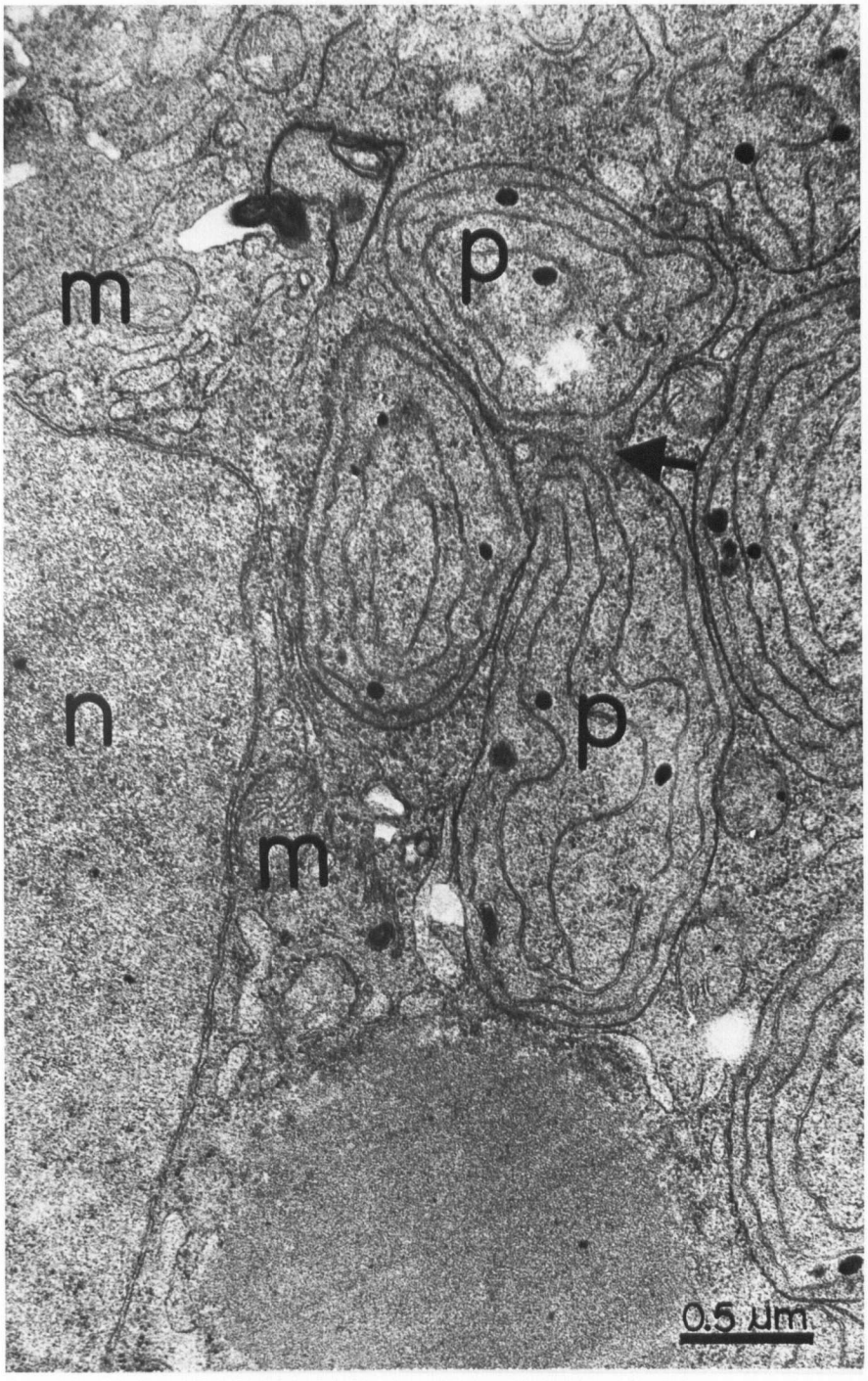

m
p
n
p
m
0.5 µm

Fig. 4. Electron micrographs of J. gardneri tetraspores (A) and carpospores (B, C) before release from the parent plants.

Plant material was fixed using method a in the Materials and Methods.

(A) Two tetraspores are present and are divided by a thick cell wall (cw). Both tetraspores contain numerous proplastids (p) which contain a single encircling thylakoid. Mitochondria (m) and floridean starch (s) are evident as are dictyosomes (d).

(B) This carpospore has numerous proplastids (p) present peripherally and centrally as well as floridean starch (s).

(C) Dividing proplastids (p) are indicated by the arrow in this section from a carpospore. Also present are floridean starch (s), mitochondria (m), and a dictyosome (d) and associated vesicles.

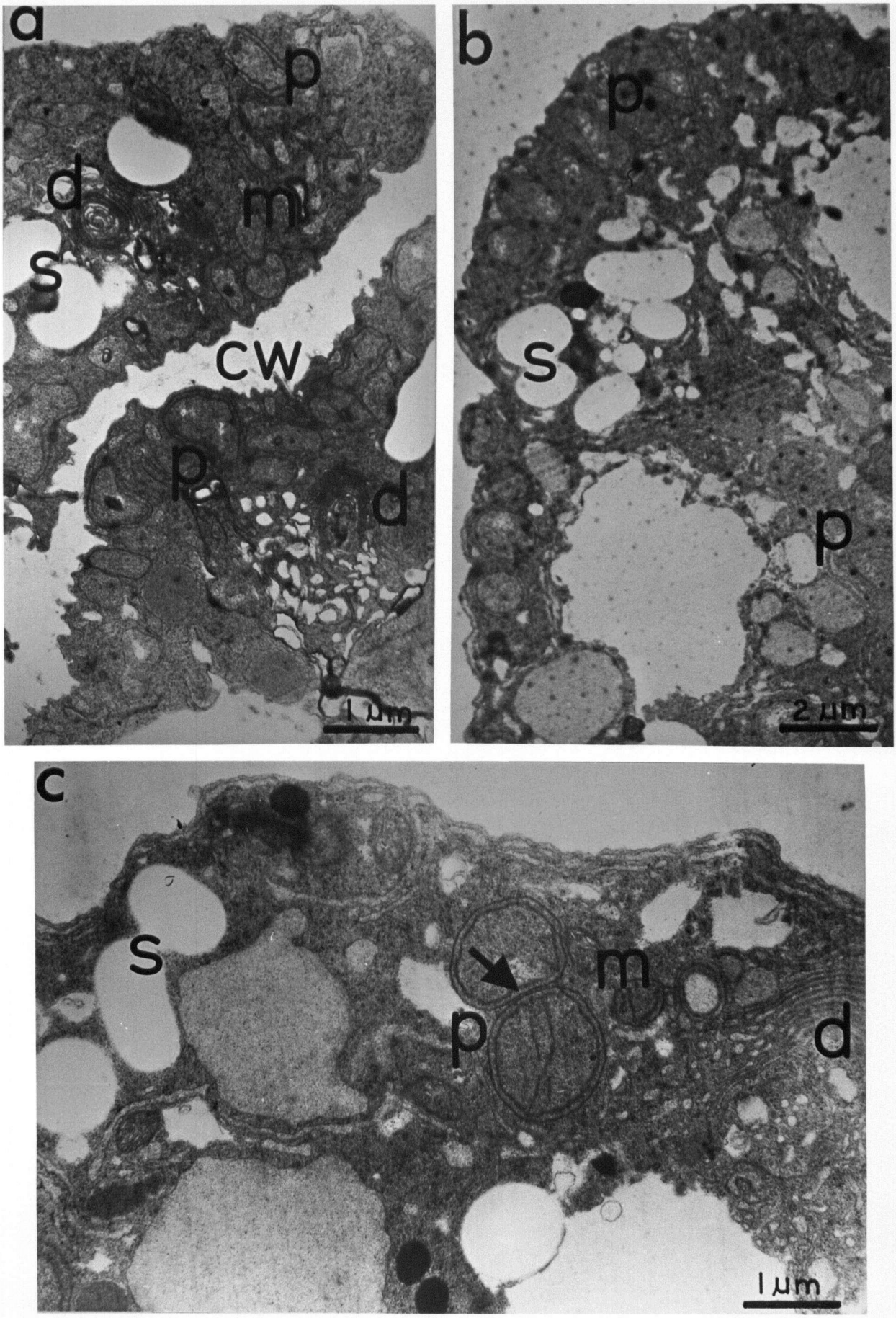

a
p
d
m
s
cw
p
d
1 µm
b
p
s
p
2 µm
c
s
m
p
d
1 µm

TABLE II

UPTAKE OF ^{14}C-LABEL BY L. SPECTABILIS AND J. GARDNERI FROM SEAWATER CONTAINING $NaH^{14}CO_3$

J. gardneri and pieces of L. spectabilis with and without attached J. gardneri were incubated in seawater containing $NaH^{14}CO_3$ for the times shown under the conditions of light or dark. The killed material was prepared for scintillation counting and the ^{14}C-label uptake was measured (see Materials and Methods). The results, corrected for background, are for a single experiment (#1) and are averages of three samples from each treatment ± the standard error of the mean. Significant differences at the 5% level were established using a T-test (SASTT computer program).

	SEPARATE DURING UPTAKE		ATTACHED DURING UPTAKE	
	L. spectabilis	J. gardneri	L. spectabilis	J. gardneri
LIGHT	(dpm/mg wet wt)	(dpm/mg wet wt)	(dpm/mg wet wt)	(dpm/mg wet wt)
10 sec	162 ± 55	64 ± 17	160 ± 18	110 ± 29
30 sec	*195 ± 3	126 ± 14	*174 ± 6	112 ± 2
5 min	1490 ± 122	484 ± 16	1094 ± 214	464 ± 183
30 min	3822 ± 503	1584 ± 403	3057 ± 232	1123 ± 80
DARK				
30 sec	15 ± 3	6 ± 2	16 ± 2	8 ± 2
30 min	60 ± 13	111 ± 21	84 ± 9	106 ± 25

* -- Significantly different from one another at the 5% level.

ment #3, when the means of separate and attached ^{14}C-uptake (62 and 156 dpm/mg wet wt, respectively) by J. gardneri in the light for 30 sec were significantly different.

B. Incorporation of Radioactive Label. All fractions prepared from the two algae in photosynthesis experiments #1 and 2 were radioactive (Table III). The quantities incorporated varied between experiments and thus the results serve only to show which groups of compounds became labeled. Most of the label was present in the neutral and cationic fractions with very little label in the lipid fractions. No attempt was made to establish the cause of the quantitative differences between the results of these two experiments. Variation in season, light intensities, extraction procedures, or severe color quenching during scintillation counting seemed most likely causes.

Figure 5 shows the radioautograms of the neutral, cationic, and anionic fractions of L. spectabilis and J. gardneri after separation by paper chromatography. The areas of ^{14}C-activity for the neutral fractions coincided with areas on the paper chromatograms where sugars were detected. Results from scintillation counting of paper strips cut from chromatograms of the neutral fractions of L. spectabilis (Fig. 5 A,B) and J. gardneri (Fig. 5 C,D) indicated that 67-70% of the ^{14}C-activity was located in areas corresponding to standards of galactose, glucose, floridoside, isofloridoside, and mannitol (Table IV), but the activity could not be specifically associated with individual sugars due to lack of resolution. The solvent a chromatograms of the anionic fractions from both algae showed faint "sugar-positive" areas corresponding to galacturonic

TABLE III

DISTRIBUTION OF ASSIMILATED ^{14}C-LABEL IN EXTRACTS AND RESIDUES FROM L. SPECTABILIS AND J. GARDNERI

The algae were exposed to the ^{14}C-label in seawater containing $NaH^{14}CO_3$ for 30 min in the light or dark. The material was extracted in ethanol and fractions were prepared (see Materials and Methods). The results, corrected for background, are from photosynthesis experiments #1 and 2.

	LIGHT*				DARK			
	L. spectabilis**		J. gardneri**		L. spectabilis		J. gardneri	
	Exp. #1	Exp. #2	Exp. #1	Exp. #2	Exp. #1	Exp. #2	Exp. #1	Exp. #2
Neutral fraction	31.8%	17.8%	36.2%	32.1%	11.6%	7.9%	27.7%	3.8%
Cationic fraction	31.8	64.5	36.8	52.9	43.8	87.1	59.4	91.0
Anionic fraction	10.9	8.4	20.2	6.2	16.0	2.7	8.5	2.2
Lipid fraction	0.4	<0.1	0.3	1.2	1.9	<0.1	0.3	<0.1
Residue	25.1	8.9	6.4	7.7	26.6	2.3	4.1	3.0

* - Light intensity: Exp. #1 - 2.6×10^5 ergs/cm^2/sec, Exp. #2 - 2.8×10^5 ergs/cm^2/sec

** - Plants from Botany Beach: Exp. #1 - 18 August 1974, Exp. #2 - 24 February 1975

Fig. 5. Radioautograms of ^{14}C-labeled fractions of L. spectabilis (A,B) and J. gardneri (C,D).

The plants were exposed to ^{14}C-label in seawater containing $NaH^{14}CO_3$ for 30 min in the light before extraction with ethanol and fractionation on ion exchange resins. Neutral, cationic, and anionic fractions of the two algae were separated by one-dimensional paper chromatography using solvent a for #5A and C, and solvent b for #5B and D. (See Materials and Methods.). The results are from photosynthesis experiment #1. Scintillation counting of paper strips cut from the chromatograms used for each radioautogram showed 67-70% of the ^{14}C-activity to be located in Zones A, B, and C corresponding to several reference sugars: mannitol, glucose, floridoside, isofloridoside, and galactose (Table IV). Zones A and B correspond to radioactive spots #1 and 2, respectively. R_{Glu} values for the radioactive spots of the radioautograms are:

Fig. 5A -- #1= 1.03, #2= 0.90, and #3= 0.14

B -- #1= 1.17 and #2= 1.02

C -- #1= 0.98, #2= 0.88, and #3= 0.13

D -- #1= 1.22 and #2= 1.05

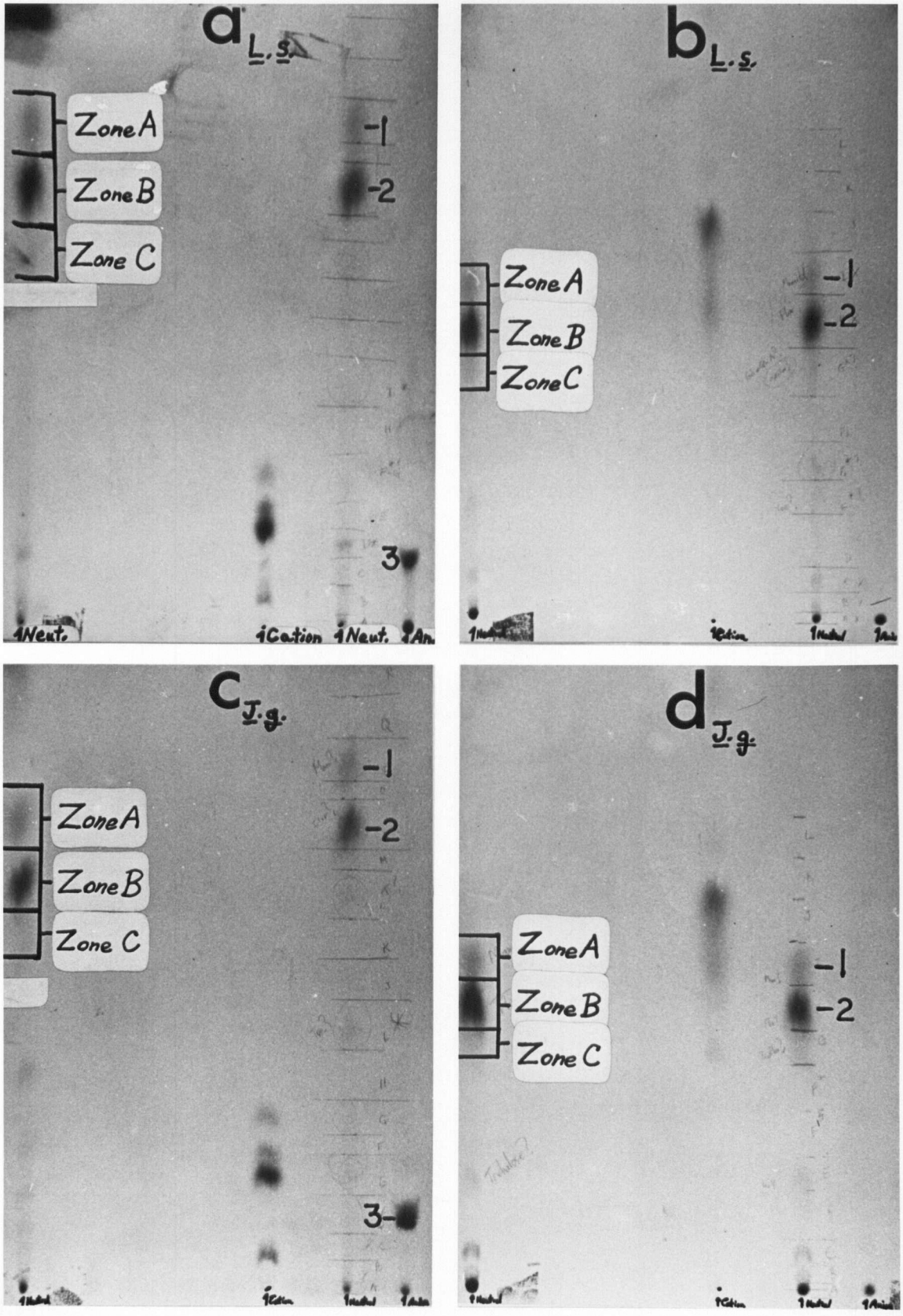

a L. s.
Zone A
Zone B
Zone C
-1
-2
3
1 Neut.
1 Cation
1 Neut.
2 An
b L. s.
Zone A
Zone B
Zone C
-1
-2
c J. g.
Zone A
Zone B
Zone C
-1
-2
3
d J. g.
Zone A
Zone B
Zone C
-1
-2

TABLE IV

R_{GLU} VALUES FOR SOME STANDARD SUGARS SEPARATED BY ONE DIMENSIONAL DESCENDING PAPER CHROMATOGRAPHY

Samples were applied to Whatman No. 1 chromatography paper and developed in either solvent a: ethyl acetate - pyridine - H_2O (10:4:3 v/v), or solvent b: ethyl acetate - acetic acid - formic acid - H_2O (18:3:1:4 v/v). The location of the sugars after chromatography was determined by an alkaline silver nitrate procedure (see Materials and Methods). The results are the average of values determined from at least two different chromatograms.

