STRAIN SPECIFICITY AND "SELF/NON-SELF" RECOGNITION IN A TROPICAL MARINE DEMOSPONGE, *Verongia longissima*.

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This thesis is dedicated to my friend and constant diving buddy, Tina Ortiz, whose support and willingness to help throughout this study contributed greatly to the completion of the work.

ABSTRACT

Grafting experiments and immunological analyses have provided direct evidence for the occurrence of strain specificity in the marine Demosponge, Verongia longissima. All autografts accepted, all xenografts rejected, and there was both acceptance and rejection of allografts. Among allografts groups of individuals could easily be identified which showed consistent acceptances and rejections of other individuals. The groups were designated as strains. Results of immunological analyses which employed rabbit antisera and sponge antigens supported this strain designation and revealed extensive antigenic polymorphism among strains of Verongia longissima. These sponges demonstrate an immunorecognition at the allogeneic level, similar to the immunological response of higher metazoans. The use of an immunofluorescence test for strain identification proved impossible when the cells of Verongia longissima were found to possess autofluorescence.

Histological preparations of graft rejections showed the development of a cuticle between the surfaces of the two sponges in contact. Results of radioautography experiments have provided the basis for the formulation of an hypothesis concerning the formation and deposition of this cuticle. The role of specificity in patterns of interactions between sponges in situ has been discussed from an ecological perspective.

RÉSUMÉ

Des expériences de greffage et des études immunologiques avec l'éponge marine Verongia longissima (Demospongia) ont révélé chez ce spongiaire une spécificité de souche. Les homogreffes sont compatibles, les xénogreffes sont rejetées et les allogreffes sont parfois compatibles, parfois rejetées. Des groupes d'individus ayant, aux allogreffes, des réactions constantes de compatibilité ou de rejet sont identifiables. Ces groupes sont appelés souche. Les résultats d'analyse immunologiques utilisant l'antisérum de lapin et des antigènes d'éponge confirme l'identification des souches et ont démontré l'existance de polymorphisme antigénique des souches de Verongia longissima.
L'identification des souches à l'aide de tests d'immunofluorescence s'est avérée impossible à cause de l'autofluorescence des cellules de Verongia longissima.

L'examen histologique de préparations de tissus provenant de greffes rejetées a révélé la formation d'une cuticule sur les zones de contact entre les deux éponges. Les résultats d'expériences de radioautographie ont permis l'élaboration d'une hypothèse concernant la formation et la déposition de cette cuticule. Le rôle de la spécificité dans les interactions entre éponges in situ est discuté du point de vue écologique.

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INTRODUCTION

One of the most definitive expressions of individuality (self-recognition) in higher organisms is the rejection of grafts between individuals. Graft rejection or acceptance is mediated by immune mechanisms considered to be different for each individual (Curtis, 1978). However, a much simpler expression of individuality is the capacity for an organism to maintain its autonomy in nature despite contacts with other individuals of the same species.

At the turn of the century Wilson pioneered sponge cell reaggregation studies with his classical work on coalescence and regeneration. When the cells of two different species of sponges were disaggregated and cultured under conditions favourable to reaggregation, Wilson (1907) observed that the cells aggregated into specific cellular masses. They appeared to be maintaining a separateness from one another, a phenomenon described by Wilson as a type of species specificity. This was the first record of this concept in the lower Metazoa, and with the possibility of gaining further insight into the general principles of species specificity and the implication of it in both lower and higher organisms, further studies of sponge cell reaggregation have been carried out by many workers (Galstoff, 1925; Curtis, 1962; Humphreys, 1970; John, et al, 1971; McClay, 1971; 1974; MacLennan, 1974).

In the past, the acceptance by an invertebrate of

material from another member of the same species was considered to be the rule (Burnet, 1971). Exceptions to that rule are now appearing in the literature. In the last decade histoincompatibility, or the capacity for an organism to distinguish "self" from "non-self" has come to be a common phenomenon among the lower invertebrates, including gorgonians (Theodor, 1970), corals (Hildemann, et al, 1975; 1977), ascidians (Oka, 1970; Mukai and Watanabe, 1974), and sponges (Humphreys, 1970; Van de Vyver, 1971 b). This "self/non-self" recognition system could be a negative reaction, where two individuals graft together unless a specific signal is exchanged to indicate incompatibility, or, it could be a positive reaction where the two tend to regard each other as foreign until a positive signal occurs enabling them to recognize each other as compatible (Burger, et al, 1978). A review of the literature related to this phenomenon reveals a lack of information of the underlying mechanisms involved in almost all cases of immunorecognition and the incompatibility reactions which follow such recognition.

The rejection of tissue transplanted from one individual to another individual of the same species, an allogeneic graft, is one aspect of an individual's ability to form an adaptive immunity response to foreign tissue (Coffaro and Hinegardner, 1977). This is a well known phenomenon among vertebrates (Warr and Marchalonis, 1978). The incompatibility responses noted in lower invertebrates

are more commonly found when transplants between species, xenogeneic grafts, are used. These incompatibility responses have been described as nonimmunological (Coffaro and Hinegardner, 1977). However, immunorecognition at the allogeneic level has been recently reported by Hildemann, et al (1977) in corals, and Hildemann, Johnson, and Jokiel (1979) in sponges. These workers have found that a highly sensitive immune system demonstrating the essential attributes of adaptive immunity is present at this lower phylogenetic level. This could represent the origins of the major histocompatibility complex and cell-mediated immunity found in higher vertebrates (Hildemann, Johnson, and Jokiel, 1979).

The first record of incompatibility between individual members of the same species (intraspecific incompatibility) in the Porifera was reported by Van de Vyver (1970) in the freshwater species, Ephydatia fluviatilis, and a marine species, Crambe crambe. This study revealed that there are a number of strain types in these two species, each strain being defined by their incompatibility with all other members of the species in contact zones, creating a discrete border, or zone of non-coalescence, separating the allogeneic individuals. Members of the same strains fused compatibly. This strain specificity appears to be carried over into the sexual larvae of Ephydatia fluviatilis. Larvae of the same mother sponge fused and gave rise to a single sponge, in contrast to larvae from different mothers

which did not fuse. It appears that the stability of a strain is preserved through early stages of sexual reproduction, but both paternity of these larvae and the time of expression of that genetic component in compatibility reactions was unknown.

Van de Vyver also noted the existence of eight different strains of a number of crossed individuals of Ephydatia fluviatilis in five different ponds containing large populations of this sponge, compared to ten different strains of the marine sponge Crambe crambe found on a single rock surface in the Mediterranean Sea. From this study it appears that the marine system offers conditions more favourable to the evolution of a higher number of strains within a species. This is substantiated by the recent work of Hildemann, Johnson, and Jokiel (1979) on the tropical Indo-Pacific sponge Callyspongia diffusa. Isografts (self grafts) of this species fused compatibly, but allografts (same species grafts) were invariably incompatible for all 200 individuals tested.

Most of our knowledge of strain specificity in sponges has come from extensive investigations on the freshwater sponges (Van de Vyver, 1970; 1971(b); 1975; Curtis and Van de Vyver, 1971; Van de Vyver and Willenz, 1975). However, as a result of the relatively recent recognition of the importance of the roles that sponges play in a coral reef system (Bergquist, 1978; Fry, 1970; Grassé, 1973;

Harrison and Cowden, 1970; Hyman, 1970; Sarà, 1970; and Vacelet, 1971), more studies are being carried out on the marine sponges.

Immunological approaches to the study of "self/non-self" recognition in sponges have extended the concept of species specificity to a more rigorous experimental arena. As a result of this approach some workers have postulated an antigen-antibody form of complementarity to account for specificity noted in reaggregation studies (Kuhns, et al, 1974). It appears that an immunological approach to strain specificity in grafting experiments of sponges is lacking in the literature to date. Perhaps if studies were to take this direction, more light could be shed on the mechanisms involved in histoincompatibility reactions occurring in allogeneic rejection, and in the sensitive immune system that these lower invertebrates possess.