	R_{GLU} VALUES	
	Solvent a	Solvent b
Mannose	1.12	1.15
Mannitol	1.03	1.25
Glucose	1.00	1.00
Floridoside	0.92	1.20
Galactose	0.85	0.94
Isoflorisoside	0.82	0.93
Sucrose	0.77	0.36
Trehalose	0.53	0.38
Glucuronic acid	0.16	1.01
Galacturonic acid	0.14	0.92

acid and glucuronic acid; both were substances (uronic acids) unlikely to adsorb to the anion exchange resin. In both algae, the radioactive spot #3 (Fig. 5 A,C) was detected after development in solvent a, but was absent in the solvent b radioautograms (Fig. 5 B,D). This spot did not correspond to any of the standard sugars examined. ^{14}C-activity in chromatograms of the cationic fractions of both algae did not correspond to any of the standard sugars, nor were any "sugar-positive" areas detected for these fractions after chromatography.

The neutral fractions were further fractionated by adsorption onto a charcoal column followed by elution with a step-wise ethanol gradient. When these "fractionated-neutral" fractions from both algae were further examined by paper chromatography and radioautography, the labeled sugars in each fraction were resolved more satisfactorily with solvent a (Fig. 6), than with solvent b. However, the radioautograms revealed that the additional fractionation procedure did not provide complete separation of labeled compounds. The major labeled compounds shown in Fig. 6 appeared to be the same (galactose, glucose, floridoside, isofloridoside, and mannitol) as shown in Fig. 5.

Since the sugar standards were not well resolved by paper chromatography (Table IV) in the solvents used, GLC was used for further separation and analysis of non-radioactive extracts from both algae. Separation of reference sugars by GLC is shown in Fig. 7. Mannitol was not detected in either alga by GLC analyses (Fig. 8). Galactose and glucose were present in trace amounts. The major GLC peak (#6) appeared to be either floridoside, isofloridoside, or both. Co-chromatography of authentic floridoside or isofloridoside with samples from both algae also

Fig. 6. Radioautograms of ^{14}C-labeled "fractionated-neutral" fractions of L. spectabilis (A) and J. gardneri (B).

The plants were exposed to ^{14}C-label in seawater containing $NaH^{14}CO_3$ for 30 min in the light before extraction with ethanol, fractionation on ion exchange resins, and further fractionation of the neutral fraction by adsorption on a charcoal column followed by elution with H_2O and then a step-wise ethanol gradient (5, 10, 20, and 35%). The samples plus ^{14}C-labeled glucose were separated by one-dimensional paper chromatography using solvent a or solvent b. The results shown are after chromatography in solvent system a of plant material from photosynthesis experiment #2. The radioactive spots appear to correspond (corroborated using solvent system b) to the reference sugars mannitol, glucose, floridoside, isofloridoside, and galactose (Table IV), but lack of resolution did not permit association of each radioactive spot with an individual sugar. The R_{Glu} values and probable corresponding sugars for the radioactive spots are:

(A) L. spectabilis -- #1= 0.98 (glucose/mannitol), #2= 0.84 (isofloridoside/galactose), #3= 0.89 (floridoside), and #4= 0.90 (floridoside).

(B) J. gardneri -- #1= 0.97 (floridoside/glucose/mannitol), #2= 0.81 (isofloridoside/galactose), #3= 1.00 (glucose/mannitol), and #4= 0.89 (floridoside).

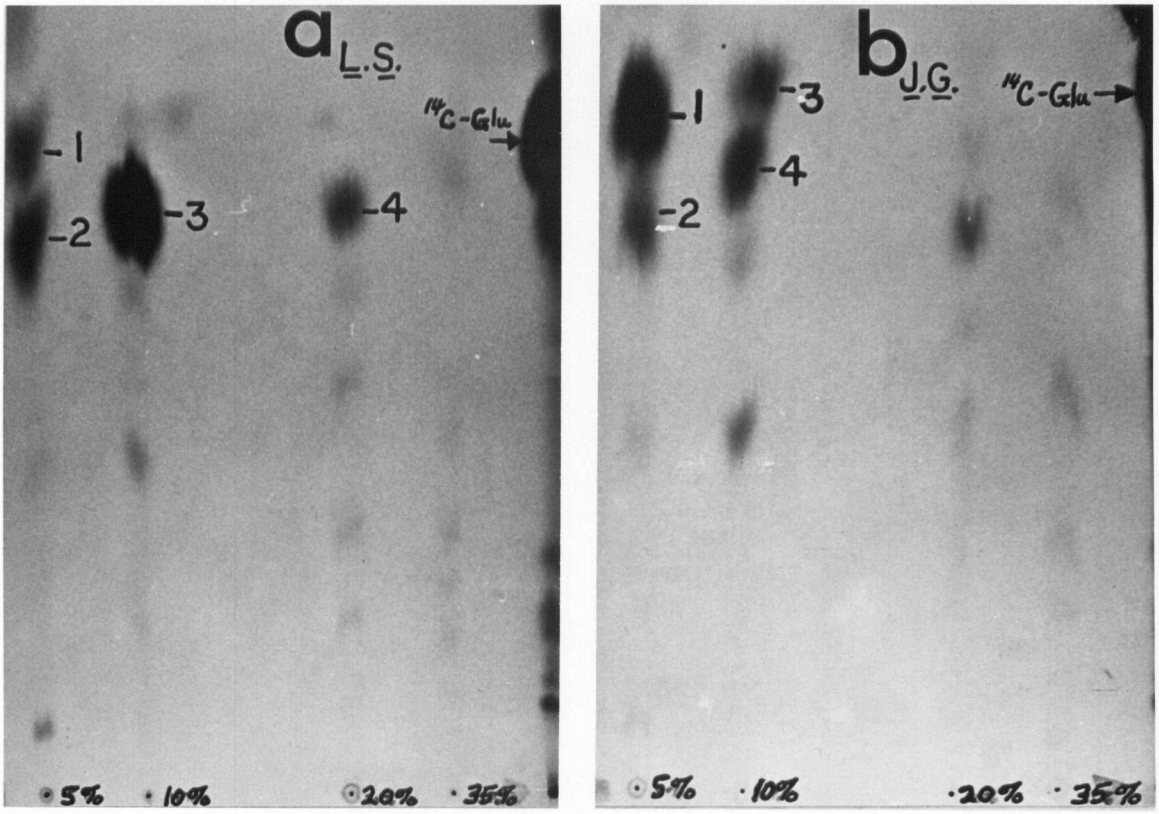
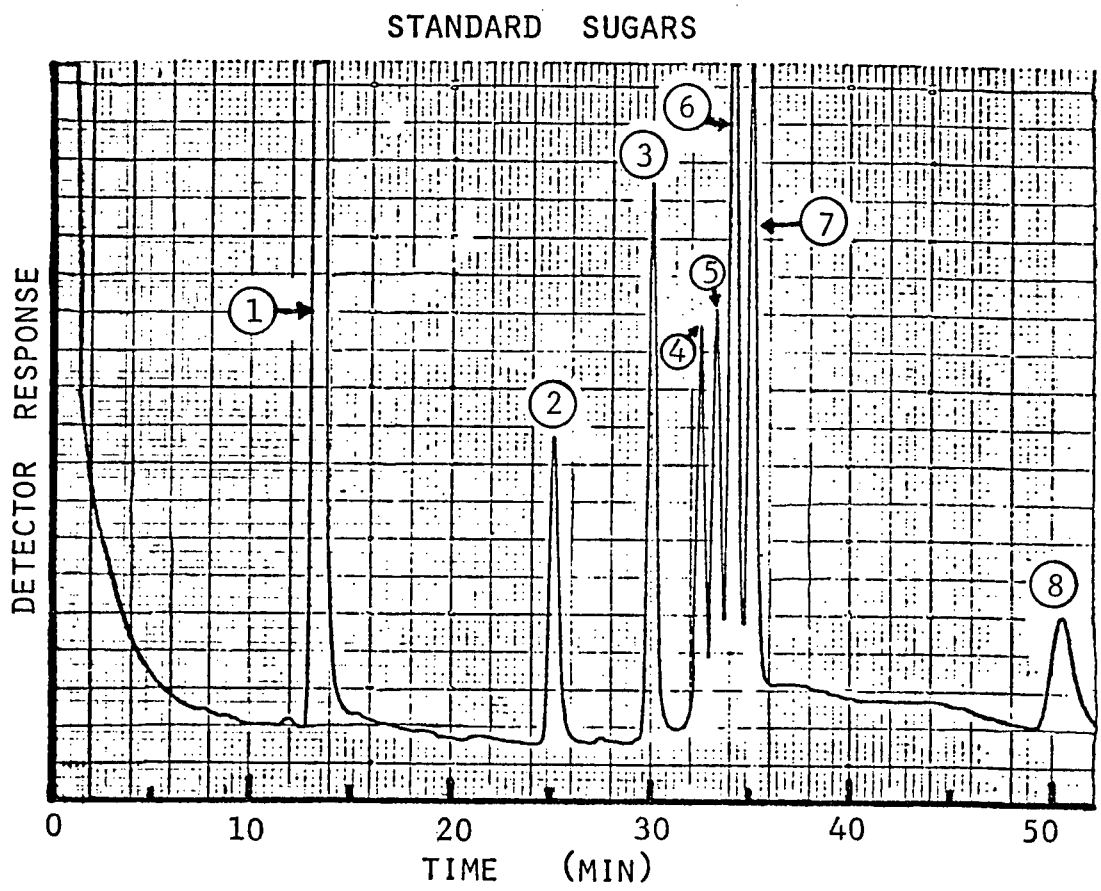

a L.S.
^{14}C-Glu
-1
-2
-3
-4
5%
10%
20%
35%
b J.G.
^{14}C-Glu
-1
-2
-3
-4
5%
10%
20%
35%

Fig. 7. Gas-liquid chromatogram of alditol acetate derivatives of some standard sugars.

The sample of sugars was reduced and, after addition of the internal standard myo-inositol, acetylated with acetic anhydride (see Materials and Methods). The sample in ethyl acetate was injected into a Hewlett-Packard Model 5710A gas chromatograph with 5% Silar 10C columns and temperature programmed from 120°-260°C at 4°/min.

FIGURE 7



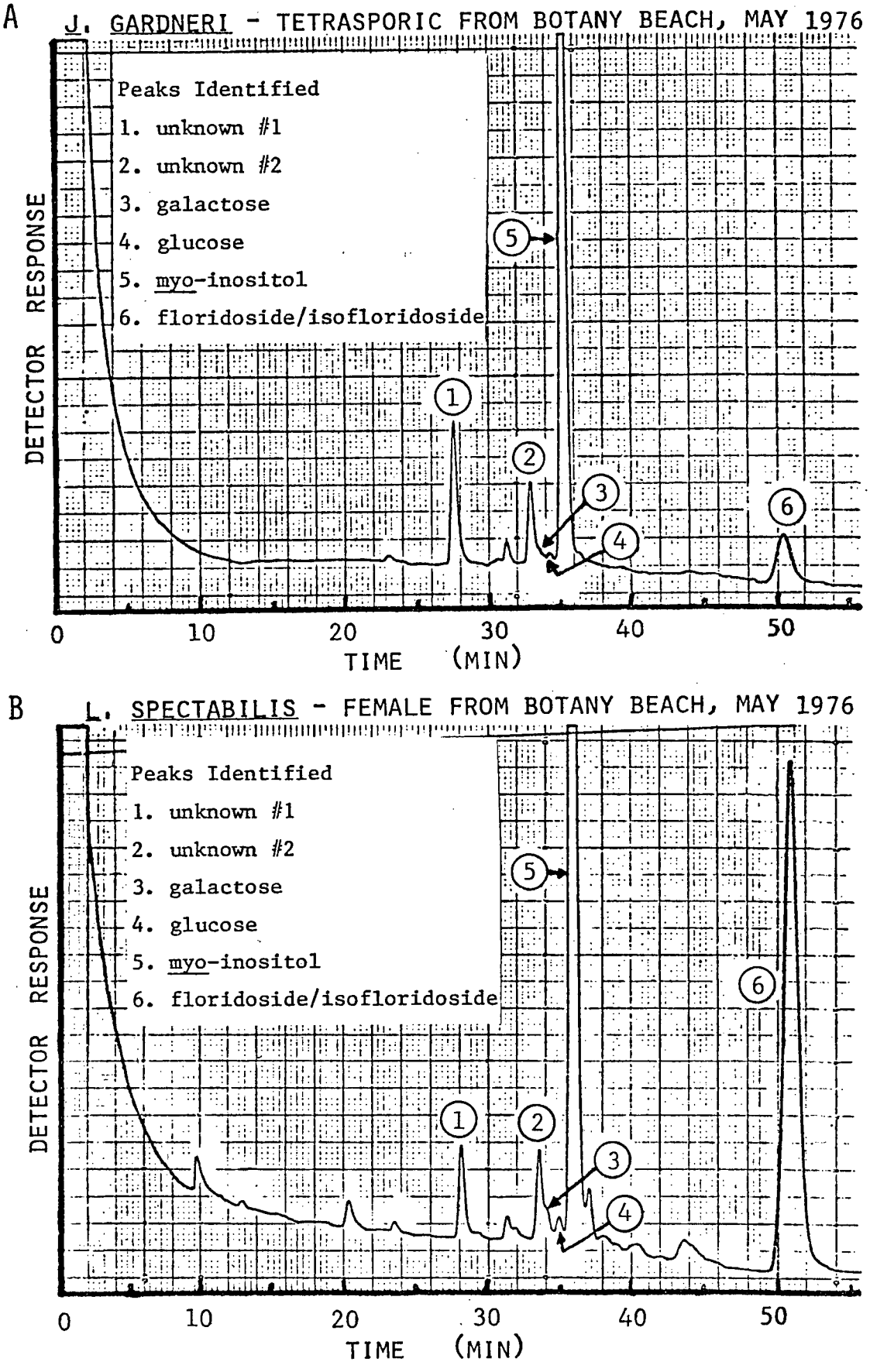
Standard Sugars Corresponding To The Peaks Are:

1. glycerol
2. fucose
3. xylose
4. mannose
5. galactose
6. glucose
7. myo-inositol
8. floridoside/isofloridoside, which are not resolved

Fig. 8. Gas-liquid chromatograms of alditol acetate derivatives of free sugars from J. gardneri (A) and L. spectabilis (B).

Plants were extracted with ethanol and a portion of the extract was dried, reduced and, after addition of the internal standard myo-inositol, acetylated with acetic anhydride (see Materials and Methods). The samples in ethyl acetate were injected into a Varian Aerograph Model 1740 gas chromatograph with 5% Silar 10C columns and temperature programmed from 120°–260°C at 4°/min.

FIGURE 8


A
J. GARDNERI - TETRASPORIC FROM BOTANY BEACH, MAY 1976
Peaks Identified
1. unknown #1
2. unknown #2
3. galactose
4. glucose
5. myo-inositol
6. floridoside/isofloridoside
DETECTOR RESPONSE
0
10
20
30
40
50
TIME (MIN)
B
L. SPECTABILIS - FEMALE FROM BOTANY BEACH, MAY 1976
Peaks Identified
1. unknown #1
2. unknown #2
3. galactose
4. glucose
5. myo-inositol
6. floridoside/isofloridoside
DETECTOR RESPONSE
0
10
20
30
40
50
TIME (MIN)

indicated that either sugar, or both, may be present. The results (Fig. 9) of analysis of neutral fractions after hydrolysis supported the hypothesis that the substance(s) was one or more galactosyl-glycerol compounds. There was a decrease in peak #6, an increase in the galactose peak, and the appearance of a glycerol peak.

Mass spectrometry was used to examine floridoside, isofloridoside, and the unknown compound(s) composing GLC peak #6 (Figs. 8, 9). The mass spectra of the two sugar standards showed that high mass peaks for the compounds were at m/e 446 for floridoside, and m/e 433 and 446 for isofloridoside, with the peak at m/e 433 being diagnostically significant (Fig. 10; see Fig. 11 for fragmentation scheme). Mass spectrometry analysis (Fig. 12) of the unknown substance(s) of peak #6 from both L. spectabilis and J. gardneri showed peaks at m/e 433 and 446, with the former being diagnostic for isofloridoside. However, a comparison of the ratio of peak intensities at m/e 433 and 446 in the known (Fig. 10) and unknown (Fig. 12) spectra revealed an enhancement of the peak at m/e 446 in the unknown spectra and illustrated the probable presence of floridoside in the samples analyzed. I conclude that both compounds were in fact present in both algae. The melting point of the crystalline samples before mass spectrometry was between 98° and 100°C, which is in agreement with the literature value of 101°C for acetylated floridoside (Putman and Hassid 1954), whereas the acetylated isofloridoside made in this study was a syrupy, non-crystalline substance. This further supports the conclusion that the unknown material was a mixture of floridoside and isofloridoside.

Fig. 9. Gas-liquid chromatograms of alditol acetate derivatives of hydrolyzed and reduced free sugars of J. gardneri (A) and L. spectabilis (B).