Investigations carried out by Vacelet (1971) on the sponge species Verongia aerophoba (Schmidt) and Verongia cavernicola (Vacelet) demonstrated the presence of a cuticle on the surface of these sponges. It was reported to be present when foreign substances came into contact with the surface, and under pathological conditions. Since the process of "self/non-self" recognition involves the recognition of "foreigness", and this process has been found to occur in allogeneic grafting studies (Curtis, 1978;

Hildemann, Johnson, and Jokiel, 1979), it is expected that the cuticle formed by Verongia species is involved in graft rejection of this genus.

It was with these considerations of the process of "self/non-self" recognition that I chose to carry out the investigations reported in this manuscript. Specifically, the objective of this study was to investigate whether strain specificity is expressed in the marine Demosponge, Verongia longissima and the significance of this specificity as demonstrated in in situ grafting experiments, immunological analyses, histological studies, and radioautographic analyses.

MATERIALS AND METHODS

The field work was conducted from May to September 1978 and December 1978, at the Bellairs Research Institute of McGill University. The institute is located on the west coast of Barbados, West Indies (Figure 1). Preliminary laboratory experiments were carried out at the institute and final analyses were performed at McGill University in Montreal.

The study was focused principally on the marine Demosponge, Verongia longissima (Carter) which is typically an irridescent blue-violet ramose sponge with branches about 1 cm in diameter (Plate I). It is a common sponge widely distributed on the west coast of Barbados at depths ranging from 5 to 25 metres. A closely related species, Verongia cauliformis (Carter) was studied less intensively. It is a bright golden-yellow ramose sponge with branches about 1.5 cm in diameter. This species is usually found only in shallow water (approximately three metres) but was observed in the study area at a depth of 13 metres. Another Verongia species, Verongia fistularis (Pallas) was also observed and used in several of the investigations. It is a golden-yellow tube sponge, ranging from a few centimetres to more than a metre in height, and is found in 5 to 25 metres of water. All three species are common, widely distributed members of general sponge fauna of Caribbean coral reefs.

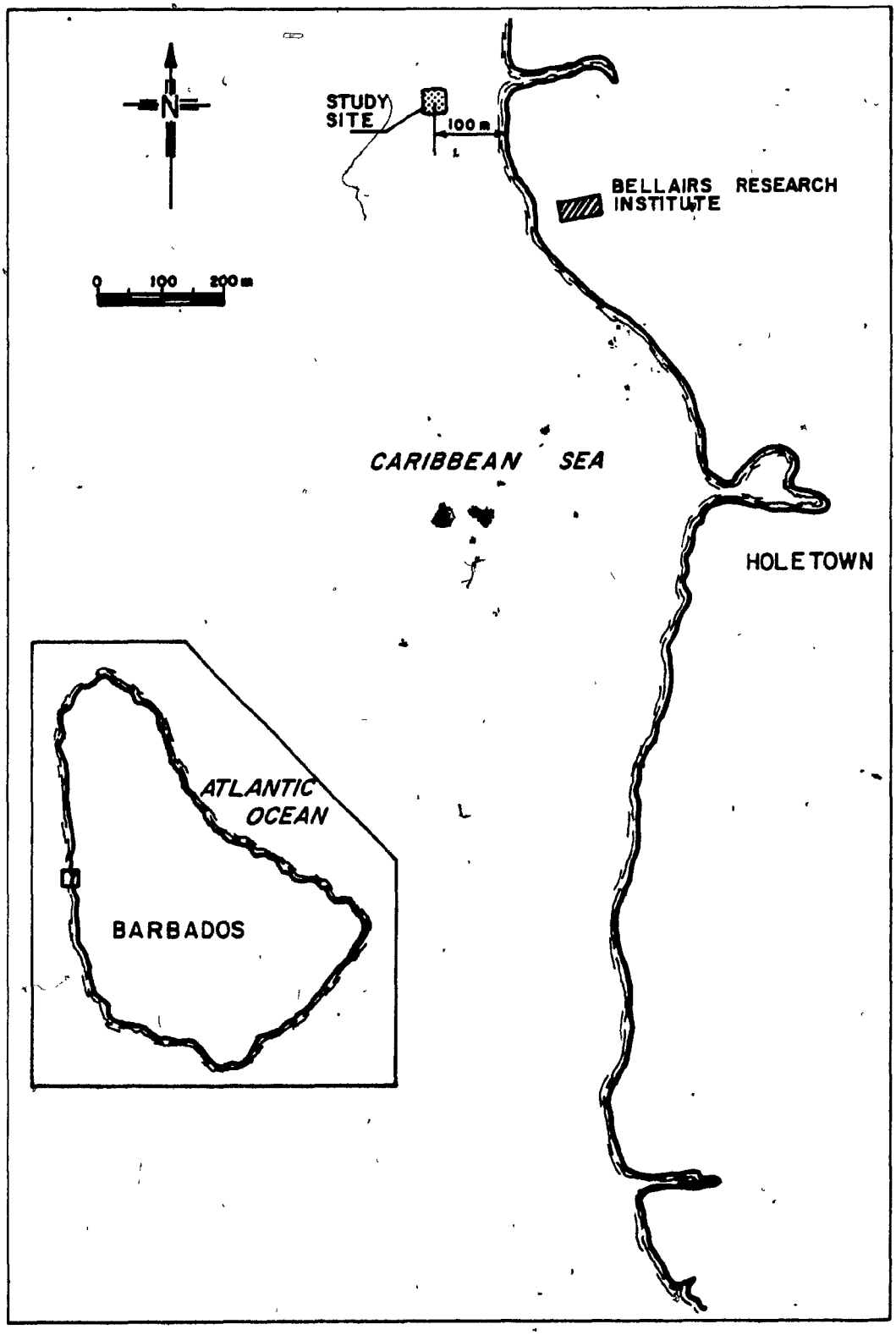
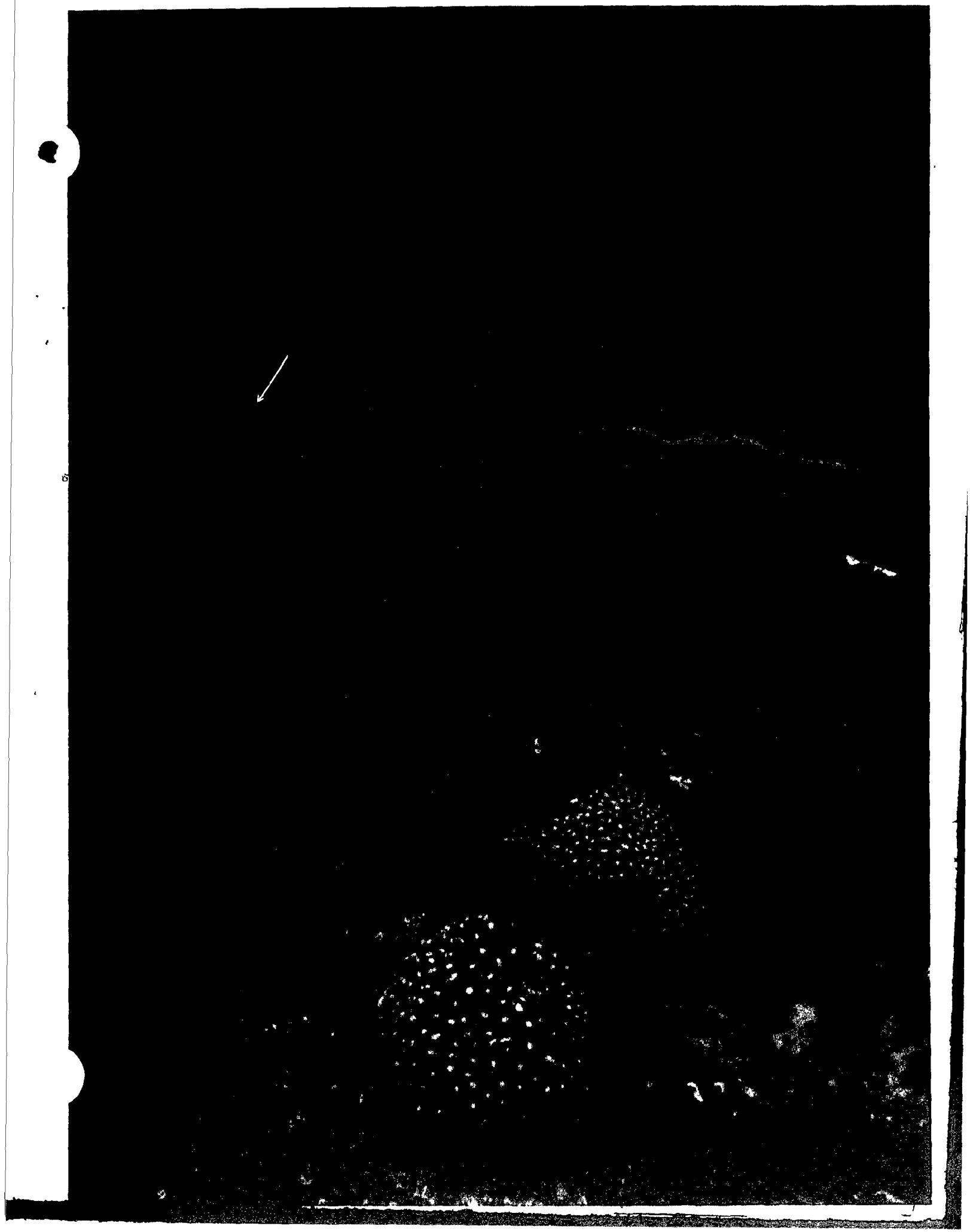