Dried ethanolic extracts containing soluble free sugars from each alga were hydrolyzed, reduced and, after the addition of the internal standard myo-inositol, acetylated with acetic anhydride (see Materials and Methods). The samples in ethyl acetate were injected in a Hewlett-Packard Model 5710A gas chromatograph with 5% Silar 10C columns. Column temperature was held for 8 min at 120°C and then progammed from 120°-260°C at 4°/min.

FIGURE 9

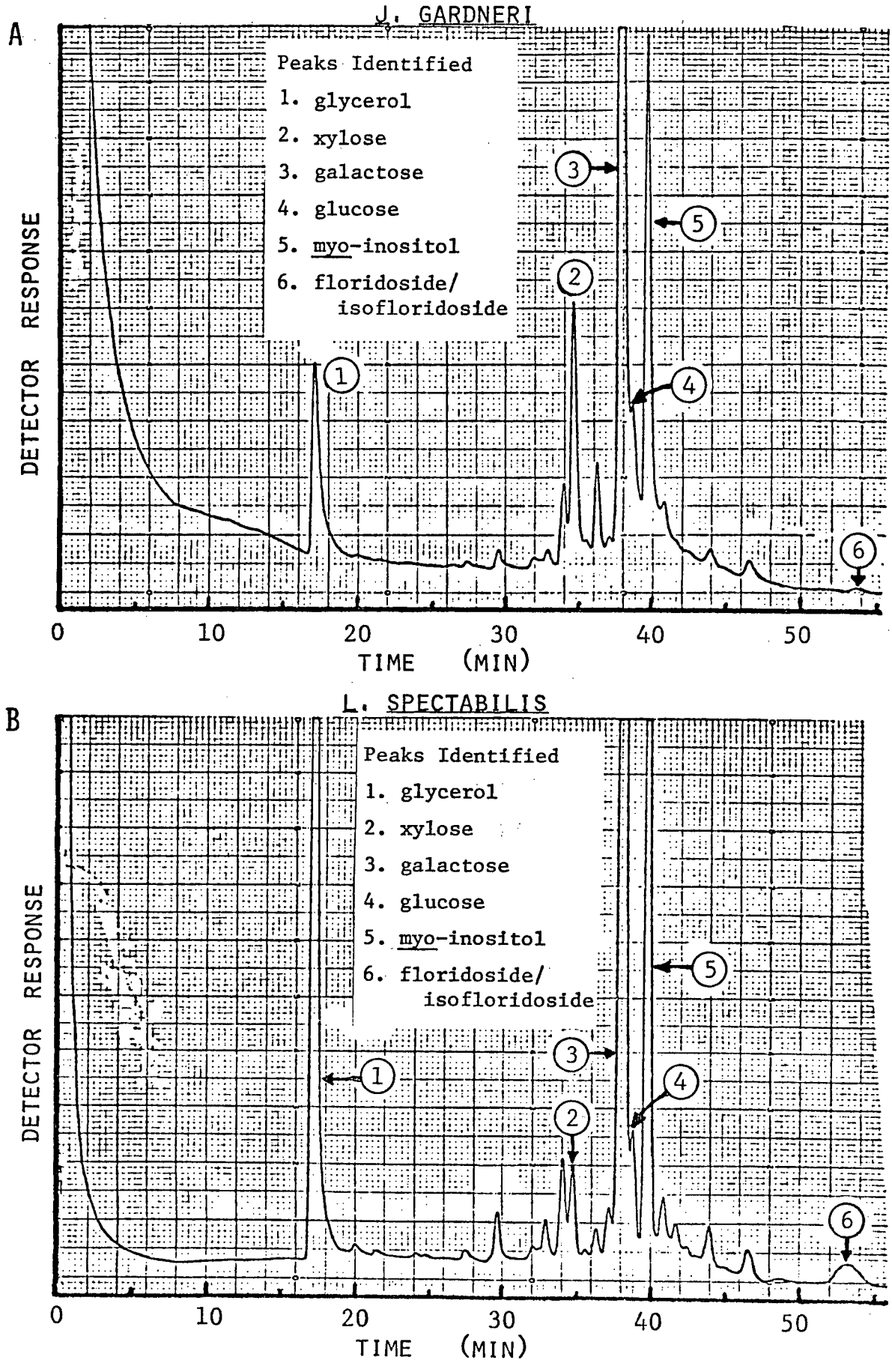

A
J. GARDNERI
Peaks Identified
1. glycerol
2. xylose
3. galactose
4. glucose
5. myo-inositol
6. floridoside/
isofloridoside
DETECTOR RESPONSE
0
10
20
30
40
50
TIME (MIN)
B
L. SPECTABILIS
Peaks Identified
1. glycerol
2. xylose
3. galactose
4. glucose
5. myo-inositol
6. floridoside/
isofloridoside
DETECTOR RESPONSE
0
10
20
30
40
50
TIME (MIN)

Fig. 10. Diagnostic portions of the mass spectra of floridoside acetate (A) and isofloridoside acetate (B).

Pure floridoside and isofloridoside (obtained from Dr. J. S. Craigie) was acetylated (see Materials and Methods) before mass spectrometry, which was performed in a Varian/MAT CH4-B mass spectrometer using the direct-probe insertion technique.

(A) Portion of the mass spectrum of reference floridoside acetate showing the high mass peaks. The probe temperature was 100°C for this analysis.

(B) Portion of the mass spectrum of reference isofloridoside acetate showing the high mass peaks, including its diagnostic peak at m/e 433. The probe temperature was 120°C for this analysis.

FIGURE 10

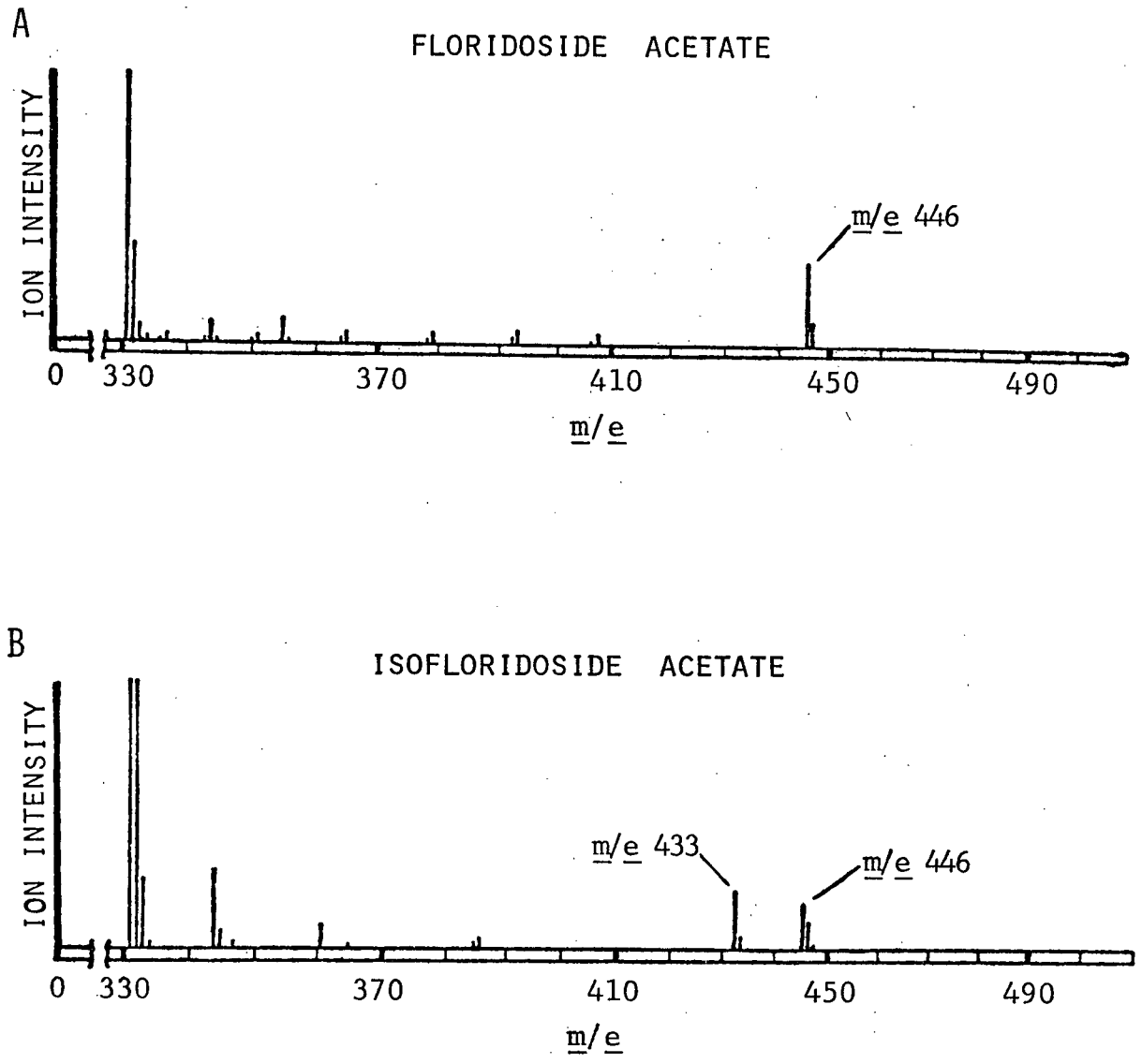

A
FLORIDOSIDE ACETATE
ION INTENSITY
m/e 446
0
330
370
410
450
490
m/e
B
ISOFLORIDOSIDE ACETATE
ION INTENSITY
m/e 433
m/e 446
0
330
370
410
450
490
m/e

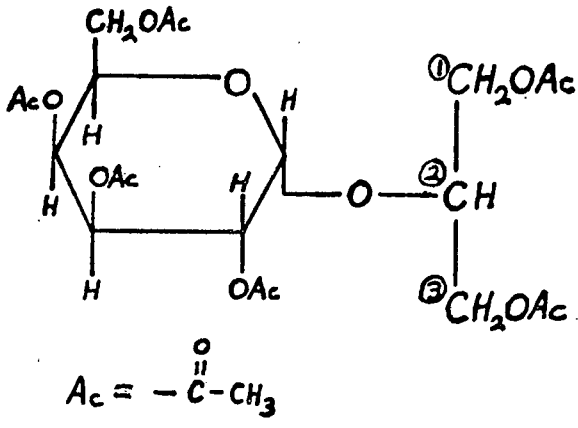
Fig. 11. Theoretical fragmentation schemes for floridoside acetate (A) and isofloridoside acetate (B) during mass spectrometry.

(A) Floridoside acetate. The elimination of acetic acid (60 mass units) from the parent molecule (MW 506) provides the high mass peak at m/e 446. Cleavage between C_1 and C_2, or C_2 and C_3 of the glycerol moiety is unlikely.

(B) Isofloridoside acetate. In addition to the high mass peak at m/e 446 due to the elimination of acetic acid (60 mass units) from the parent molecule, cleavage occurs more readily between C_2 and C_3 of the glycerol moiety which eliminates CH_2OAc (73 mass units) from the parent molecule (MW 506) to produce the high mass peak at m/e 433. The peak at m/e 433 is diagnostically significant for isofloridoside acetate.

FIGURE 11

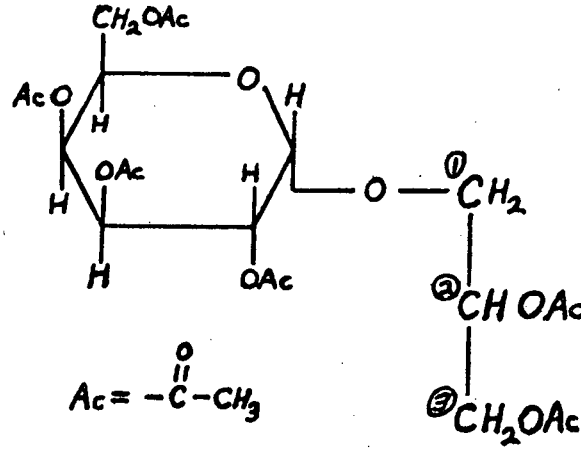
A.



$\xrightarrow[\text{(60 mass units)}]{-AcOH}$ m/e 446

FLORIDOSIDE ACETATE (MW 506)

B.



$\xrightarrow[\text{(60 mass units)}]{-AcOH}$ m/e 446

$\xrightarrow[\text{(73 mass units)}]{-CH_2OAc}$ m/e 433

ISOFLORIDOSIDE ACETATE (MW 506)

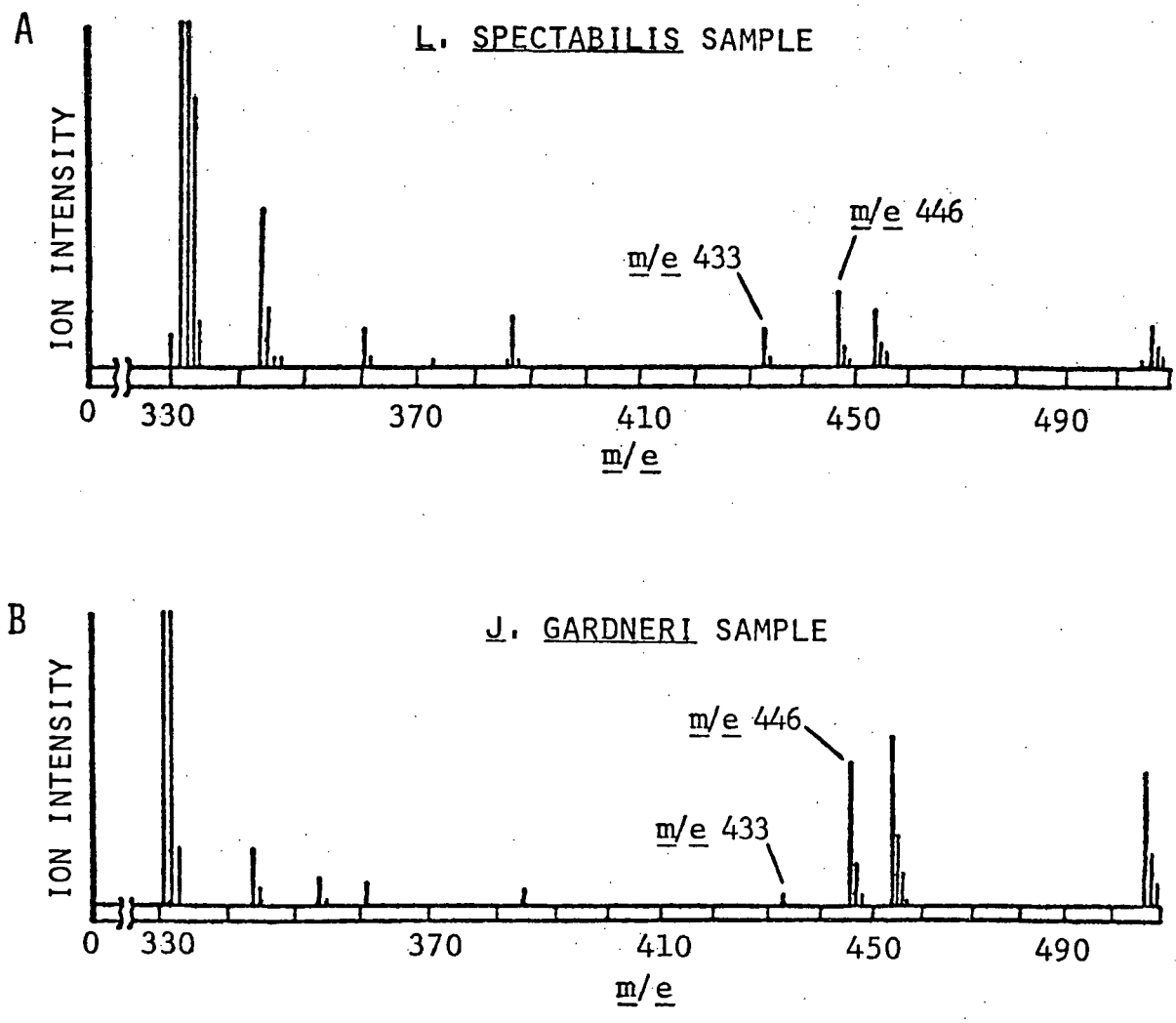
Fig. 12. Diagnostic portions of mass spectra of suspected floridoside/isofloridoside GLC peaks from L. spectabilis (A) and J. gardneri (B).

Samples from ethanolic extracts of L. spectabilis and J. gardneri were derivatized as alditol acetates followed by GLC using a preparative column of 5% Silar 10C (see Materials and Methods). Samples of suspected floridoside/isofloridoside peaks were collected in glass capillary tubes for mass spectrometry, which was performed in a Varian/MAT CH4-B mass spectrometer using the direct-probe insertion technique.

(A) Spectrum of the unknown GLC peak from L. spectabilis showing the peak at m/e 433 considered diagnostic for isofloridoside acetate. Note the difference in the ratio of peak intensities of m/e 433 to 446 for this spectrum compared with the reference spectrum (Fig. 10B). The probe temperature was 100°C for this analysis.

(B) Spectrum of the unknown GLC peak from J. gardneri showing the peak at m/e 433 considered diagnostic for isofloridoside acetate. Note the difference in the ratio of peak intensities of m/e 433 to 446 for this spectrum compared with the reference spectrum (Fig. 10B). The probe temperature was 120°C for this analysis.

FIGURE 12


A
L. SPECTABILIS SAMPLE
ION INTENSITY
m/e 446
m/e 433
0
330
370
410
450
490
m/e
B
J. GARDNERI SAMPLE
ION INTENSITY
m/e 446
m/e 433
0
330
370
410
450
490
m/e

The conclusions from the paper chromatography, radioautography, gas-liquid chromatography, and mass spectrometry results are that the major labeled sugars of both algae were galactose, glucose, isofloridoside, and floridoside.

The results after the thin-layer chromatography of the organic acid fractions (anionic fractions) of both algae were ambiguous because of the low level of detectable radioactivity and the low concentration of organic acids. There were faint "acid-positive" spots which had mobilities corresponding to citrate, malate, and glycolate. These organic acids have been reported from other red algae (Bean and Hassid 1955; Kremer and Vogl 1975).

Figure 13 is a radioautogram made of the cationic fractions of J. gardneri and L. spectabilis after separation by high voltage paper electrophoresis. The unknown ^{14}C-labeled amino acids had mobilities corresponding to those of alanine, aspartic acid, glutamic acid, glycine, and serine. The presence of these five amino acids in both algae was confirmed by analyses of non-radioactive cationic fractions from the algae using an amino acid analyzer.

TRANSLOCATION EXPERIMENTS

The results of the three translocation experiments are given in Table V. There was no significant (α=0.05) net movement of the ^{14}C-label in either direction between the attached algae during the translocation period for each experiment. This conclusion holds regardless of whether the pretreatment condition before labeling was light or dark, whether the algae

Fig. 13. Radioautogram of the cationic fractions of J. gardneri and L. spectabilis after separation using paper electrophoresis.

Plants were exposed to the ^{14}C-label in seawater containing $NaH^{14}CO_3$ for 30 min in the light (l) or dark (d) before extraction with ethanol and fractionation using ion exchange resins. The amino acids in the cationic fractions were resolved using high voltage paper electrophoresis at ca. 52.6 V/cm of paper length for 40 min at pH 2.0 (see Materials and Methods). This radioautogram was from photosynthesis experiment #2. The amino acids detected in fractions from plants labeled in the light were glycine (gly), alanine (ala), serine (ser), glutamic acid (glu), and aspartic acid (asp). Aspartic acid was the major labeled amino acid from plants exposed to the label in the dark.

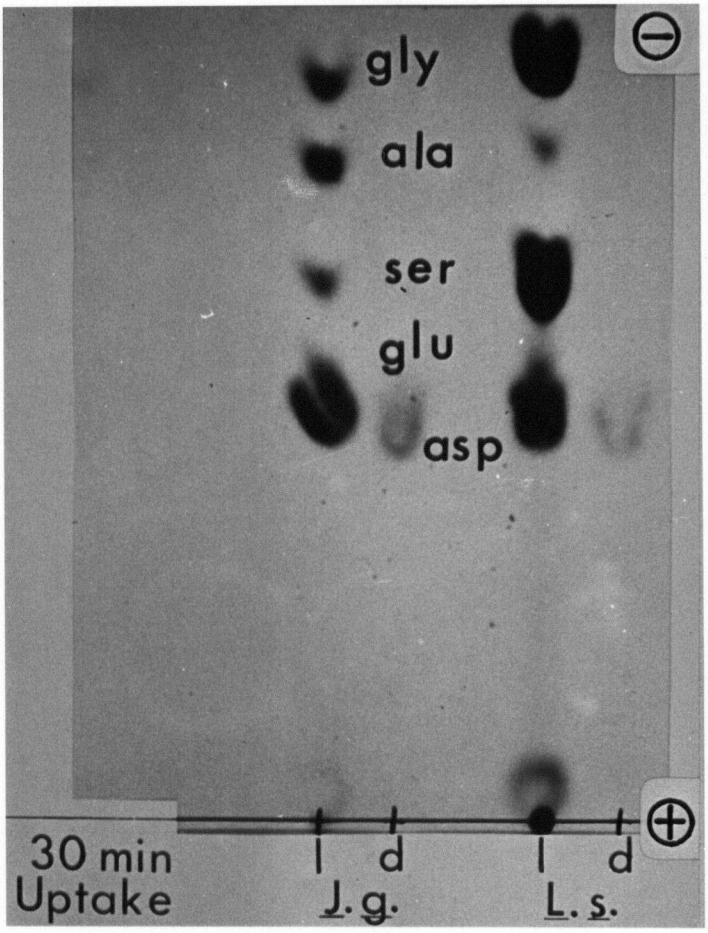

gly
ala
ser
glu
asp
⊖
⊕
30 min
Uptake
l
d
J. g.
l
d
L. s.

TABLE V

RESULTS OF ^{14}C-PULSE-CHASE TRANSLOCATION EXPERIMENTS WITH *L. SPECTABILIS* AND *J. GARDNERI*

Pieces of *L. spectabilis* with attached *J. gardneri* were incubated in seawater containing $NaH^{14}CO_3$, then transferred to non-radioactive "chase" seawater for various translocation periods. A summary of conditions for the experiments is presented in Table I. The algae were killed at the end of each of the translocation periods and were prepared separately for scintillation counting (see Materials and Methods). These values, corrected for background are an average of 3 samples (except for the 0 hr values for experiment #3 which are based on 6 samples) ± SEM. An analysis of variance, performed by the ANVAR computer program (see Materials and Methods), determined that the values for each alga during the translocation periods for each experiment did not differ significantly ($\alpha = 0.05$).

		TRANSLOCATION PERIOD				
EXPERIMENT		0 hr	2 hr	4 hr	8 hr	12 hr
		dpm/mg wet wt	dpm/mg wet wt	dpm/mg wet wt	dpm/mg wet wt	dpm/mg wet wt
#1	*J. gardneri*	1428 ± 225	1855 ± 513	2221 ± 158	2348 ± 258	- - - - - -
	L. spectabilis	4393 ± 1658	5460 ± 1426	4786 ± 753	6930 ± 1941	- - - - - -
#2a	*J. gardneri*	2826 ± 465	2418 ± 444	2178 ± 202	2026 ± 255	2721 ± 526
	L. spectabilis	5259 ± 819	5281 ± 864	5714 ± 1087	6457 ± 479	6585 ± 393
#2b	*J. gardneri*	- - - - - -	5094 ± 416	6102 ± 1835	5441 ± 714	4210 ± 367
	L. spectabilis	- - - - - -	11739 ± 2082	12264 ± 812	13181 ± 1523	13280 ± 1501
#3a	*J. gardneri*	1470 ± 165	1658 ± 166	1738 ± 156	1813 ± 105	1786 ± 79
	L. spectabilis	4019 ± 438	6444 ± 998	5838 ± 666	5181 ± 135	5268 ± 173
#3b	*J. gardneri*	1470 ± 165	1651 ± 233	1424 ± 54	1424 ± 215	1406 ± 72
	L. spectabilis	4019 ± 438	4815 ± 721	4609 ± 46	4317 ± 83	3127 ± 382

took up the label for 30 or 60 min, or whether the algae were allowed to translocate for a short (2 hr) or long (12 hr) period.

Figure 14 shows that the amount of label in J. gardneri after an initial 30 min labeling pulse remained steady during a light or dark translocation period. L. spectabilis material also remained generally steady after the initial labeling pulse except for some change during the first 4 hours of the light translocation period and the last 4 hours of the dark translocation period. However, Scheffe's Test for Multiple Comparisons indicates no significant difference (α=0.10) between the various light or dark results.

The data used to determine the values in Table V were also expressed as a ratio of J. gardneri value/L. spectabilis value. These ratios (Table VI) were also compared by the ANVAR computer program. If the ratio of J. gardneri/L. spectabilis tended toward 1.000, the label was assumed to be moving into J. gardneri, while a shift towards 0.000 indicates the label is moving into L. spectabilis. The results showed there was no tendency (α=0.10) for the ratio to shift in either direction during the three experiments. Thus, ^{14}C-labeled photosynthetic products did not move between the algae under the experimental conditions used in this study.

The translocation experiments also established the amount of label passing from the algae to the "chase" seawater during the translocation period. The results for experiments #2 and 3 (Table VII) showed that the amount of label released to the seawater did not increase during the translocation period. Since the label was apparently not continuously released into the chase seawater and only accounted for about 5% or less

Fig. 14. Radioactivity of L. spectabilis and attached J. gardneri after 30 min uptake of $NaH^{14}CO_3$ followed by translocation periods.

Plants pretreated with light were exposed to the ^{14}C-label in seawater containing $NaH^{14}CO_3$ for 30 min in the light before rinsing and placing them into the "chase" seawater (except the plants sampled at zero translocation time). The plants were killed at the end of each translocation period and prepared for scintillation counting (see Materials and Methods). The graph presents data from experiments #3a and 3b which had light and dark translocation periods, respectively. The means are an average of 3 samples, except the initial readings which are based on 6 samples. Bars = SEM

FIGURE 14

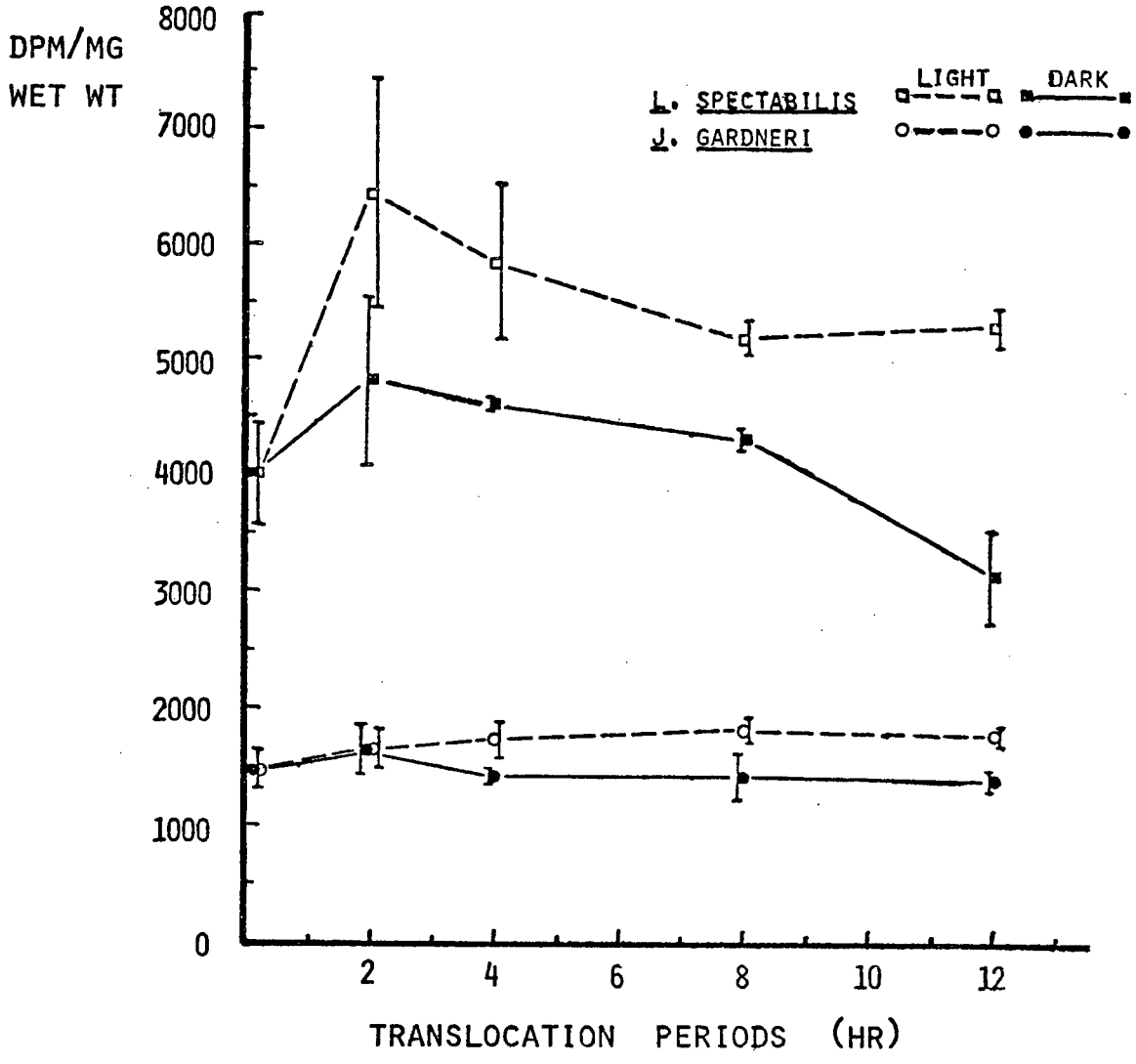

DPM/MG
WET WT
8000
7000
6000
5000
4000
3000
2000
1000
0
L. SPECTABILIS
J. GARDNERI
LIGHT
DARK
2
4
6
8
10
12
TRANSLOCATION PERIODS (HR)

TABLE VI

RATIOS OF J. GARDNERI/L. SPECTABILIS ^{14}C-PULSE-CHASE TRANSLOCATION VALUES

The ^{14}C-pulse-chase translocation data used to calculate these ratios were the same as those used to calculate the results reported in Table V. Each ratio was obtained by dividing a J. gardneri value by its corresponding L. spectabilis value and is the mean of 3 such ratios for each treatment, except for the 0 hr ratios of experiment #3 which are an average of 6 ratios. An analysis of variance, performed by the ANVAR computer program (see Materials and Methods), determined that the ratios during the translocation period for each experiment did not differ significantly (α= 0.10).

	RATIOS FOR TRANSLOCATION PERIOD (hr)				
EXPERIMENT	0 hr	2 hr	4 hr	8 hr	12 hr
#1 (Light)*	0.379	0.335	0.483	0.465	- - -
#2a (Light)	0.544	0.454	0.421	0.311	0.424
#2b (Dark)	- - -	0.471	0.488	0.422	0.321
#3a (Light)	0.374	0.261	0.300	0.351	0.340
#3b (Dark)	0.374	0.345	0.309	0.330	0.457

* - Condition of light or dark during translocation period.

TABLE VII

RADIOACTIVITY OF "CHASE" SEAWATER FROM THE ^{14}C-PULSE-CHASE TRANSLOCATION EXPERIMENTS #2 AND 3

The amount of ^{14}C-label released by the algae into the "chase" seawater during each translocation period of translocation experiments #2 and 3 was determined by scintillation counting, corrected for background, and divided by the wet wt of algal material present (see Materials and Methods). These results are an average of 2 samples taken from the chase seawater.

EXPERIMENT	TRANSLOCATION PERIOD							
	2 hr		4 hr		8 hr		12 hr	
	dpm/mg wet wt		dpm/mg wet wt		dpm/mg wet wt		dpm/mg wet wt	
#2a (Light)*	61	(0.8%)**	53	(0.7%)	55	(0.7%)	95	(1.0%)
#2b (Dark)	328	(2.0)	363	(2.0)	291	(1.6)	368	(2.1)
#3a (Light)	74	(1.0)	87	(1.2)	79	(1.1)	79	(1.1)
#3b (Dark)	225	(3.5)	327	(5.4)	250	(4.4)	233	(5.1)

* - Condition of light or dark during translocation period.

** - Percentage of the total recovered label present in chase seawater.

of the total ^{14}C-activity recovered (based on the results of Table V and VII), the label probably got into the medium from the surface of the algae despite the careful rinsing procedure.

FREE SUGARS AND AMINO ACIDS OF LIFE HISTORY STAGES

The ethanol-soluble free sugar content of material from each life history stage of both algae was determined after analysis by GLC. The same sugars (galactose, glucose, and floridoside/isofloridoside, which co-chromatograph) and several unknowns were detected after analysis of extracts from L. spectabilis male and female (Fig. 8B) gametophyte, and tetrasporophyte stages from Botany Beach (April, May 1976) and Cable Beach (May 1976). Male gametophyte and tetrasporophyte (Fig. 8A) stages (no female plants were available) of J. gardneri from Botany Beach (April, May 1976) also had these same sugars as well as several unknown substances.

The ethanol-soluble free amino acids from each life history stage of the two algae (except for female J. gardneri) are shown in Tables VIII and IX. The major free amino acids were detected in approximately similar amounts in each life history stage examined for each alga from both Botany Beach collections. The same free amino acids were detected in L. spectabilis collected in May at both Botany Beach and Cable Beach, with most amino acids found in similar quantities except alanine, aspartic acid, and glutamic acid.

TABLE VIII

COMPOSITION OF RECOVERED FREE AMINO ACIDS OF L. SPECTABILIS

Plants were collected at Botany Beach (16 April, 14 May 1976) and at Cable Beach (13 May 1976). Before extraction of ethanol-soluble free amino acids (see Materials and Methods), the plants were grouped according to life history stages. The results are from one analysis. Tr = trace, less than 0.1% of recovered amino acids.

	TETRASPORIC			MALE			FEMALE		
	Botany Beach		Cable Beach	Botany Beach		Cable Beach	Botany Beach		Cable Beach
	April	May	May	April	May	May	April	May	May
Asp	3.5%	4.8%	13.2%	4.6%	3.7%	23.5%	7.8%	7.4%	13.8%
Thr	0.7	0.9	1.6	0.7	1.1	2.9	1.0	1.0	1.9
Ser	2.0	3.3	4.8	2.0	3.0	8.5	3.3	2.8	5.8
Glu	75.1	74.9	61.4	73.0	76.2	42.8	73.6	74.5	59.0
Pro	1.1	1.2	3.0	1.0	1.1	2.4	1.6	1.2	1.7
Gly	0.6	0.7	0.6	0.7	0.6	0.6	0.9	0.8	0.4
Ala	13.7	11.2	4.7	14.8	10.2	3.6	7.1	9.0	4.9
Val	0.7	0.5	3.1	0.7	1.0	3.6	1.1	0.8	3.0
Met	0.1	Tr	0.3	0.2	Tr	0.4	0.2	Tr	Tr
Ile	0.5	0.5	2.1	0.4	0.7	2.9	0.7	0.7	2.4
Leu	0.5	0.6	2.8	0.5	0.8	4.1	0.9	0.7	3.2
Tyr	0.5	0.6	1.0	0.5	0.7	1.7	0.7	0.6	1.9
Phe	0.1	0.4	1.1	0.1	0.5	1.8	0.2	0.2	1.4
Lys	0.4	0.2	0.3	0.3	0.2	0.9	0.4	0.2	0.3
His	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.1
Arg	0.2	0.1	0.1	0.2	0.2	0.3	0.3	0.1	0.1

TABLE IX

COMPOSITION OF RECOVERED FREE AMINO ACIDS OF J. GARDNERI

Plants were collected at Botany Beach (16 April, 14 May 1976). Before extraction of ethanol-soluble free amino acids (see Materials and Methods), the plants were grouped according to life history stages (no female plants were collected). The results are from one analysis. Tr = trace, less than 0.1% of recovered amino acids.