FIG. 1 : LOCATION OF STUDY AREA ON WEST COAST OF BARBADOS

PLATE I

Verongia longissima. *In situ* photograph of this typically irridescent blue-violet ramose sponge growing attached to a piece of dead coral. The branches are about 1 cm in diameter. It is a common marine sponge widely distributed on the west coast of Barbados at depths ranging from 5 to 25 metres. Note the natural grafting occurring (indicated by the arrow) when branches of the individual come into contact, and join together.

Photograph by H. M. Reiswig.



GRAFTING EXPERIMENTS:

The grafting was carried out *in situ* using SCUBA equipment. Two sites were selected at a depth of 13 metres off the mid-west coast of the island in a coral rubble area of the "seaward slope of the fringing reef" (Lewis, 1960; Figure 2). Each site covered an area of approximately 9 square metres and were one metre apart. Nylon rope was used to mark the boundaries of each site and a moored buoy to identify their position. The two sites were selected because they each had a high concentration of *Verongia longissima* individuals (Plate II-A). A total of 91 specimens were numbered and tagged according to site, for later identification.

Grafting experiments involved removing small pieces from donors, attaching them in close contact to hosts, and scoring results of the ensuing interaction. Grafts were removed from donors by cutting off a small piece of a branch tip, approximately 1 cm in length, with a diving knife. The grafts were immediately tied to a host individual by a tagged piece of nylon thread used to identify the donor (Plate II-B, and II-C). All grafts were examined *in situ* after three days, and after one month; a portion of the early grafts were re-examined after six months. Grafts were scored as an acceptance if:

1) it could not be separated from the host by gentle pulling.

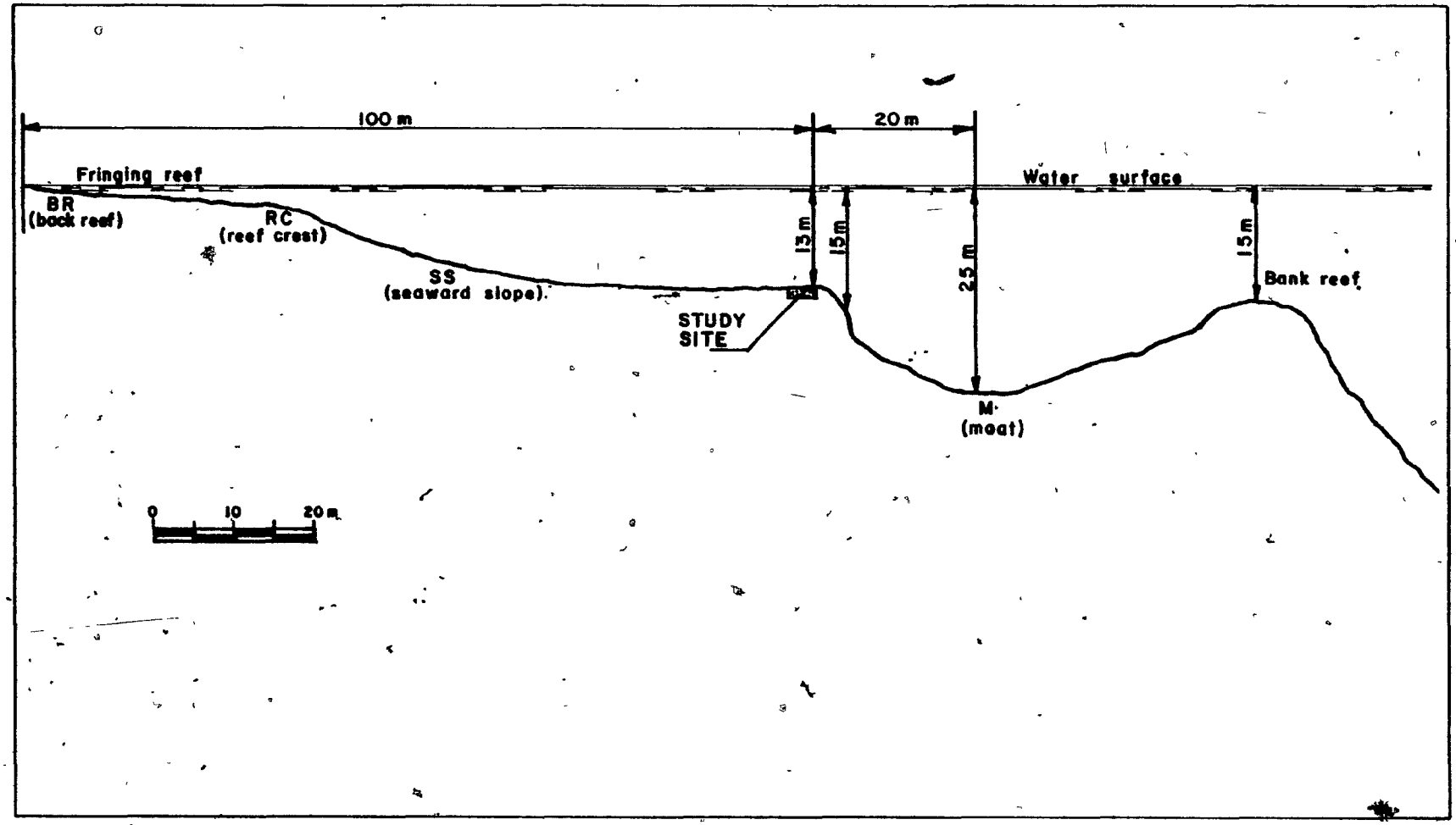


FIG. 2 REPRESENTATIVE BOTTOM PROFILE THROUGH STUDY SITE LOCATED ON WEST COAST OF BARBADOS.

PLATE II

A. In situ photograph of a corner of one of the study sites. The sites were 9 square metres in area and the boundaries were marked by nylon rope.

B. In situ photograph of the grafting procedure. A small piece of a branch was cut off from a donor sponge and tied to a host sponge by a tagged piece of nylon thread.

C. Close-up photograph of the tieing procedure. A tagged piece of nylon thread was used to tie a small piece (approximately 1 cm in length) of a donor sponge (g) to a host sponge (h).

A

B

C

2) it was joined to the host by a continuous superficial epithelium - the pinacoderm.

and 3) it was in good health (i.e. colour, presence of a pinacoderm).

Grafts were scored as a rejection if:

1) it could be easily separated from the host by touch.

2) there was a clear, evident gap between the host and graft.

and 3) a ridge had formed on each edge of the sponges in contact (noted after 1 month).

Plate III shows typical examples of acceptances and rejections. Control autografts (self grafts) were made for all tagged individuals in both sites.