	TETRASPORIC		MALE	
	Botany Beach		Botany Beach	
	April	May	April	May
Asp	4.4%	4.4%	4.0%	6.7%
Thr	2.2	1.6	2.4	2.3
Ser	6.9	4.6	7.8	6.4
Glu	39.5	56.2	46.1	41.1
Pro	2.2	2.3	2.7	1.5
Gly	11.2	1.6	4.5	3.3
Ala	26.8	21.6	24.1	28.2
Val	1.6	2.0	1.8	2.6
Met	0.3	0.5	0.1	Tr
Ile	1.6	1.4	1.6	2.1
Leu	1.9	1.6	2.2	2.4
Tyr	0.3	0.6	1.7	1.1
Phe	0.3	0.7	0.7	0.8
Lys	0.3	0.4	0.1	0.1
His	0.3	0.3	0.1	1.0
Arg	0.3	0.1	0.1	0.1

CULTURE OF SPORES

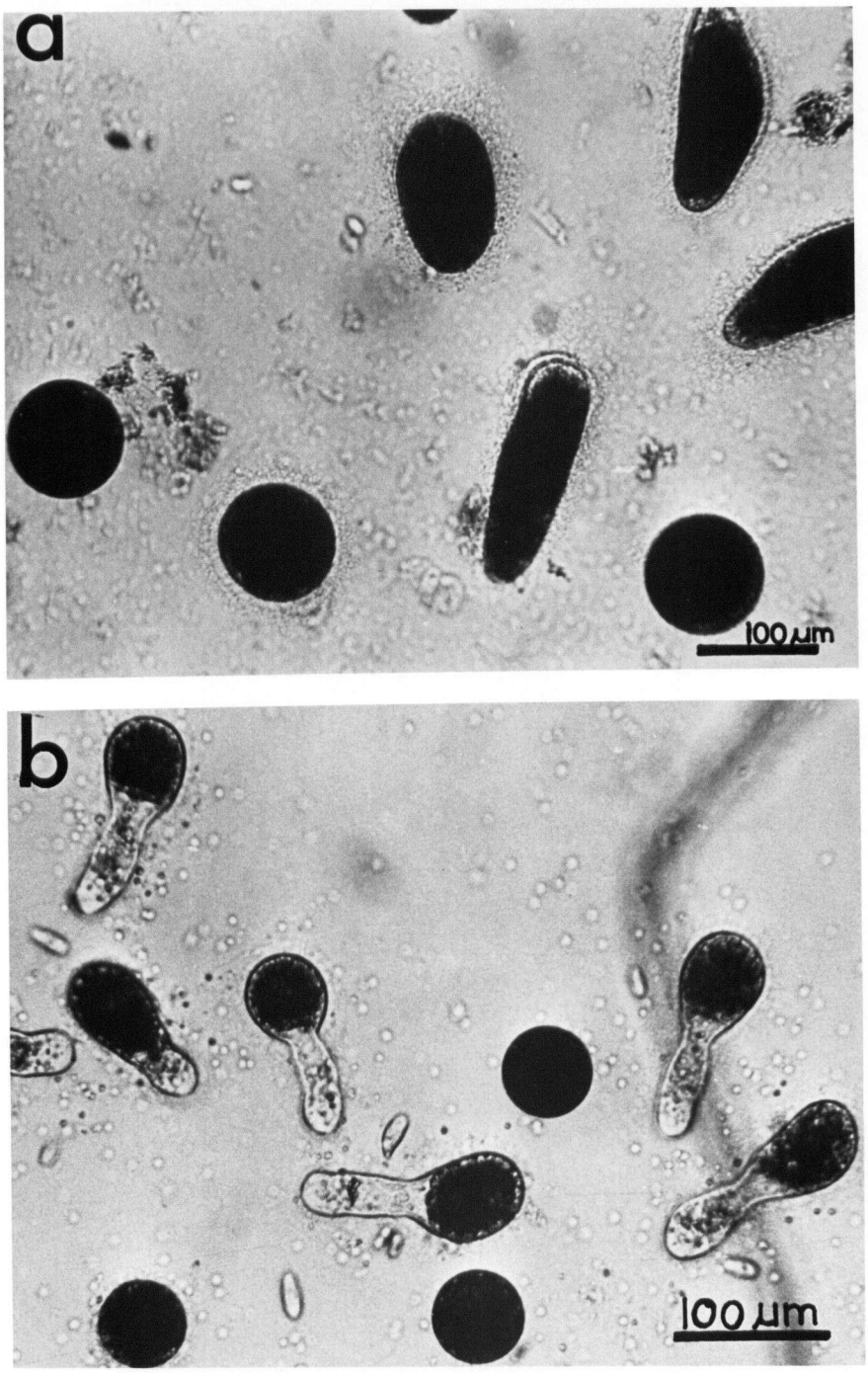
Material containing carposporangia and tetrasporangia of both genera when placed in petri plates with seawater medium readily released spores which adhered to cover slips and bottoms of petri plates and then became rounded (Fig. 15). Germination of the reddish L. spectabilis spores was inconsistent as only tetraspores germinated from plants collected at Victoria (12 July 1976), whereas only carpospores germinated from the material obtained at Botany Beach (11 July 1976). The L. spectabilis spores which germinated produced a 2-4 cell colorless rhizoid (sometimes branched). The upper part of the spores would divide several times to form between 8-60 colored cells. The medium was changed every 2-4 days and the germlings appeared healthy (no loss of pigmentation or bacterial contamination) for 1-2 weeks.

The spores of J. gardneri when released were pinkish in color. The only J. gardneri spores to germinate were the carpospores (Fig. 15B) from a Botany Beach collection (11 July 1976). The J. gardneri germlings (Fig. 15B) did not survive as long as those of L. spectabilis. They did form a rhizoid of one or two cells, but there was no division of the upper, pigmented part of the spores. Most of the germlings lost their color in less than 1 week and bacteria became apparent despite changes of medium, addition of pieces of L. spectabilis, or both. This experience was similar to that reported by Kugrens (1971), except that in his study tetraspores, not carpospores, germinated.

Fig. 15. Carpospores of L. spectabilis (A) and J. gardneri (B) in culture.

(A) Elongate carpospores released from female plants of L. spectabilis (collected at Victoria 28 June 1976) attached by their "tails" and then became rounded. These did not germinate.

(B) Elongate carpospores released from female plants of J. gardneri (collected at Botany Beach 11 July 1976) attached and became rounded within 24 hr of their release. These germinated about three days after their release, with no further development observed.


a
100 µm
b
100 µm

DISCUSSION

The results show that mature J. gardneri is capable of photosynthesis. The alga has a pinkish to reddish hue (see Frontispiece) probably due to the presence of r-phycoerythrin which has been identified from J. gardneri extracts (Kugrens 1971). It possesses "typical" red algal chloroplasts (Fig. 3) also reported by Kugrens (1971) and it is capable of light-dependent carbon fixation from $NaH^{14}CO_3$. The rate of ^{14}C-uptake by the symbiont under light conditions (Table II) was lower than that of its host. The conditions chosen for this study were the same for both organisms and may not have been as close to optimum for J. gardneri as for L. spectabilis. On the other hand, the symbiont is much smaller in size than the host (see Frontispiece) and may have a slower metabolism, and consequently a lower photosynthetic rate. The ^{14}C-label was detected in amino acid-, lipid-, organic acid-, and sugar-containing fractions (Table III) obtained from both algae after they had been separated and then exposed to the radioactive precursor. The labeled amino acids and sugars were the same in both algae. These results indicated that J. gardneri was capable of utilizing photosynthetically-fixed carbon for the biosynthesis of the various compounds which one would expect to isolate from a "typical" self-sufficient Ceramialian red alga such as L. spectabilis.

The major ^{14}C-labeled amino acids identified (Fig. 13) after photosynthesis by both algae were alanine, aspartic acid, glutamic acid, glycine, and serine. The major ^{14}C-labeled sugars were floridoside, usually the most intensive radioactive spot (Fig. 5, 6), isofloridoside,

galactose, and glucose. These compounds have been identified as some of the photosynthetic products of other red algae (Bean and Hassid 1955; Bidwell 1958; Majak, Craigie and McLachlan 1966; Craigie, McLachlan and Tocher 1968; Fiege 1973; Kremer and Vogl 1975). Mannitol was not detected by gas-liquid chromatography in either alga, which is contrary to reports for some other red algae, including members of the Ceramiales (Majak, Craigie and McLachlan 1966; Fiege 1973). This lack of mannitol may support the claim of Kremer (1976) that mannitol should not be "regarded as a natural metabolite (assimilate) of Rhodophyceae"; however, its absence in this study as an assimilate may be due to a seasonal decrease or disappearance not noted because analyses were not conducted throughout a one year period. Interestingly, Kremer did accept the positive identification of mannitol from the parasitic red alga Holmsella pachyderma (Evans, et al. 1973), but he suggested that the alga's parasitic habit may have caused it not to "exhibit the normal metabolic situation" for a red alga.

Some dark-assimilation of the ^{14}C-label occurred for both algae, with the majority of the label detected in the cationic fractions (Table III). The rate for this process, unlike that for photosynthesis, was similar for both algae (Table II). For both algae, the only product sufficiently labeled to be detected by radioautography was aspartic acid (Fig. 13). This amino acid was also identified as the major dark-assimilation product for the red algae Gigartina canaliculata (Joshi, et al. 1962) and Polysiphonia lanosa (Craigie 1963). The apparent similarities of the dark assimilation process for J. gardneri and L. spectabilis do not

suggest a special role for it in either alga. Yet, the amount of label taken up by J. gardneri during 30 min in the dark was about 7-10% of the amount taken up during 30 min in the light which suggests that this process is of some importance to the alga for assimilation of carbon.

The pulse-chase translocation experiments showed no significant movement of assimilated ^{14}C-label from one alga to the other during the translocation periods used (Tables V and VI). This result was obtained even when the algae were kept in the dark for several hours before being exposed to the radioactive label and then given time for translocation (if any) to occur. Translocation did not occur regardless of whether the algae were provided with light or dark conditions for the translocation periods. The maximum translocation period of 12 hr used for these experiments was selected because it was longer than the 8 hr period which was used by Evans, et al. (1973) to establish that translocation did occur from Gracilaria verrucosa to its parasite Holmsella pachyderma.

The observation (Fig. 2) of host-penetrating filaments from J. gardneri confirms that by Setchell (1914), but is contrary to that of Kugrens (1971) who could not distinguish them. The results of the translocation experiments suggested that the only function of the filaments of mature J. garnderi was for attachment to the host. This is unlike the filaments of the non-photosynthetic red algal parasites Holmsella pachyderma (Evans, et al. 1973) and Harveyella mirabilis (Goff 1975) which appeared to function in absorption of translocates from their hosts. However, it is evident that these translocation experiments do not rule out a small localized exchange of ^{14}C-labeled compounds between the symbiont's

endophytic filaments and adjacent cells of L. spectabilis, nor do the results provide any insight about the possible exchange of non-labeled substances between the two plants.

Examination of ^{14}C-label passing from the algae into the chase seawater (Table VII) showed that usually only 2% or less of the total label present leached out of the algae. This did not appear to be a continuous phenomenon since the amount detected for each translocation period of an experiment remained similar, even under dark conditions when light was not present to enable reassimilation of the label. The similarities in the amounts of "leachate" during the translocation periods of each experiment suggested that the presence of this label in the seawater was not a result of leaching. Instead, it may be from unincorporated ^{14}C-label which was present in or on the surfaces of the cell walls of the algae after the uptake period and was not rinsed away before placing the algae in the chase seawater. It was also noticed that the presence of light during the translocation periods did reduce the amount of label in the chase seawater. However, the small amounts of ^{14}C-label which did appear to be reassimilated suggest a minor role for this process. This contrasts with results from studies on the epiphytic red alga Smithora naiadum which assimilated leached ^{14}C-, ^{15}N-, and ^{32}P- containing compounds from its seagrass hosts (Harlin 1971b; McRoy and Goering 1974).

Electron microscope examination and culture of spores both suggested that J. gardneri had the potential to be self-sufficient. Both carpospores and tetraspores had a pinkish color when released from parent plants in culture, which contrasts with the report of colorless spores for J.

verrucaeformis (Feldmann and Feldmann 1958). Both kinds of spores from J. gardneri were shown by electron microscopy (Fig. 4) to contain dictyosomes, floridean starch reserves, mitochondria, and proplastids. These observations indicated that these spores had the potential to germinate and become photosynthetic. Indeed, carpospores obtained from J. gardneri plants collected in July 1976 at Botany Beach germinated in culture (Fig. 15B). Tetraspores were not germinated in this study, but Kugrens (1971) was successful in this endeavour. In both studies the germlings died within two weeks despite changes of culture media and the presence of pieces of host plants. The amount of development seen for the carpospores in Fig. 15B was similar to that obtained by Feldmann and Feldmann (1958) for J. verrucaeformis 3-6 days after germination upon glass slides. The J. verrucaeformis spores also deteriorated at this stage and was apparently concomitant with the absence of any more floridean starch reserves in the spores. Whether a similar occurrence prevented further development of J. gardneri spores is not known since the presence of starch reserves was not checked. But, at this stage (Fig. 15B) the J. gardneri spores were still intact, with light pigmentation and various cell contents.

Recently Nonamura (pers. comm.), working at the University of California, Berkeley, succeeded in "infecting" L. spectabilis with J. gardneri. He also succeeded in "infecting" L. nipponica from Japan with its parasitic symbiont J. morimotoi. He observed that both J. gardneri and J. morimotoi germlings had an inhibitory effect on the growth of infected segments (ca. 1-5 cm long) of host material. Although this effect may be due to a wound response, Nonamura suggested that the plants

were deriving foodstuffs from their hosts because germlings of both plants change color from pinkish to whitish after a few days growth and continue to increase in size. After about three weeks, the plants become pigmented again. It would seem from these observations that both J. gardneri and J. morimotoi are parasitic as germlings. However, the evidence is circumstantial, based on morphological features, since the necessary physiological studies of photosynthesis and translocation are lacking as is an electron microscopic examination of the "whitish" plants for chloroplasts.

Nonamura's culture work (pers. comm.) did provide some insight into the host specificity of a Janczewskia species. He was able to infect plants of a Laurencia species which is not normally a host for J. mormitoi as it grows in other parts of Japan outside of the distribution range of the parasite. This result like the report of J. gardneri parasitizing L. splendens (Abbott and Hollenberg 1976) suggests that species of Janczewskia are not necessarily restricted to one host species. Numerous potential hosts, including several species of Laurencia, are found within the distribution ranges of both J. gardneri and J. morimotoi. It may be that in nature the two algae are restricted to only one or two specific species of Laurencia by other factors, for example, the type of surrounding substrate (sand vs. rock), the amount of wave action, the temperature or salinity of surrounding waters, or the amount of exposure to desiccation. The factors (such as temperature or wave action) could operate in a general manner by limiting only a few potential hosts to the same locality as the symbionts, or the factors (such as desiccation or substrate) could operate in a particular locality to limit the vertical distribution of potential hosts and symbionts.

There was no preferential selection by J. gardneri for a particular life history stage of L. spectabilis as determined by examination of fresh, preserved, and herbarium specimens. There was no difference in the nutrient pool (Table VIII) of free (ethanol-soluble) amino acids and sugars from L. spectabilis at Botany Beach and at Cable Beach, but the occurrence of J. gardneri upon L. spectabilis was rare at Cable Beach [recorded once during this study (May 1976); not recorded previously at Cable Beach and vicinity (Scagel 1973)] and common year-round at Botany Beach. This suggests that some factor(s) other than the host's life history stage or nutrient pool of free amino acids and sugars accounted for the rare occurrence of J. gardneri upon L. spectabilis at Cable Beach.

The aim of this research was to determine the type of symbiotic relationship which exists between J. gardneri and its host L. spectabilis. Most of the information sought was physiological, including the abilities of both algae to photosynthesize and to translocate photosynthetic products to one another. It was established that both algae photosynthesize, that ^{14}C-label is assimilated into similar compounds and that the labeled compounds are not translocated between the algae. These physiological studies indicate that mature J. gardneri is not a parasite, but an obligate epiphyte. The culture work by Nonamura suggests that the relationship between the two algae is even more complex, with morphological observations suggesting that the germlings of J. gardneri are parasitic on host material; however, the necessary confirmatory physiological studies are lacking. Until J. gardneri is shown to be a physiological parasite, it is probably best to consider it an obligate epiphyte like Smithora naiadum (Harlin 1971b, 1973a). J. gardneri and S. naiadum are photosynthetic, germinate in the

absence of the host, and appear to need a factor, probably chemical, from the host to enable normal development and completion of their life history. The factor for J. gardneri may not be a host photosynthetic metabolite since its spores are pigmented and contain proplastids as well as floridean starch reserves.

The basis for the specificity of J. gardneri for L. spectabilis is unclear. If J. gardneri should require a factor from its host for successful development from a germling to a mature plant, such a factor (if specific to L. spectabilis) could explain this association. Also, as discussed earlier, certain environmental factors may limit only a few potential hosts to the same localities or intertidal areas where J. gardneri may grow successfully. Another possibility is that J. gardneri will only develop successfully after germination if it has certain specific recognition interactions with some surface macromolecule from its host; a possibility which was examined in the second part of this thesis.