IMMUNOLOGICAL STUDIES:

Eighteen tissue samples were collected, from tagged individuals within the two sites, for immunological studies. Branches of the test specimens were cut from the donor and transferred to plastic bags underwater. The bags were filled with seawater, tied, and immediately returned to the laboratory. Here the tag numbers of the specimens were recorded and the specimens were then used in the preparation of antigens (sponge cell suspensions) for immunization, agglutination tests, and cross-absorption tests.

PLATE III

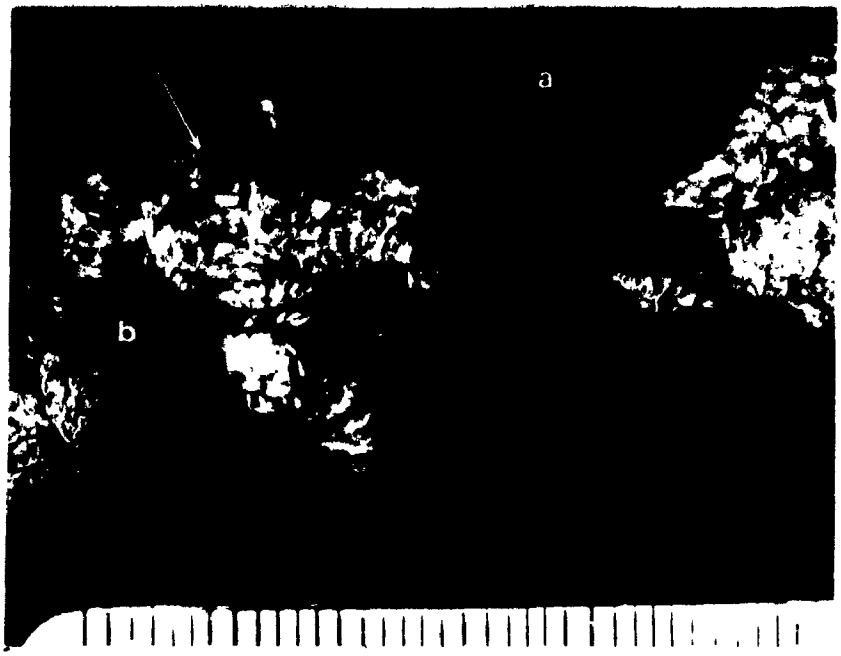
A. In situ photograph of a graft from Verongia cauliformis that has been accepted (a), and a graft from Verongia longissima that has been rejected (r). The host (indicated by the arrow) is Verongia cauliformis.
(a) represents an allograft,
(b) represents a xenograft.

B. Close-up photograph of a control autograft acceptance of Verongia longissima. The graft was cut from the branch of the same host individual. Tagged nylon thread identifies the graft. Scale is in millimetres.

C. Close-up photograph of two rejected grafts. The upper graft (a) was taken from a Verongia cauliformis individual, and the lower graft (b) was taken from a Verongia longissima individual. The host (indicated by the arrow) is Verongia longissima. (a) represents a xenograft, (b) represents an allograft. Scale is in millimetres.

A

B



C

a) Preparation of antigens:

The specimens were dissociated according to the procedure of Humphreys, Humphreys, and Moscona (1960) with slight modifications. Approximately 10 g of the specimens were cut into small pieces (1 cm^3) and placed in 100 ml of calcium-magnesium-free seawater (CMF-SW) containing 20 mM ethylenediamine tetra-acetate (EDTA) for 1 hour. These preparations were then mechanically dissociated using a Waring blender. The resulting suspensions were pressed through cheese cloth and the dissociated cells were collected in 30 ml of CMF-SW. Cell counts were taken for all the suspensions using a Neubauer haemocytometer. Merthiolate (0.3 ml of a 5% solution) was added to each of the suspensions to retard bacterial growth. Six of the suspensions were then divided into 0.1 ml aliquots to be used for immunization, and 10 ml aliquots to be used in the agglutination and cross-absorption tests. The other 12 suspensions were divided into 10 ml aliquots to be used only in the agglutination and cross-absorption tests.

b) Immunization:

Six young adult rabbits weighing 4 to 6 kg were used in the production of antisera. The rabbits were injected subcutaneously in the hind region with 0.1 ml of the sponge antigen emulsified with 0.2 ml of Freund's complete adjuvant. The injection was administered using a 26 guage, 2.5 cm long hypodermic needle (Carpenter, 1975). The

following immunization schedule was used for the production of sponge antisera in the rabbits:

1st day: Injection 1
3rd day: Injection 2
5th day: Injection 3
7th day: Injection 4
9th day: Injection 5
17th day: Injection 6
19th day: Injection 7
21st day: Injection 8
23rd day: Injection 9
31st day: Bleed from the heart

Blood (100 cm^3) was collected via cardiac puncture. The blood was allowed to clot for 30 min and then centrifuged at 3,000 rpm for 5 minutes. Serum (50 cm^3) was removed, heated at 55 $^{\circ}C$ for 30 min to inactivate complement components present in the sera, and then frozen in 5 ml aliquots for later use in the agglutination and cross-absorption tests. These tests were performed 2 to 6 months after the collection and frozen storage of the antisera and antigens (sponge cell suspensions).

c) Agglutination tests:

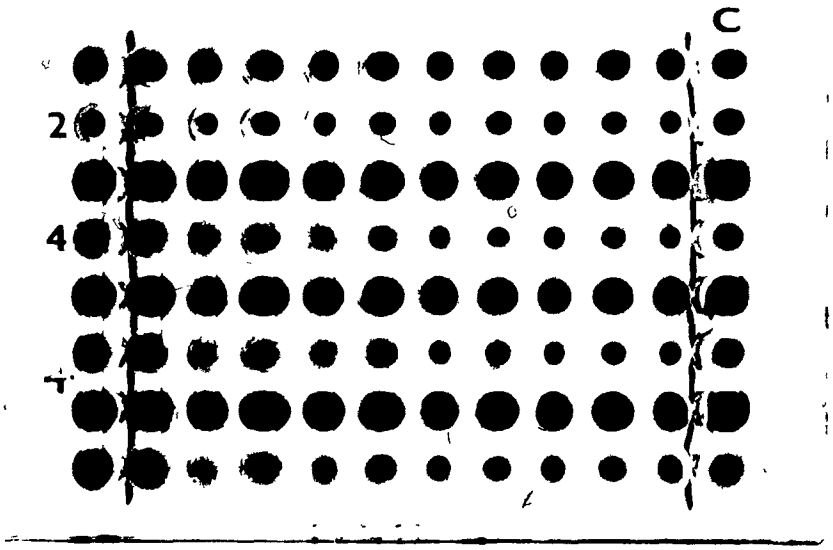
Microtitre plates (Plate IV-A) containing U-wells were used for the agglutination reactions. Each reaction well contained 25 λ of diluted antisera and 25 λ of diluted antigen (sponge cell suspension). The antisera in the wells were serially diluted by doubling dilutions, of CMF-SW and antiserum, ranging from 1:1 to 1:256. The antigens were diluted 1:1 with CMF-SW. Control wells contained only 25 λ of the diluted antigen and 25 λ of CMF-SW. The plates were incubated at room temperature for 8 hours and then

PLATE IV

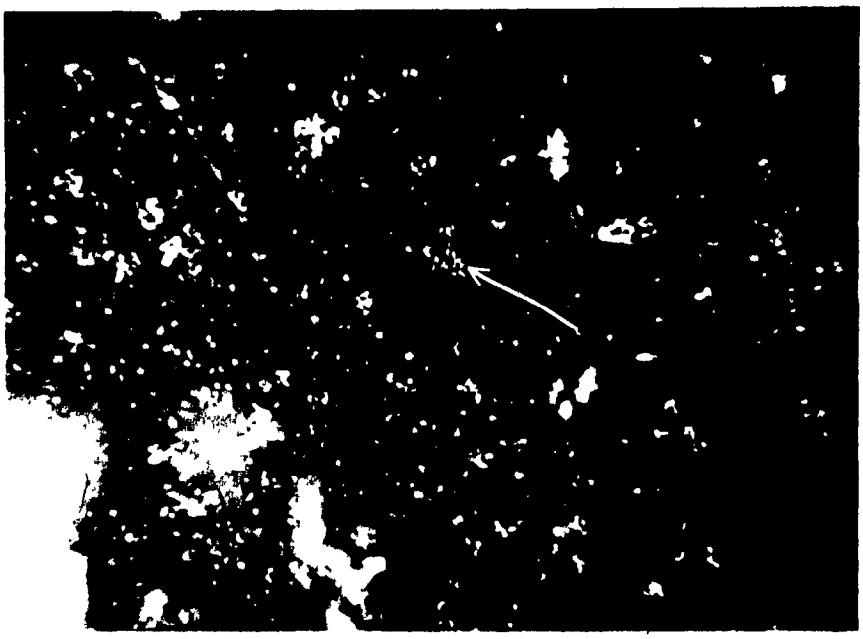
A. Photograph of a microtitre plate showing macroscopic results of the agglutination reactions. The far right hand column of wells (c) contain antigen only and demonstrate the pellet formation of these controls. The second row from the top (2) shows a definite pellet (like that in the control) in all 11 wells. This was scored as a negative reaction (no agglutination). The first 6 wells (from left to right) of the fourth row from the top (4) show a cloudy mixture. This was scored as a positive reaction (agglutination). The remaining 5 wells in this row show a pellet (like that of the control for this row). This was scored as a negative reaction (no agglutination).