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PART II

ISOLATION AND PARTIAL CHARACTERIZATION OF A PROTEOGLYCAN FROM *LAURENCIA SPECTABILIS*

INTRODUCTION

A wide range of proteoglycans (major component carbohydrate), as distinct from glycoproteins (major component protein), are known from higher plants and algae. Hydroxyproline-rich arabinogalactan-proteins have been isolated from wheat endosperm by extraction in water (Fincher and Stone 1974), from cell walls of Chlamydomonas using the chaotropic agent sodium perchlorate (Catt, Hills and Roberts 1976), from various plant cell walls by alkaline extraction (Lamport 1967; Miller, et al. 1974; Monro, Bailey and Penny 1974), and from numerous higher plant sources after extraction in dilute buffer-NaCl solution (Jermyn and Yeow 1975; Anderson, et al. 1977). Acid-soluble proteoglycans from brown algae have been shown to have fucose, mannose or glucuronic acid as the predominant monosaccharide (Larsen, Haug and Painter 1966; Abdel-Fattah, Hussein and Salem 1973; Abdel-Fattah and Edrees 1977; Medcalf and Larsen 1977) and, in a proteoglycan from Ascophyllum nodosum (Medcalf and Larsen 1977), to contain a small amount (ca. 0.2%) of hydroxyproline in the protein moiety. In the red algae, two different water-soluble extracellular proteoglycans have been isolated from the culture media of the unicellular algae Porphyridium cruentum (Jones 1962; Heaney-Kieras and Chapman 1976; Heaney-Kieras, Rodén and Chapman 1977) and Rhodella maculata (Evans, et al. 1974). Both proteoglycans contained xylose (major sugar), galactose, glucose, uronic acids and sulfate as well as a protein moiety (1.5-16% by weight). Another red algal proteoglycan, a

hexose oxidase, was extracted in aqueous buffer from Chondrus crispus and contained galactose (major sugar), xylose, and a protein moiety (ca. 20% of the molecule) (Sullivan and Ikawa 1973). Hydroxyproline was not reported in the protein components of these three red algal proteoglycans. The isolation and partial characterization of a proteoglycan from the red alga Laurencia spectabilis Postels & Ruprecht (Ceramiales) was the subject of this research. This was an attempt to obtain a macromolecule which might have cell recognition properties similar to those for the macromolecular "lectins" in higher plants (Callow 1975; Clarke, Knox and Jermyn 1975; Sharon 1977) and which might allow the red alga Janczewskia gardneri Setchell & Guernsey (Ceramiales), an obligate symbiont of L. spectabilis (Smith 1969; Abbott and Hollenberg 1976), to recognize its host plant.

Lectins

The term "lectin" (from the Latin legere, to choose or pick out) was originally used by Boyd and Sharpleigh (1954) to refer to substances of plant origin which agglutinated red blood cells exhibiting blood group specificity. Later authors (Liener 1976; Kauss and Bowles 1976; Marx 1977; Sharon 1977) considered lectins, such as concanavalin A (Con A), ricin, wheat germ agglutinin and various Phaseolus lectins, to be a class of proteins or glycoproteins which had at least one of two properties: agglutination of cells, including red blood cells, lymphocytes, fibroblasts, spermatozoa or bacteria; or mitogenicity of cells, especially of lymphocytes. The word "lectin" was used by Jermyn and Yeow (1975) to describe any macromolecule that was capable of specific non-covalent

binding to carbohydrates. I will use this broader definition. There is no implication about the chemical nature of the macromolecule, nor about its interactions with various cells. The definition is in agreement with Callow (1975) who also recognized the past problems of the narrow definition and use of the term lectin. He suggested that "in the broadest sense, lectins might simply be defined as those proteins, or glycoproteins, of plant, animal, or bacterial origin, which bind to cell surfaces through specific carbohydrate-containing receptor sites".

β-Lectins

Many of the hydroxyproline-rich arabinogalactan-proteins isolated from higher plant sources have been characterized as β-lectins (Jermyn and Yeow 1975; Anderson, et al. 1977; Jermyn 1977). This group of "all-β" lectins was characterized by Jermyn and Yeow (1975) as having binding specificity for β-D-glycosyl linkages when reacted with Yariv artificial β-glycosyl antigens (Yariv, Rapport and Graf 1962). All the β-lectins so far described contain both a protein and a carbohydrate moiety. The proportion of protein in the β-lectin preparations ranges from 1-50%; hence, β-lectins may be referred to as proteoglycans. Typically, preparations of β-lectins from seeds contain 10-20% protein, and those from leaves, 3-5% protein. The protein is described as "hydroxyproline-rich" because hydroxyproline is a major component found by analysis after acid hydrolysis, usually accounting for 10-25% of the protein composition. The carbohydrate portion of β-lectins contains about 90% galactose (the major sugar) plus arabinose.

β-lectins have been isolated from 91 of 104 higher plant families, including six gymnosperm, eight monocotyledon and 78 dicotyledon families (Jermyn and Yeow 1975). These lectins have been detected from various plant parts, including seeds, stems and leaves, and, in the case of angiosperms, from flower and fruit parts (Jermyn and Yeow 1975; Knox, et al. 1976; Knox and Clarke 1977). This general distribution of β-lectins among higher plant families suggests that there may be physiological roles for these macromolecules in the plants. There is histological evidence that β-lectins are associated with the cell membrane and are concentrated in cell walls and intercellular spaces of bean cotyledon tissue (Clarke, et al. 1975). It was suggested that they "may function as self-recognition factors in plant cells, determining the relationships between vegetative cells in tissues and organs" and that they may "fulfill some role in cell communication". Leaves, petioles, and stems of a number of species contained determinants which bound to the reddish-colored Yariv artificial β-glucosyl antigen and were generally associated either with the cell membrane interfaces, including phloem tissue of veins and midribs, and secretory cells, or with lumen material in secretory canals (Knox and Clarke 1977). The pistils and pollen grains of Gladiolus gandavensis contained β-glucosyl determinants associated with stigma surfaces, mucilage in style secretory canals (which act as pollen tube guides to the ovary), and peripheral cytoplasm of style canal cells and pollen grains (Knox, et al. 1976; Knox and Clarke 1977). Knox, et al. (1976) suggested that the β-lectins may "function as a nutrient source and physical support for the developing pollen tube" of G. gandavensis "as well as acting as an

adhesive for capturing pollen grains at the stigma surface".

Objectives For This Study

I could not find any descriptions of lectins nor any reports of attempts to isolate lectins from any alga. Since β-lectins have proven to be of general occurrence in higher plants of a broad taxonomic range, I decided to investigate the presence of these or related molecules in the algae.

The alga Laurencia spectabilis Postels & Ruprecht was chosen as the study organism because it is the only host along the northeastern Pacific Ocean coastline of the obligate symbiont Janczewskia gardneri Setchell & Guernsey (Smith 1969; Abbott and Hollenberg 1976), and I speculated that some surface macromolecule had a role in specific recognition interactions with might lead to establishment of the symbiosis. If such types of interactions occur, they may be similar to those proposed for β-lectins in higher plants, and L. spectabilis could be a good source of macromolecules like the β-lectins. The use of L. spectabilis as the study organism offered other favorable circumstances, including year-round availability of the organism for analysis, and my familiarity with several aspects of the physiology and biochemistry of both it and J. gardneri. The objectives for this preliminary study were:

(1) to isolate proteoglycans from L. spectabilis using methods for obtaining β-lectins.

(2) to determine, using Yariv artificial antigens, if any "β-lectins" were isolated.

(3) to determine the chemical composition of any β-lectins or other proteoglycans which were isolated.

(4) to determine microscopically the *in situ* locations of any β-lectins in *L. spectabilis* as well as the symbiont *J. gardneri*.

MATERIALS AND METHODS

CHEMICALS AND SOLVENTS

Chemicals and solvents, reagent grade ACS or better, were obtained from the suppliers as indicated: acetic anhydride (J.T. Baker Chemical Co., Phillipsburg, New Jersey); Beckman Amino Acid Calibration Mixture Type 1 (Beckman Instruments, Inc., Spinco Division, Palo Alto, California); barbitone, D-galactose, D-galactosamine HCl, sodium borohydride (British Drug Houses Ltd., Poole, England); all amino acids, p-nitrophenyl-β-D-glucopyranoside (Calbiochem, Los Angeles, California); caprylic acid, chloramine T, N,N-dimethyl-m-phenylenediamine di-HCl, N,N-dimethyl-p-phenylenediamine HCl, m-phenylphenol (m-dihydroxydiphenyl), trifluoroacetic acid (Eastman Kodak Co., Rochester, New York); alcian blue 8GS, barbital sodium, D-mannose, D-mannitol, Phenol Reagent Solution (Folin-Ciocalteau Reagent), phloroglucinol, toluidine blue O (Fisher Scientific Co., Fair Lawn, New Jersey); sodium glucuronate (Koch-Light Laboratories Ltd., Colnbrook, England); p-dimethylamino benzaldehyde, platinum oxide (Matheson Coleman & Bell, Norwood, Ohio); D-arabinose, D-galacturonic acid (monohydrate), D-glucose, D-xylose (Nutritional Biochemicals Corp., Cleveland, Ohio); methyl cellosolve, ninhydrin (Pierce Chemical Co., Rockford, Illinois); bovine serum albumin (crystallized 1x and lyophilized), D-glycosamine HCl, myo-inositol, Trizma Base (Sigma Chemical Co., St. Louis, Missouri). D-galactitol, D-glucitol, D-mannitol and their acetates, D-arabitol pentaacetate, and D-xylitol pentaacetate were provided by Dr. J.N.C. Whyte, Fisheries and Marine Service, Vancouver, British Columbia. All other chemicals and solvents were obtained locally and were of reagent grade ACS quality or better.

PLANT MATERIAL

Laurencia spectabilis Postels & Ruprecht was collected at Botany Beach near Port Renfrew on Vancouver Island, British Columbia in April and November 1976. The fresh material was kept on ice in plastic bags filled with seawater during transportation back to the laboratory. The material was cleaned, blotted dry, and stored at -15°C.

PREPARATION OF PLANT MATERIAL

Plant material (*ca.* 300 gm fresh wt) was thawed and homogenized in small portions in a Waring Blendor in *ca.* 800 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 1% (w/v) NaCl and 3 mM NaN_3 (sodium azide). Additional buffer (500-700 ml) was added and the slurry stirred for 2-6 hr at 5°C then filtered through four layers of cheesecloth. The initial extract was stored at 5°C and the residue was re-extracted in 10 mM potassium phosphate buffer (pH 7.0) containing 5% (w/v) NaCl and 3 mM NaN_3, and filtered. The two extracts were combined. $(NH_4)_2SO_4$ was added stepwise to 40, 70, and 100% saturation at room temperature, and insoluble material was removed after each addition by centrifugation at 4800 x *g* for 15 min. The resulting 100% $(NH_4)_2SO_4$ saturated supernatant was dialyzed against running tap water for 48 hr and concentrated, but not dried, by rotary evaporation at 45°C. It was important that complete drying of the sample be avoided at this step and at later isolation steps because the solubility of substances, including the proteoglycan later isolated, in aqueous solutions was effected.

GEL AND ION EXCHANGE CHROMATOGRAPHY

Samples (10-20 ml) were applied to a column (50 x 3.4 cm) of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 1% (w/v) NaCl and 3 mM NaN_3. The column was eluted with the same buffer. The eluate was monitored for carbohydrate (Dubois, et al. 1956) and for protein (Lowry, et al. 1951; Eggstein and Kreutz 1955). Fractions from the second carbohydrate-containing peak (which contained the proteoglycan material) were pooled, dialyzed against running tap water for 24 hr, concentrated (but not dried) by rotary evaporation, and re-chromatographed on Sepharose 4B. The fractions containing carbohydrate and protein were pooled, desalted on a column (50 x 2.3 cm) of Sephadex G-25-300 (Sigma Chemical Co., St. Louis, MO.), and concentrated (but not dried) by rotary evaporation. Samples (2-5 ml) in deionized water were applied to a column (25-27 x 1.7 cm) of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals) equilibrated with 200 mM sodium phosphate buffer (pH 8.0) containing 3 mM NaN_3. The column was eluted by a linear salt gradient with the flow rate maintained between 15-20 ml/hr. The linear gradient was formed by mixing 300 ml of equilibration buffer with 300 ml of equilibration buffer containing 1.8 M NaCl. The molarity of the eluate was monitored using a conductivity meter (Type COM 2d No. 56606, Radiometer, Copenhagen, Denmark). Column elution was stopped when the eluate molarity reached 0.5 M. Eluate samples were monitored for the presence of carbohydrate. Usually, only one peak of carbohydrate activity was detected. The homogeneity of the carbohydrate-containing fractions was monitored routinely by cellulose acetate strip electrophoresis.

CELLULOSE ACETATE STRIP ELECTROPHORESIS

Samples (5-10 µl) of the carbohydrate-containing fractions from the ion exchange column were applied to 15.2 x 2.5 cm Sephaphore III cellulose polyacetate strips (Gelman Instrument Co., Ann Arbor, MI.). Electrophoresis was performed at 300 V for 40 min at room temperature in a Gelman electrophoresis chamber (model No. 51170-1) using 60 mM Tris-barbital-sodium barbital buffer (pH 8.8). The strips were cut in half and were stained with alcian blue (method a in Reid, et al. 1972) to detect polyanionic substances or with high iron diamine (Reid, et al. 1972) to detect half sulfate esters. The fractions which contained one band of similar electrophoretic and staining behavior were combined, desalted by passage through Sephadex G-25-300, and concentrated (but not dried) by rotary evaporation to 5-10 ml. Some of this solution was kept for testing with Yariv artificial antigen for the presence of a β-lectin, while the remainder was lyophilized prior to chemical analysis.

β-LECTIN DETECTION

Several unsuccessful attempts were made to produce the β-glucosyl form of Yariv artificial antigen using the method described by Yariv, et al. (1962) with some attempts under conditions for the synthesis suggested by Professor Bruce A. Stone (pers. comm.). The gel diffusion cross-precipitation test for β-lectins with a β-glucosyl determinant (Jermyn and Yeow 1975) was done by Dr. Robin L. Anderson (formerly of Professor Stone's Laboratory). An approximate 1% (w/v) solution of the L. spectabilis proteoglycan was provided for the sample test solution, and pea cell

supernatant solution and soybean culture medium were used as controls. A 0.1% (w/v) solution of Yariv artificial β-glucosyl antigen was used for the tests.

ANALYTICAL PROCEDURES

General analysis. Total carbohydrate was determined by the phenol-sulfuric acid method (Dubois, et al. 1956) using galactose as standard.

Total protein was determined by modification (Eggstein and Kreutz 1955) of the Folin-Lowry procedure (Lowry, et al. 1951) using bovine serum albumin as standard.

Total uronic acids was determined using the method of Blumenkrantz and Asboe-Hansen (1973) with a 1:1 mixture of galacturonic and glucuronic acids as standard.

Total sulfate was determined by the method of Nader and Dietrich (1977).

Neutral sugar analysis. Samples (1.5-4 mg) were hydrolyzed with 1 ml of 2 N trifluoroacetic acid in sealed tubes at 110°C for 1 hr. The neutral sugars were analyzed as alditol acetate derivatives (Albersheim, et al. 1967) by gas-liquid chromatography, using a Varian Aerograph dual column gas chromatograph Model 1740 (Varian Aerograph, Walnut Creek, CA.) equipped with flame ionization detectors. The flow rates of N_2 and H_2 were 25 ml/min and of air were 250 ml/min. Derivative samples or standard sugars in chloroform were injected into stainless steel columns (1.8 m x 3 mm o.d.) of 5% (w/w) Silar 10C (Alltech Associates, Arlington Heights, IL.) on 100/120 mesh Gas Chrom Q (Applied Sciences Lab. Inc., State

College, PA.) which were temperature programmed linearly from 120°C (at injection) to 260°C at 2°C/min.

Amino acid and amino sugar analyses. Samples (1-4 mg) were hydrolyzed in vacuo with ca. 0.5 ml of 6 N HCl at 110°C for 20 hr. Hydrolyzates were dried in vacuo and analyzed by the method of Spackman, Stein and Moore (1958) using a Beckman Amino Acid Analyzer Model 120C Instruments, Inc., Palo Alto, CA.). The basic amino acids and the amino sugars were resolved on a 16 x 0.9 cm column (Cameron and Taylor 1976). The acidic and neutral amino acids were resolved on a 58 x 0.9 cm column.

Hydroxyproline was determined after acid hydrolysis using the spectrophotometric method of Bergman and Loxley (1970).

RESULTS

PURIFICATION OF A PROTEOGLYCAN

Figure 16 shows the carbohydrate and protein profile for the elution of L. spectabilis 100% $(NH_4)_2SO_4$ saturated supernatant from Sepharose 4B. Carbohydrate was located in three peaks (A, B, and C). Protein was detected in peaks B and C, but only peak B contained proteoglycan material. After re-chromatography of peak B, the fractions forming the single carbohydrate peak were pooled. The material was shown to contain at least six components when analyzed by cellulose acetate strip electrophoresis (Fig. 17). All the bands (No. 4 and 6) reacted with the high iron diamine stain (sulfate groups present). When this mixture was separated by ion exchange chromatography, a single carbohydrate peak was obtained (Fig. 18). However, electrophoretic examination of the fractions in this peak revealed that the front of the peak (Fig. 18, fractions No. 27-31) contained two alcian blue reactive substances. The major one was a proteoglycan (band No. 2). Electrophoretic analysis of fractions which emerged later revealed only a single component which corresponded to the proteoglycan. Only those fractions shown to be homogeneous by electrophoresis were used for β-lectin detection and for chemical analysis. The minimum yield of the purified proteoglycan was approximately 32 mg dry wt from 800 gm fresh wt of alga.