B. Photomicrograph of a positive reaction in the agglutination test. The sponge cells aggregate together in small clumps (indicated by arrow). 210X

C. Photomicrograph of a negative reaction in the agglutination test. The sponge cells remain evenly distributed (similar to the control wells) and no clumping of cells occurs. 210X



A



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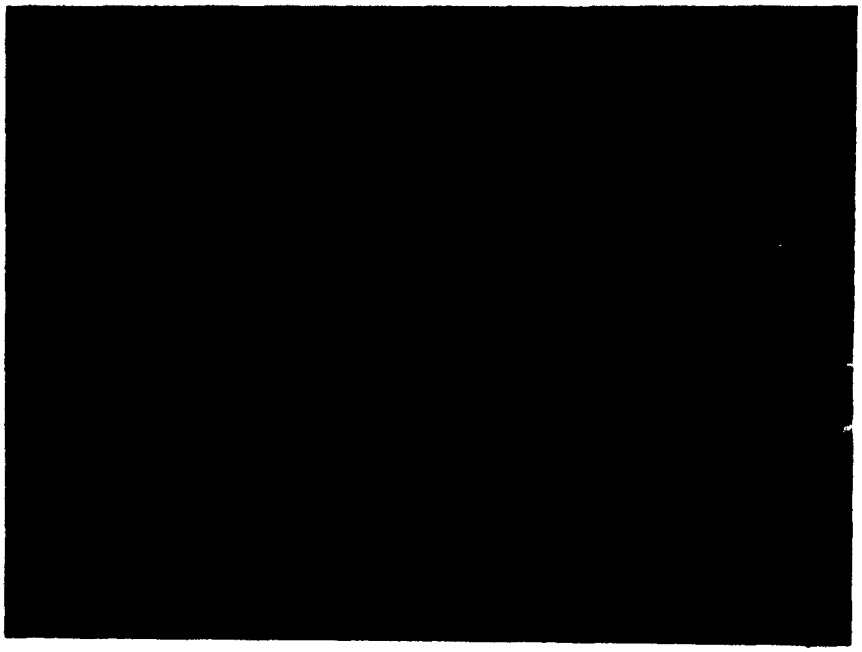


C

macroscopic and microscopic observations of the contents of the wells were recorded.

For macroscopic analyses a positive reaction (agglutination) was scored if a well containing the mixture of antiserum and antigen was cloudy and showed no signs of definite pellet formation such as occurred in the control well containing the antigen only. A negative reaction (no agglutination) was scored if a well containing the mixture of antiserum and antigen showed a definite pellet like that of the control well (Plate IV-A).

For microscopic analyses the contents of each well were gently mixed and placed on a slide for observation under the compound microscope at 200X magnification. A positive reaction (agglutination) was scored if the sponge cells had formed clumps and aggregates which had not been disturbed during the gentle mixing, and were not observed in the control wells (Plate IV-B). A negative reaction (no agglutination) was scored if the sponge cells had not clumped or aggregated together, and the mixture of antiserum and antigen appeared similar to that in the control (Plate IV-C).

It should be noted here that macroscopic and microscopic observations agreed 90% of the time, and when there was a disagreement the microscopic observations were always recorded. The reciprocals of the last dilutions of antisera that showed agglutination were recorded.

This procedure was carried out for each of the 6 different antisera against each of the 18 antigens (sponge cell suspensions) prepared earlier.

d) Cross-absorption tests:

Cross-absorption tests were performed on two of the antisera to confirm the agglutination tests. Varying cell numbers of each of several antigens (sponge cell suspensions) were spun down at 7,000 rpm for 20 min in an RC-5 Sorvall centrifuge. The supernatant was discarded and the cells in the pellet were washed and resuspended once in CMF-SW and again spun down at 7,000 rpm for 10 minutes. The pellet was then mixed with 0.3 ml of the antiserum and incubated at room temperature for 1 hour. Each mixture was spun down at 7,000 rpm for 10 min and the supernatant was collected and used in the agglutination reaction to test for the amount of antibody absorbed (removed). Agglutination was performed as before (see IMMUNOLOGICAL STUDIES: c) Agglutination tests) testing the absorbed antiserum (supernatant) against its homologous antigen.

The reciprocals of the highest dilutions of antisera that showed agglutination were plotted against the various cell numbers of each of the tested antigens.

e) Immunofluorescence experiment:

In this experiment, the rabbit antisera prepared earlier were utilized in conjunction with goat anti-rabbit

immunoglobulin fluorescein (FITC) conjugated antiserum which was to act as a specific label. The antibodies in the antisera directed against the sponge cells bind to their homologous antigens and then the goat anti-rabbit immunoglobulin fluorescein (FITC) binds to the antibodies. When observed under ultraviolet light the bound label will fluoresce.

A mixture of 0.1 ml of antiserum (diluted 1:10 with CMF-SW) and 0.9 ml of antigen (diluted 1:10 with CMF-SW) was incubated for 30 min at 5°C, and spun down at 3,000 rpm. The pellet was washed 3 times by resuspending in CMF-SW and spinning at 3,000 rpm for removal of excess antiserum. The pellet was then incubated for 30 min at 5°C with 0.1 ml of the goat anti-rabbit immunoglobulin fluorescein (FITC) conjugated antiserum diluted 1:4 with CMF-SW. After incubation the mixture was spun down at 3,000 rpm and washed 3 times to remove excess label. The cells were examined microscopically at low and high power using phase and fluorescence optics.

HISTOLOGICAL STUDY:

Sample specimens of graft acceptances and rejections were collected and returned to the laboratory where they were preserved in small vials of Bouin's fixative until they were processed for histological examination two months later. The samples were dehydrated, embedded in paraffin and sectioned. The specimens were sectioned through the

zone of contact at a thickness of 8 μm . The sections were mounted on albumin-coated slides, deparaffinized, and stained with Masson trichrome stain for differentiation (Humason, 1962). The sections were examined under the compound microscope at low (100X) and high (400X) power.