β-LECTIN DETERMINATION OF THE PROTEOGLYCAN

The L. spectabilis proteoglycan solution did not form cross-precipitation lines with the Yariv artificial β-glucosyl antigen. Only the control

Fig. 16. Sepharose 4B gel chromatography of L. spectabilis 100% saturated $(NH_4)_2SO_4$ supernatant.

A 15 ml sample of L. spectabilis extract (see Materials and Methods for preparation) was subject to gel chromatography on a Sepharose 4B column (50 x 3.4 cm). The running buffer was 10 mM potassium phosphate (pH 7.0) containing 1% (w/v) NaCl and 3 mM NaN_3. Carbohydrate (Dubois, et al. 1956; ●——●) and protein (Lowry, et al. 1951 as modified by Eggstein and Kreutz 1955; O——O) were detected in the eluate.

V_o = void volume V_t = total volume

FIGURE 16

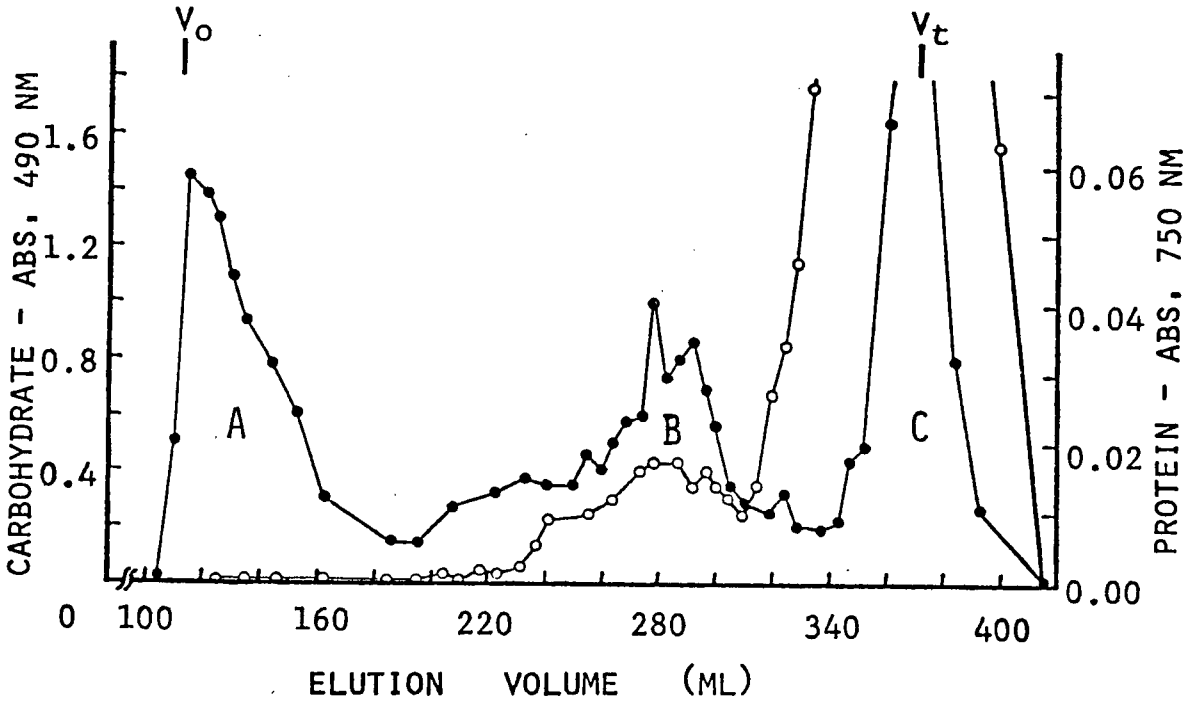

V_o
V_t
A
B
C
CARBOHYDRATE - ABS. 490 NM
PROTEIN - ABS. 750 NM
0
0.4
0.8
1.2
1.6
0.00
0.02
0.04
0.06
0
100
160
220
280
340
400
ELUTION VOLUME (ML)

Fig. 17. Cellulose acetate strip electrophoresis of L. spectabilis proteoglycan after re-chromatography on Sepharose 4B.

The proteoglycan after re-chromatography on Sepharose 4B was subject to electrophoresis and then stained with alcian blue or with high iron diamine (see Materials and Methods). This drawing shows the results after staining of the strips. Band No. 2 is the proteoglycan investigated in this study.

FIGURE 17

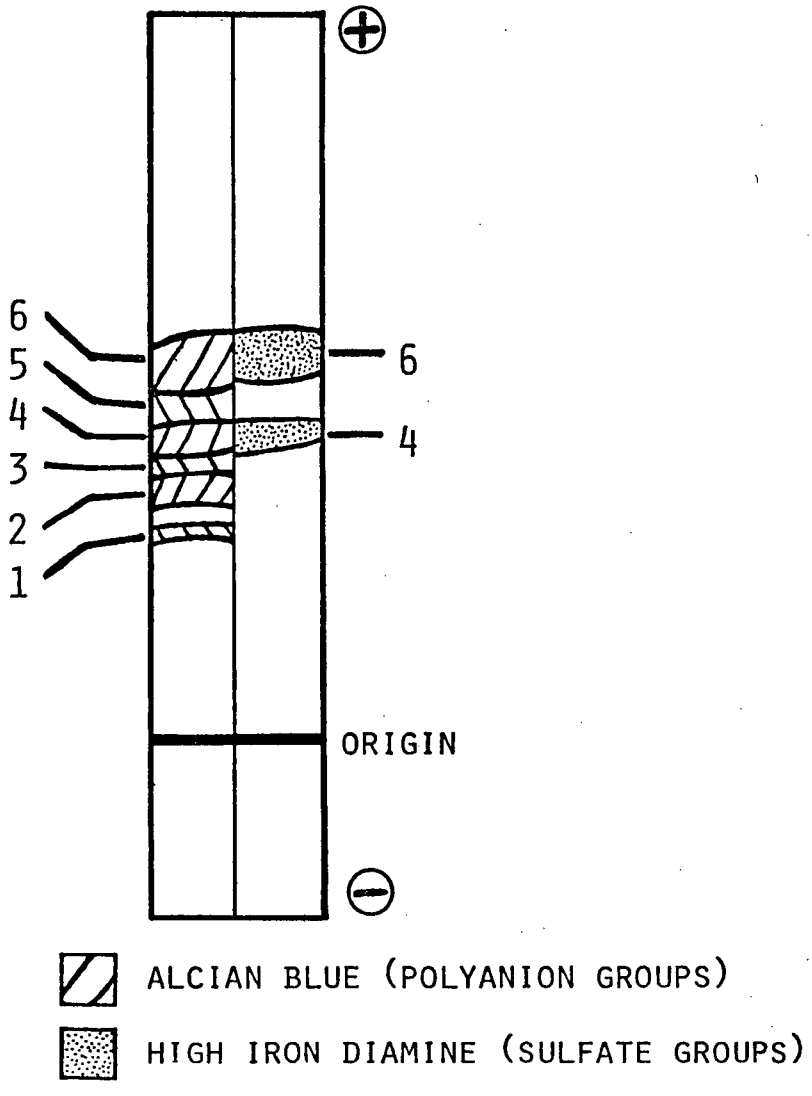
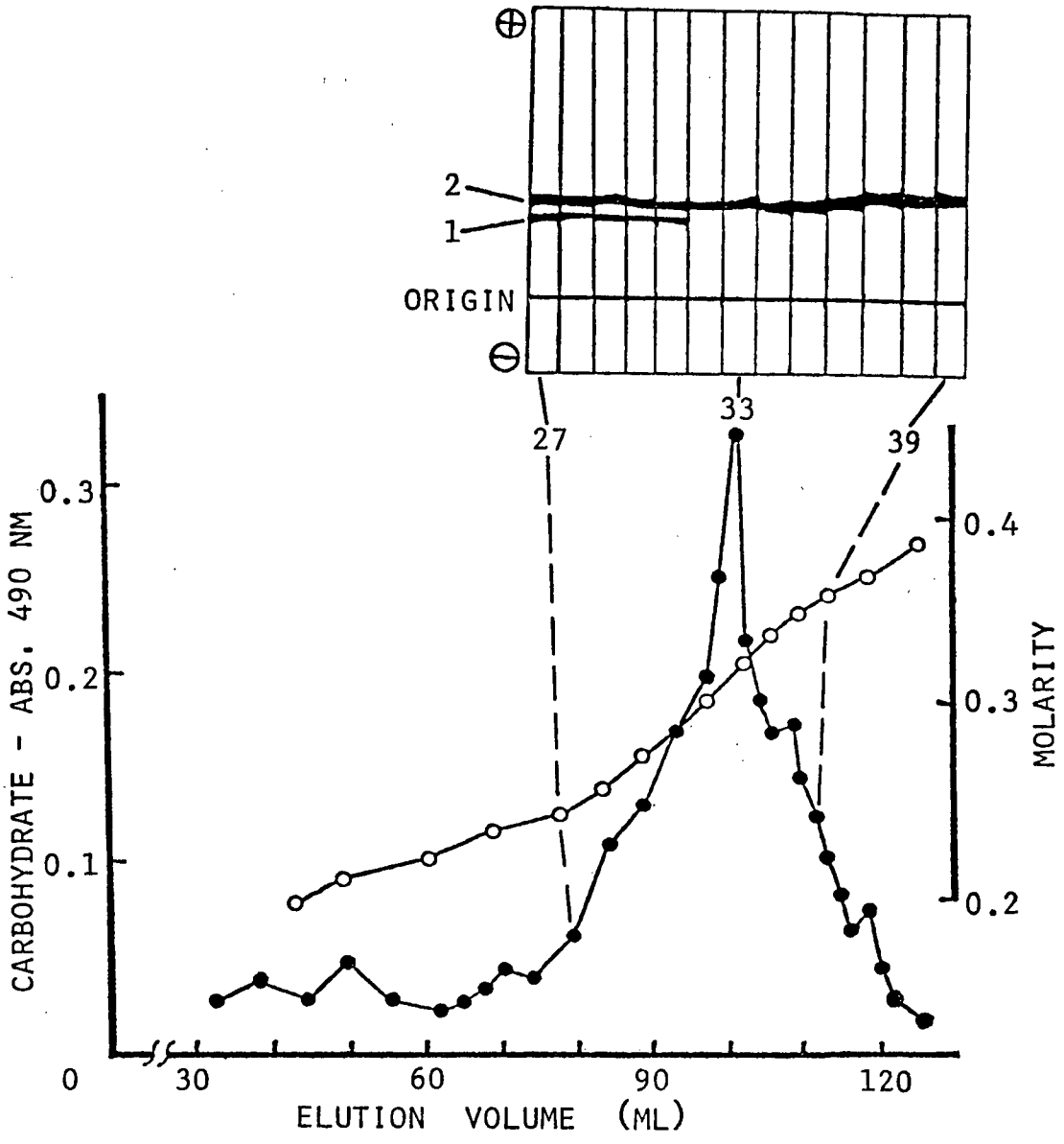

6
5
4
3
2
1
6
4
ORIGIN
ALCIAN BLUE (POLYANION GROUPS)
HIGH IRON DIAMINE (SULFATE GROUPS)

Fig. 18. Salt-gradient elution of L. spectabilis proteoglycan from DEAE-Sephadex A-50 and cellulose acetate strip electrophoresis of fractions.

A proteoglycan sample purified by gel chromatography was applied to the anion exchange column (27 x 1.7 cm) and eluted by a salt gradient. The carbohydrate content (●——●) and the salt molarity (○——○) were determined. Electrophoresis was performed on carbohydrate-containing fractions followed by staining with alcian blue and high iron diamine. Details in the Materials and Methods. The drawing shows results of the electrophoresis and staining with alcian blue of fractions No. 27-39. No substances reacted with high iron diamine. Band No. 2 is the proteoglycan investigated in this study.

FIGURE 18


⊕
2
1
ORIGIN
⊖
27
33
39
CARBOHYDRATE - ABS. 490 NM
0.3
0.2
0.1
0
30
60
90
120
ELUTION VOLUME (ML)
0.4
0.3
0.2
MOLARITY

solutions of pea cell supernatant and soybean culture medium formed cross-precipitation lines. Even when the L. spectabilis proteoglycan solution was concentrated six-fold, no precipitation lines were formed. The proteoglycan did not have a β-glucosyl determinant β-lectin.

CHEMICAL ANALYSIS OF THE PROTEOGLYCAN

The composition of the apparently homogeneous proteoglycan is shown in Table X. The preponderance of carbohydrate over protein confirmed the view that the compound isolated from L. spectabilis was a proteoglycan. Uronic acids accounted for 8.4% of the total composition. Sulfate was not detected by either the assay of Nader and Dietrich (1977) or, after electrophoresis of the proteoglycan, reaction with high iron diamine stain (minimum detection limit of stain ca. 20 μg of sulfate when determined by the Nader and Dietrich assay).

The neutral sugar compositions of two different proteoglycan preparations are shown in Table XI. The proteoglycan was apparently a galactan with small quantities of xylose and glucose in addition to the uronic acids (see Fig. 19). The arabinose detected in both preparations was present in only trace quantities.

The amino acids and amino sugars recovered (see Fig. 20) from the hydrolyzates of two different preparations of the proteoglycan are given in Table XII. The acidic amino acids aspartate, threonine, serine, and glutamate accounted for approximately 60% of the recovered amino acids. Hydroxyproline and several other amino acids were detected in small (less than 1.5%) quantities, while neither preparation contained detectable

TABLE X

COMPOSITION OF PURIFIED PROTEOGLYCAN FROM LAURENCIA SPECTABILIS

Results are the mean of four analyses (see Materials and Methods) for one preparation.

Carbohydrate	92%	Uronic Acids	
Protein	8%	Total weight	8.4%
		Total carbohydrate	9.1%
Carbohydrate/ Protein Ratio	12:1	Sulfate	N.D.*

* N.D. - not detected; if present, less than 2.0% of total weight.

TABLE XI

NEUTRAL SUGAR COMPOSITION OF PURIFIED LAURENCIA SPECTABILIS PROTEOGLYCAN

Results for each preparation are the average of two analyses using gas-liquid chromatography (see Materials and Methods).

	Preparation No. 1	Preparation No. 2
Arabinose	1.8% (1.5%)*	2.3% (1.9%)
Xylose	6.4 (5.4)	7.4 (6.2)
Galactose	84.6 (70.7)	84.6 (70.7)
Glucose	7.3 (6.1)	5.7 (4.8)

* Results in ( ) are the proportions of each neutral sugar by total weight of the proteoglycan.

Fig. 19. Gas-liquid chromatogram of alditol acetate derivatives of sugars from purified L. spectabilis proteoglycan.

A sample of purified proteoglycan was hydrolyzed for 1 hr in 2 N trifluoroacetic acid, reduced and, after addition of the internal standard myo-inositol, acetylated with acetic anhydride (see Materials and Methods). The sample in chloroform was injected into a Varian Aerograph gas chromatograph with 5% SilarCl0C columns and temperature programmed from 120°-260°C at 2°/min.

FIGURE 19

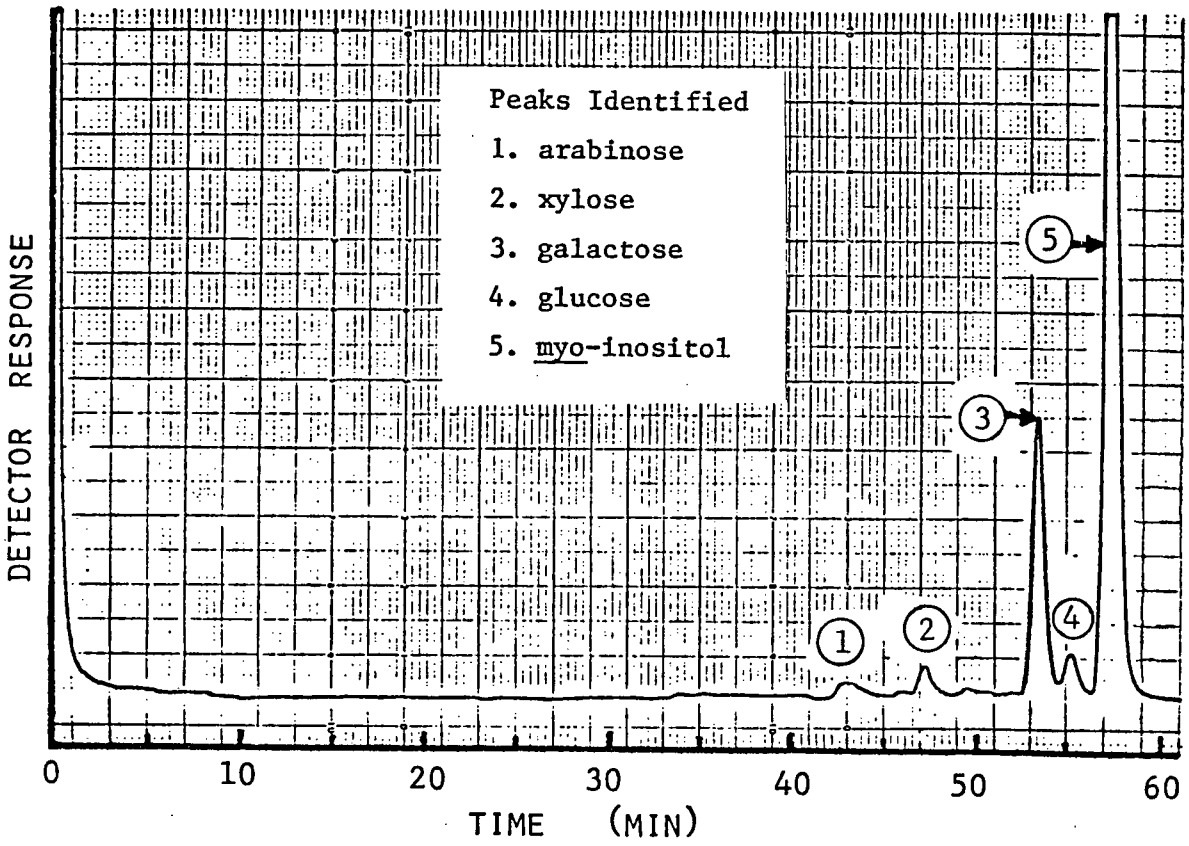

Peaks Identified
1. arabinose
2. xylose
3. galactose
4. glucose
5. myo-inositol
DETECTOR RESPONSE
0
10
20
30
40
50
60
TIME (MIN)

Fig. 20. Chromatograms of (A) amino sugars and basic amino acids and (B) acidic and neutral amino acids of purified L. spectabilis proteoglycan.