RADIOAUTOGRAPHIC STUDIES:

The radioautographic studies were performed on a host and donor, from within one of the study sites, that had been grafted earlier and scored as a rejection.

a) Incorporation of labelled proline:

Two branches, 30 cm long, were cut from one specimen and each branch was tied by nylon thread at 2.5 cm intervals to a branch of another specimen. This procedure was carried out in situ. One graft branch was left in contact in the field for six hours and the other for nine hours. After field incubation, the grafts were cut into 12 pieces (2.5 cm) marking the donor individual with a notch; six of these grafts were placed in Millipore filtered seawater plus ^{3}H-proline at a final concentration of 0.125 mg/l for four hours, and the remaining six pieces were placed in a control fluid containing Millipore filtered seawater plus unlabelled proline at a final concentration of 0.125 mg/l for four hours. After this four hour period all the graft pieces were placed in individual, circulating chase solutions of Millipore filtered seawater plus unlabelled

proline at a final concentration of 1.25 mg/l. Control graft pieces and labelled graft pieces for radioautography were removed from the chase solution after 6, 12, 24, 36, 48, and 62 hours and fixed in Bouin's fixative.

b) Histological processing:

After being fixed in Bouin's for 24 hours, the graft pieces were dehydrated, embedded in paraffin, and stored until sectioned. The grafts were sectioned 3 months after embedding. The sections were cut through the zone of contact at 8 μm, mounted on albumin-coated slides, dried and processed for radioautography. The remaining paraffin block of each graft piece was trimmed, deparaffinized, and embedded in Spurr epoxy. The grafts were again sectioned through the zone of contact, at 0.5 μm. These sections were mounted in a drop of distilled water on a slide, dried, and processed for radioautography.

c) Radioautographic procedure:

The procedures of Kopriwa and Leblond (1962) were followed and were carried out in total darkness. The slides were dipped into Kodak NTB-2 photographic emulsion for 3 seconds, drained and dried for 2 hours. Coated slides were stored in dry, light-tight containers at 2°C and were exposed for 2 to 4 weeks. The emulsion was developed in Kodak D-19, and fixed. Slides with paraffin sections were stained with haemotoxylin-eosin (Humason, 1962), and slides

with epoxy sections were stained with 1% toluidine blue in borax (Humason, 1962). Silver grains were localized and quantified under oil immersion. Features of contact surfaces were examined for deposition of cuticle material (Vacelet, 1971).

RESULTS AND OBSERVATIONS

GRAFTING EXPERIMENTS:

Intraspecific acceptance and rejection of individual Verongia longissima specimens were observed to occur commonly in nature. A natural acceptance was noted when two branches had joined and totally fused at the point of contact, and a natural rejection was noted when each branch retained its separate integrity and, after extended contact, had formed a ridge on each of the surfaces in the zone of contact. Acceptance was also observed when branches of one individual came into contact with themselves (Plate I). The branches had united and become one at the point of contact. Natural auto-rejection was never observed to occur in these circumstances.

A total of 91 individuals consisting of 29 specimens of Verongia longissima, and 13 specimens of Verongia cauliformis found in site A, and 49 specimens of Verongia longissima found in site B were used for in situ grafting experiments. In order to test all combinations of grafts between individuals and self/self controls 8,372 (91 x 92) operations would be required. A random grafting protocol was initiated to allow provisional strain identification of some individuals. These were then grafted to single members of other strains to insure strain identification of all individuals with a minimum number of required operations. The results of the grafting within each site are presented

in Figures 3a and 3b. The results of all the 758 grafts that were performed within each site and between sites are presented in Figure 3c.

The results clearly show that all autografts (self grafts) of both species accepted, all xenografts (different species grafts) rejected, and there was both acceptance and rejection observed in allografts (same species grafts) of Verongia longissima (Plate III-A, and III-C). Among the allografts groups of individuals could easily be identified which always accepted grafts from individuals within their group and always rejected all other individuals. These groups were designated as strains (Figure 3c).

A preliminary investigative xenograft, not included in the grafting results, was performed between Verongia longissima and Verongia fistularis which was scored as a rejection.

The consistent specificity of within and between strain grafts observed in the early stages of the study allowed development of assumptions concerning the acceptance or rejection of one individual by another individual. For example, if individual A rejected individual B and accepted individual C, and individual C accepted individual D and rejected individual B, then it was assumed that individual A would accept individual D and individual D would reject individual B. Individuals A, C, and D were assumed to be of the same strain, and of a different strain than individual B.

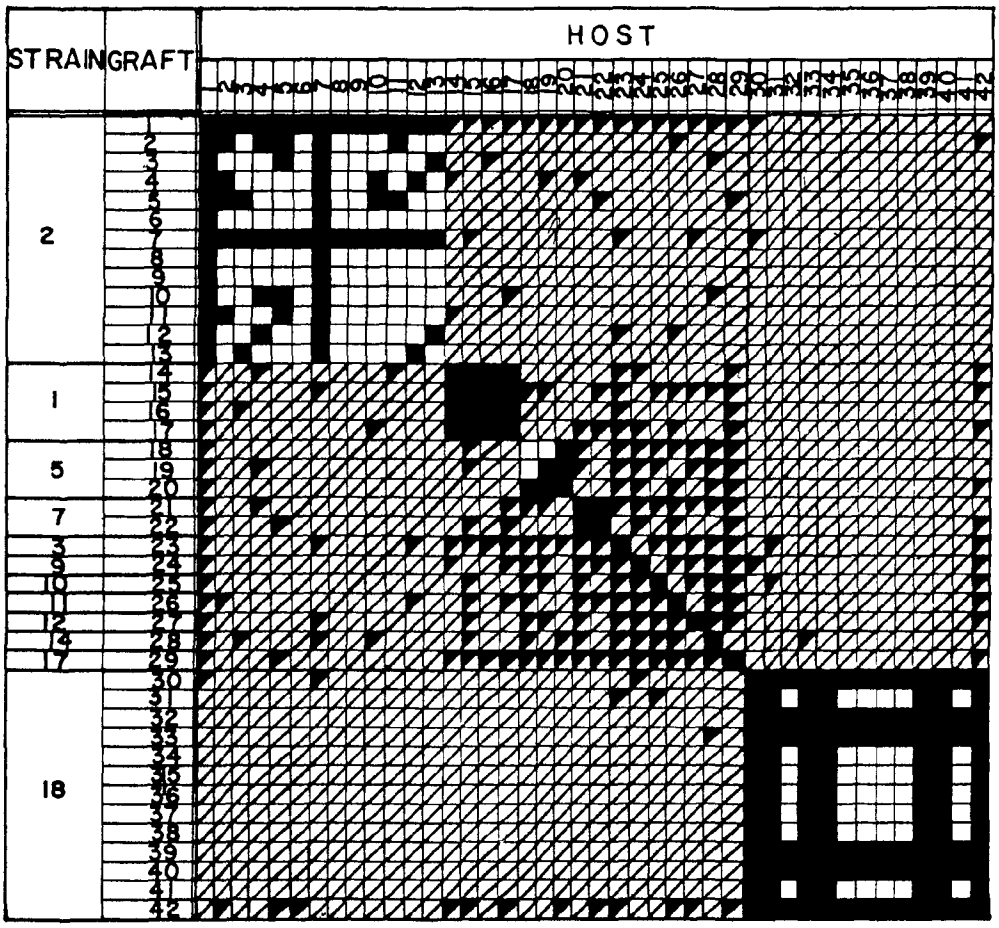


FIG. 3a: GRAFTING SITE A

Legend:

- Acceptance
- Assumed acceptance on basis of actual acceptance of members of same strain
- Rejection
- Assumed rejection on basis of actual rejection of members of same strain

Note: Results of *in situ* grafting experiments in site A. Dates of testing, May – Sept. 1978, and Dec. 1978. Numbers 1–29, *Verongia longissima*. Numbers 30–42, *Verongia cauliformis*.

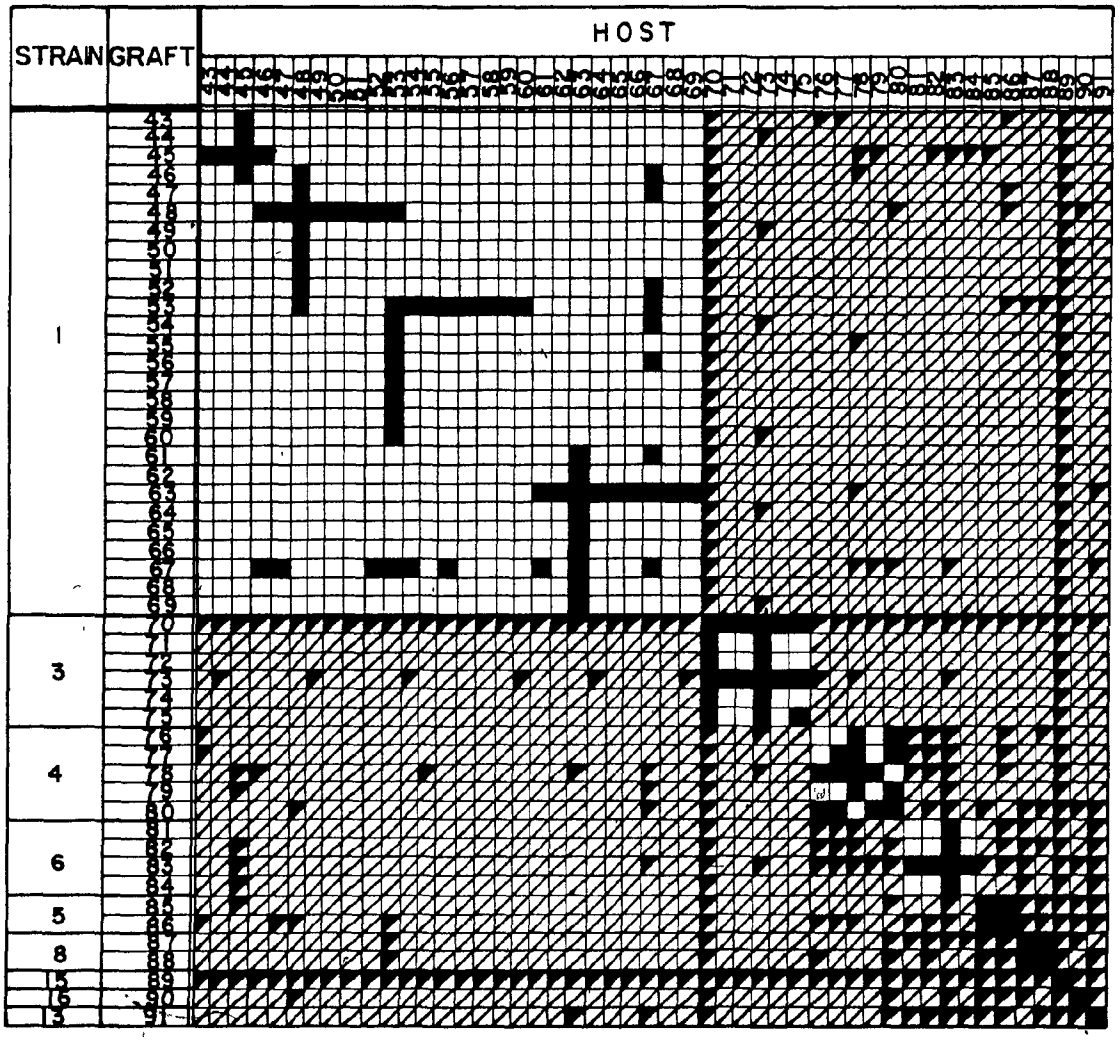


FIG. 3b: GRAFTING SITE B

Legend:

- Acceptance
- Assumed acceptance on basis of actual acceptance of members of same strain
- Rejection
- Assumed rejection on basis of actual rejection of members of same strain

Note: Results of *in situ* grafting experiments in site B. Dates of testing, May —Sept. 1978, and Dec. 1978. All individuals are *Verongia longissima*.

FIG. 3c: SUMMARY OF GRAFTING BOTH SITES

LEGEND

Acceptance. ----------

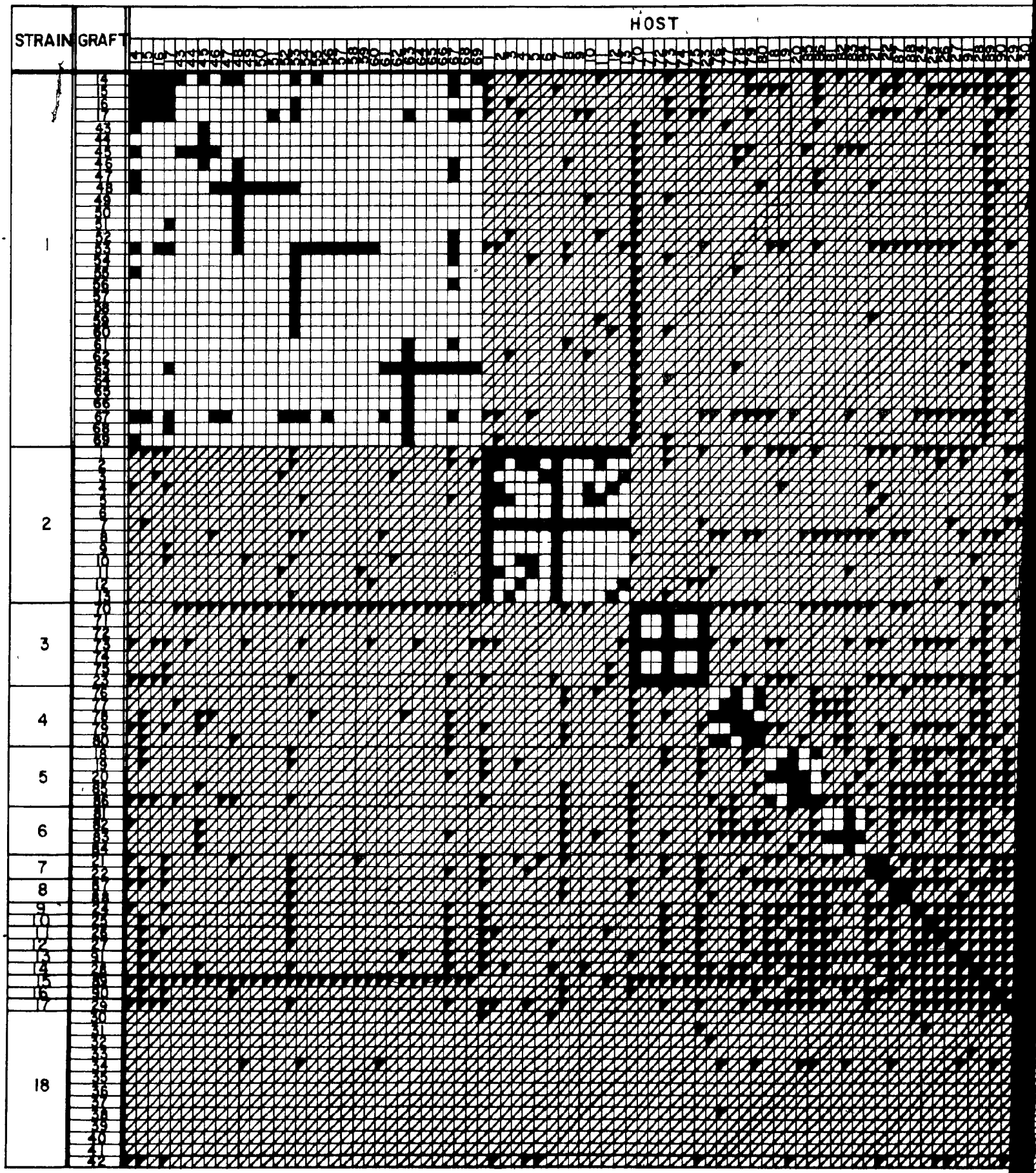
Assumed acceptance on basis of actual acceptance of members of same strain. ----------

Rejection. ----------

Assumed rejection on basis of actual rejection of members of same strain ----------

NOTE

Results of all in situ grafting experiments in both site A and site B.
Dates of testing, May - Sept. 1978, and Dec. 1978.
Strains 1 - 17, Verongia longissima
Strain 18 Verongia cauliformis