(A) A sample of purified proteoglycan was hydrolyzed for 20 hr in 6 N HCl before analysis of amino sugars and basic amino acids using an amino acid analyzer, with a running buffer of pH 5.25 (see Materials and Methods). AGPA (α-amino-β-guanidinopropionic acid) was added to the sample as an internal standard. The detected substances are;

(1) acidic and neutral amino acids
(2) Phe
(3) GlcN
(4) GalN
(5) Lys
(6) His
(7) NH_3
(8) AGPA
(9) Arg

(B) Sample details for the analysis of acidic and neutral amino acids were as in (A). The running buffer was pH 3.25 until peak (9), when the buffer was changed to pH 4.30. Norleucine was added to the sample as an internal standard. The detected substances are:

(1) Asx
(2) Thr
(3) Ser
(4) Glx
(5) Pro
(6) Gly
(7) Ala
(8) Val
(9) buffer change peak
(10) Met
(11) Ile
(12) Leu
(13) Nle
(14) ATyr
(15) Phe

FIGURE 20

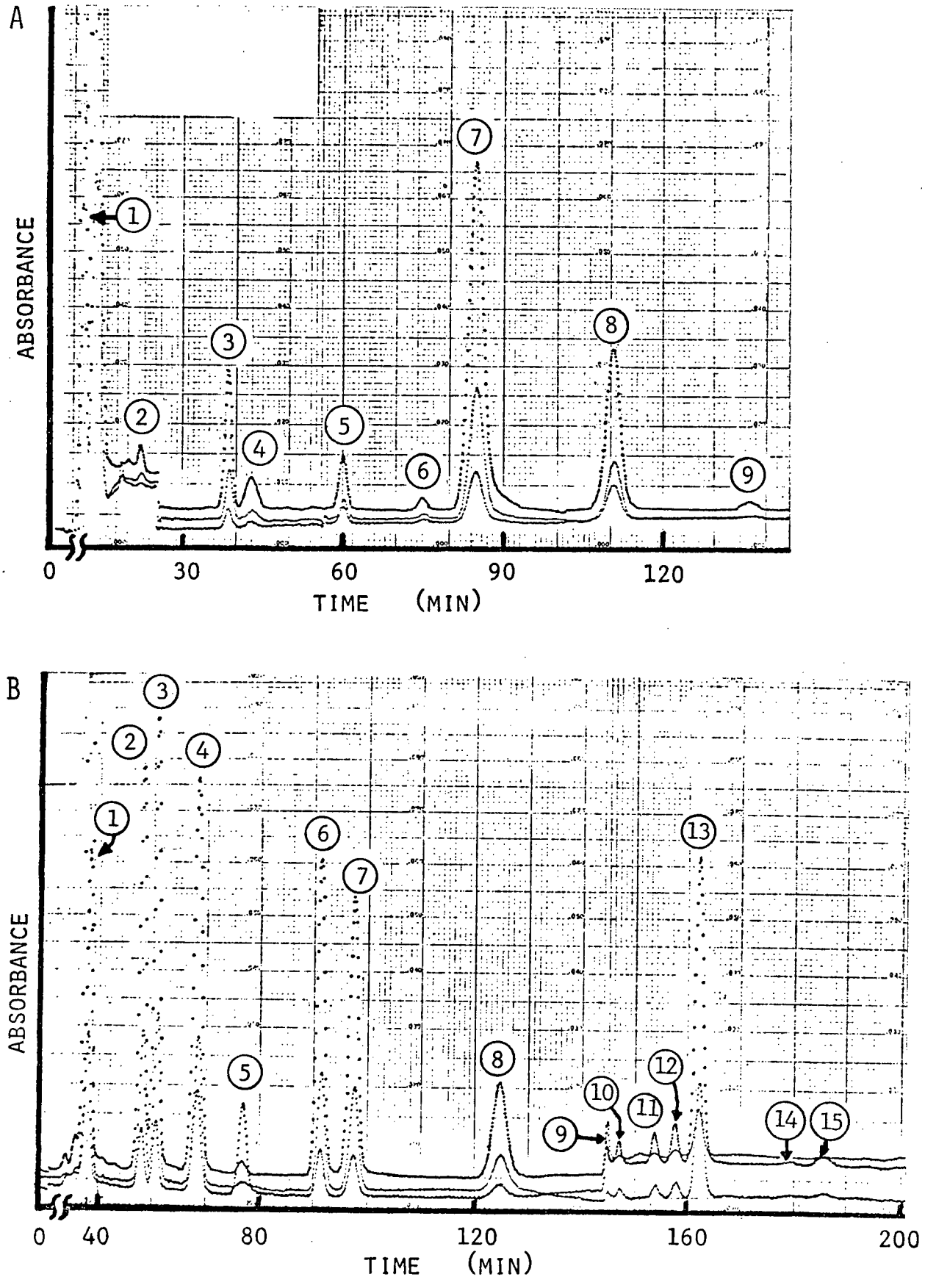

A
ABSORBANCE
1
2
3
4
5
6
7
8
9
0
30
60
90
120
TIME (MIN)
B
ABSORBANCE
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
0
40
80
120
160
200
TIME (MIN)

TABLE XII

COMPOSITION OF AMINO ACIDS AND AMINO SUGARS RECOVERED FROM HYDROLYZATES OF L. SPECTABILIS PROTEOGLYCAN

Two preparations of purified proteoglycan were hydrolyzed for 20 hr in 6 N HCl before analysis of amino acids and amino sugars using an amino acid analyzer (see Materials and Methods). The results for each preparation are the average of two analyses.

	Prep. No. 1	Prep. No. 2		Prep. No. 1	Prep. No. 2
Asx	23.9%		Lys	1.7%	2.7%
Thr	9.9	9.5	His	0.6	0.5
Ser	8.8	8.0	Arg	0.8	1.0
Glx	15.4	17.7			
Pro	11.2	11.7			
Gly	5.1	6.5	GlcN	4.9	2.1
Ala	6.6	5.9	GalN	2.3	0.5
Cys	0	0			
Val	5.0	4.3			
Met	0.6	0.5	Hyp*	0.3	0.3
Ile	0.8	0.7			
Leu	0.8	0.7			
Tyr	0.3	0.3			
Phe	0.8	1.2			

*Determined spectrophotometrically

amounts of cyst(e)ine. Glucosamine and galactosamine were present in both preparations, although the recovery of both amino sugars was lower in preparation No. 2.

DISCUSSION

The procedures used to obtain the isolated proteoglycan from L. spectabilis included extraction of the plant material with a dilute buffer-NaCl solution, followed by purification steps involving solubility in 100% saturated $(NH_4)_2SO_4$ and gel chromatography on Sepharose 4B. These procedures were used to obtain β-lectins from higher plant sources (Jermyn and Yeow 1975; Anderson, et al. 1977). The use of a dilute buffer-NaCl solution for extraction was of importance since this mild condition, compared with more rigorous acid or base extraction conditions, was more likely to leave proteoglycans intact. An additional procedure involving ion exchange chromatography with very careful gradient elution was needed to obtain the purified L. spectabilis proteoglycan. Cellulose acetate strip electrophoresis was used to monitor the progress of purification and to determine the point of emergence of the single proteoglycan component from the DEAE-Sephadex A-50 ion exchange column (Fig. 18). Clear resolution of the "contaminant" component from the proteoglycan was obtained only when a "slow" continuous salt gradient (total vol. 600 ml) formed part of the ion exchange chromatography procedure. Earlier attempts using a step-wise or a "steep" continuous gradient (total vol. less than 300 ml) did not provide resolution of the two substances.

Only one major proteoglycan fraction (peak B, Fig. 16), containing a single proteoglycan (band No. 2, Fig. 17), was obtained from L. spectabilis. The other fraction containing Folin-Lowry positive material was peak C, which consisted of substances eluted at the total volume (V_t) of

the Sepharose 4B column. The "contaminant" component (band No. 1, Fig. 18) which was resolved from the proteoglycan by ion exchange chromatography appeared to be a polysaccharide composed mainly of uronic acids, arabinose, and glucosamine, but without any detectable protein (according to electrophoretic, sugar, and amino acid analyses of fractions No. 27-31 in Fig. 18). The four additional substances (bands No. 3-6, Fig. 17) detected after electrophoresis of the proteoglycan fraction were not characterized because they were not recovered after ion exchange chromatography, even when the molarity of the elution buffer was raised to 2 M.

Some methods used to obtain β-lectins have proven successful for isolating a proteoglycan from a red alga. However, the results of the gel diffusion cross-precipitation studies of the purified proteoglycan with the Yariv artificial β-glucosyl antigen indicated that the proteoglycan was not a β-lectin.

The chemical analyses also indicated that the isolated proteoglycan was not a β-lectin. The β-lectins characterized to date (Jermyn and Yeow 1975; Anderson, et al. 1977; Jermyn 1977) have been composed primarily of a carbohydrate component of galactose and arabinose (ca. 4:3 ratio of galactose:arabinose) and a smaller protein moiety "rich" in hydroxyproline (usually greater than 10% of the amino acid composition). The isolated proteoglycan from L. spectabilis had a carbohydrate:protein ratio in the range of those for β-lectins and it contained galactose and arabinose, but the ratio of the two sugars (85:2) and the low amount of hydroxyproline (0.3% of the total amino acid composition) indicated that the molecule differed substantially from β-lectins. It has been noted that hydrox-

yproline plays an important role in the active site of β-lectins (Jermyn 1977; R.L. Anderson, pers. comm.); thus a substance containing the low amount of hydroxyproline observed in the molecule from L. spectabilis was very unlikely to react with a Yariv artificial antigen. The composition and relative amounts of sugars in the carbohydrate portions of the two proteoglycans reported from the red algae Porphyridium cruentum (Jones 1962; Heaney-Kieras and Chapman 1976; Heaney-Kieras, et al. 1977) and Rhodella maculata (Evans, et al. 1974) were similar, but only the amount of uronic acids was determined quantitatively for both molecules (8.5% by weight for P. cruentum and 12% for R. maculata). The quantitative neutral sugar analysis (by total weight) of the molecule from P. cruentum showed that there was approximately 44% xylose, 38% galactose, and 18% glucose (Heaney-Kieras and Chapman 1976; Heaney-Kieras, et al. 1977). The carbohydrate portion of the L. spectabilis proteoglycan (Tables X, XI) had sugars and uronic acid content (8.4%) similar to the other two molecules; however, galactose was by far the major neutral sugar (70.7% by total weight) and a trace amount of arabinose was present. The hexose oxidase proteoglycan from the red alga Chondrus crispus also contained galactose as the predominant sugar and xylose, but no uronic acids were present (Sullivan and Ikawa, 1973). The neutral sugars galactose and glucose, plus trace amounts of arabinose and xylose, were the main constituents of the carbohydrate portions of glycoproteins from the red algae Phyllophora nervosa (Medvedeva and Selich 1968; Medvedeva and Kaganovich 1970; Medvedeva, et al. 1973) and Furcellaria fastigiata (Krasil'nikova and Medvedeva 1975). The main neutral sugar constituents of L. spectabilis cell walls were also

galactose (ca. 69% of the neutral sugars), glucose (9%), and xylose (6%), plus mannose (5%) (Court 1972).

Protein accounted for 1.5-7% (by weight) of the Porphyridium cruentum proteoglycan (Jones 1962; Heaney-Kieras and Chapman 1976; Heaney-Kieras, et al. 1977), for about 16% of the Rhodella maculata proteoglycan (Evans, et al. 1974), and for about 20% of the Chondrus crispus proteoglycan (Sullivan and Ikawa, 1973). The published amino acid compositions of the proteoglycans from P. cruentum and C. crispus does not contain hydroxyproline, but the authors did not report using any of the specific assays available to detect this imino acid. The L. spectabilis proteoglycan contained 8% (by weight) protein and hydroxyproline was present. The presence of hydroxyproline in red algal protein was reported by Lewis and Gonzalves (1962) and Lewis (1973), but not in the analyses presented by Gotelli and Cleland (1968). My report of hydroxyproline in a red alga is based upon results from a more sensitive assay (Bergman and Loxley 1970) than used by previous workers as well as upon analysis of a purified substance rather than a whole plant extract. Either or both factors could account for the disagreement with the results of Gotelli and Cleland (1968) whose paper is often cited as the most authoritative source on hydroxyproline distribution in algal proteins.

The low amount of hydroxyproline detected in the L. spectabilis proteoglycan suggests that any role which it may have in cell wall growth or structure differs at least quantitatively from the roles suggested for hydroxyproline in the hydroxyproline-rich proteoglycans from higher plants (Lamport 1965, 1970) or green algae (Thompson and Preston 1967;

Miller, Lamport and Miller 1972; Miller, et al. 1974). The linkage between hydroxyproline and arabinose, or galactose in a green alga (Miller, et al. 1972), is the major protein-carbohydrate linkage in these proteoglycans. The protein-carbohydrate linkage of Porphyridium cruentum has been investigated and evidence for an O-glycosidic linkage of serine or threonine to xylose was obtained (Heaney-Kieras, et al. 1977). Although these authors claim this report to be the "first such report in the plant kingdom" of a "protein-carbohydrate linkage involving serine and threonine", Russian workers (Krasil'nikova and Medvedeva 1975) had earlier found an O-glycosidic bond between serine and glucose in a glycoprotein from the red alga Furcellaria fastigiata. In addition, other reports (Medvedeva and Selich 1968; Medvedeva, et al. 1973) contained evidence for protein-carbohydrate bonds through the β-carboxyl group of aspartic acid or the hydroxyl group of tyrosine to galactose in other red algal glycoproteins. Since the L. spectabilis proteoglycan contains large quantities of aspartic acid (some of which could be a hydrolysis product from asparagine), serine, threonine, galactose, glucose and xylose, these compounds, rather than hydroxyproline and arabinose, probably form the major protein-carbohydrate linkages of this molecule. Similarly in the brown alga Ascophyllum nodosum, an ascophyllan-like proteoglycan, which contained a small amount of hydroxyproline (and no arabinose), appeared to have the majority of protein-carbohydrate linkages through serine and threonine (Medcalf and Larsen 1977).

The presence of sulfate in red algal galactans has been documented (Percival and McDowell 1967), although some non-sulfated galactans are known, for example, that from the mucilage of Batracho-

spermum sp. (Turvey and Griffiths 1973). The acidic proteoglycan from Porphyridium cruentum contained 9-10% (by weight) ester sulfate (Jones 1962; Heaney-Kieras and Chapman 1976; Heaney-Kieras, et al. 1977), whereas that from Rhodella maculata had 10% sulfate (Evans, et al. 1974). Sulfate was not detected in the proteoglycan from Chondrus crispus (Sullivan and Ikawa 1973). Sulfate was also absent from the acidic L. spectabilis proteoglycan; thus, the uronic acids in the molecule accounted for its reaction with alcian blue stain and its behavior during both ion exchange chromatography and cellulose acetate strip electrophoresis.

It has been established that a proteoglycan can be isolated from the red alga L. spectabilis by methods used for obtaining β-lectins and that the isolated proteoglycan is not a β-lectin. The chemical composition of the proteoglycan has been determined. These findings accomplish the first three objectives of this study and, since no β-lectins were isolated, the fourth goal has been obviated. The location and function of the isolated proteoglycan in the alga remains to be resolved.

There are five possible general locations for this proteoglycan in L. spectabilis: in an extracellular mucilage (as the two proteoglycans from Porphyridium cruentum and Rhodella maculata), in the cell wall, in the cell membranes, in the cytoplasm, or in any combination of these places. The firm, non-slimey texture of L. spectabilis plants seems to eliminate location in extracellular mucilage, whereas the large carbohydrate component and the water-soluble property of the proteoglycan seems to exclude its location in cell membranes. The proteoglycan may be located in the cytoplasm, possibly as an enzyme. Since its neutral sugar composition is

similar to the neutral sugar composition of the cell wall, it also seems reasonable to speculate that some of the molecule is located in the cell wall. It is premature to comment on possible relationships of the proteoglycan with the protein-rich "cuticle", possibly glycoprotein in nature, which has been reported from some red algae (Hanic and Craigie 1969; Lichtlé 1975; Gerwick and Lang 1977).

Possible functions for the proteoglycan include as a structural component of the cell wall, as a non-sulfated galactan "precursor" for synthesis of a sulfated galactan, as another type of lectin than a β-lectin, or as an enzyme. The apparently small amount of proteoglycan isolated from the alga (minimum of 32 mg dry wt from 800 gm of fresh alga), suggests a non-structural role and a non-involvement as a precursor for sulfated galactans, typical components of red algal cell walls (Percival and McDowell 1967). I do not know of any reports concerning other lectin types in the red algae. An enzymatic function was reported for the non-sulfated proteoglycan from Chondrus crispus as well as for two glycoproteins from the red algae Rhodymenia pertusa (Fredrick 1971) and Cyanidium caldarium (Fredrick 1972). Future work should consider a possible enzymatic function for the L. spectabilis proteoglycan.

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