HOST
STRAIN
GRAFT
1
2
3
4
5
6
7
8
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15
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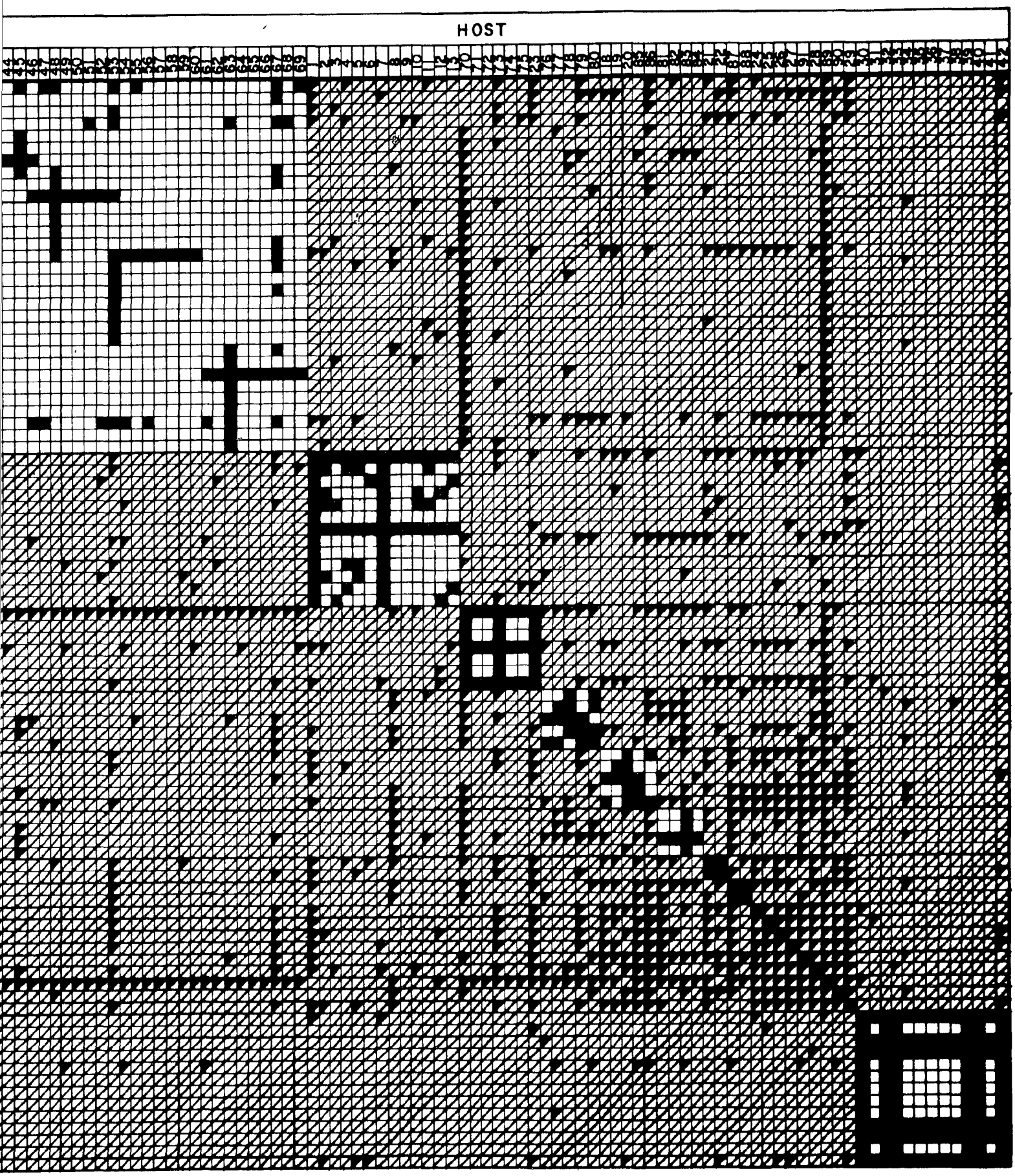
17
18



2 of 2

All assumed strain identifications which were randomly chosen to be tested were proven to be correct when the actual grafts were performed. It is concluded then that because of absolute specificity of strain identities, the individual graft reactions assumed in Figure 3c would prove correct should the indicated grafts be carried out.

The grafting experiments showed 11 strains among 29 individuals of Verongia longissima, and 1 strain among 13 individuals of Verongia cauliformis in site A and 9 strains among 49 individuals of Verongia longissima in site B. The strains are observed as the blocks of acceptances and assumed acceptances recorded in Figures 3a and 3b. Percent abundance of strains in each site is presented in Figures 4a and 4b.

The results of all the grafting experiments (Figure 3c) demonstrate the existence of 17 strains among 78 individuals of Verongia longissima, and one single strain among all 13 individuals of Verongia cauliformis. Total percent abundance of strains from both sites is presented in Figure 4c. The incidence of strain occurrence, in the species Verongia longissima investigated in this study may be used to calculate the probable number of strains that one would expect to find in other similar sites sampled. The following statistical formulae have been applied (Bailey, 1959):

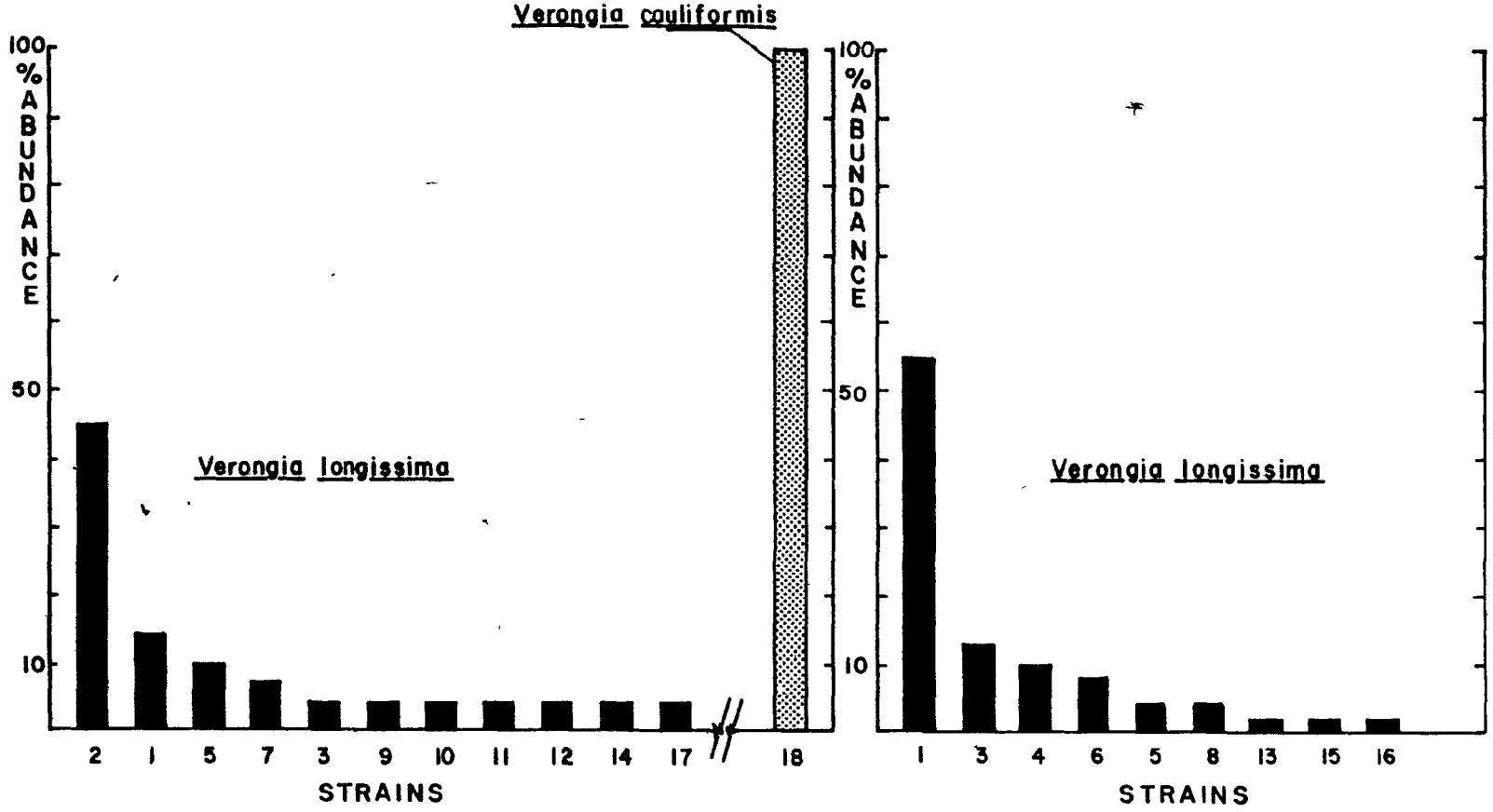


FIG. 4a: SITE A

FIG. 4b: SITE B

% ABUNDANCE OF STRAINS IN EACH SITE

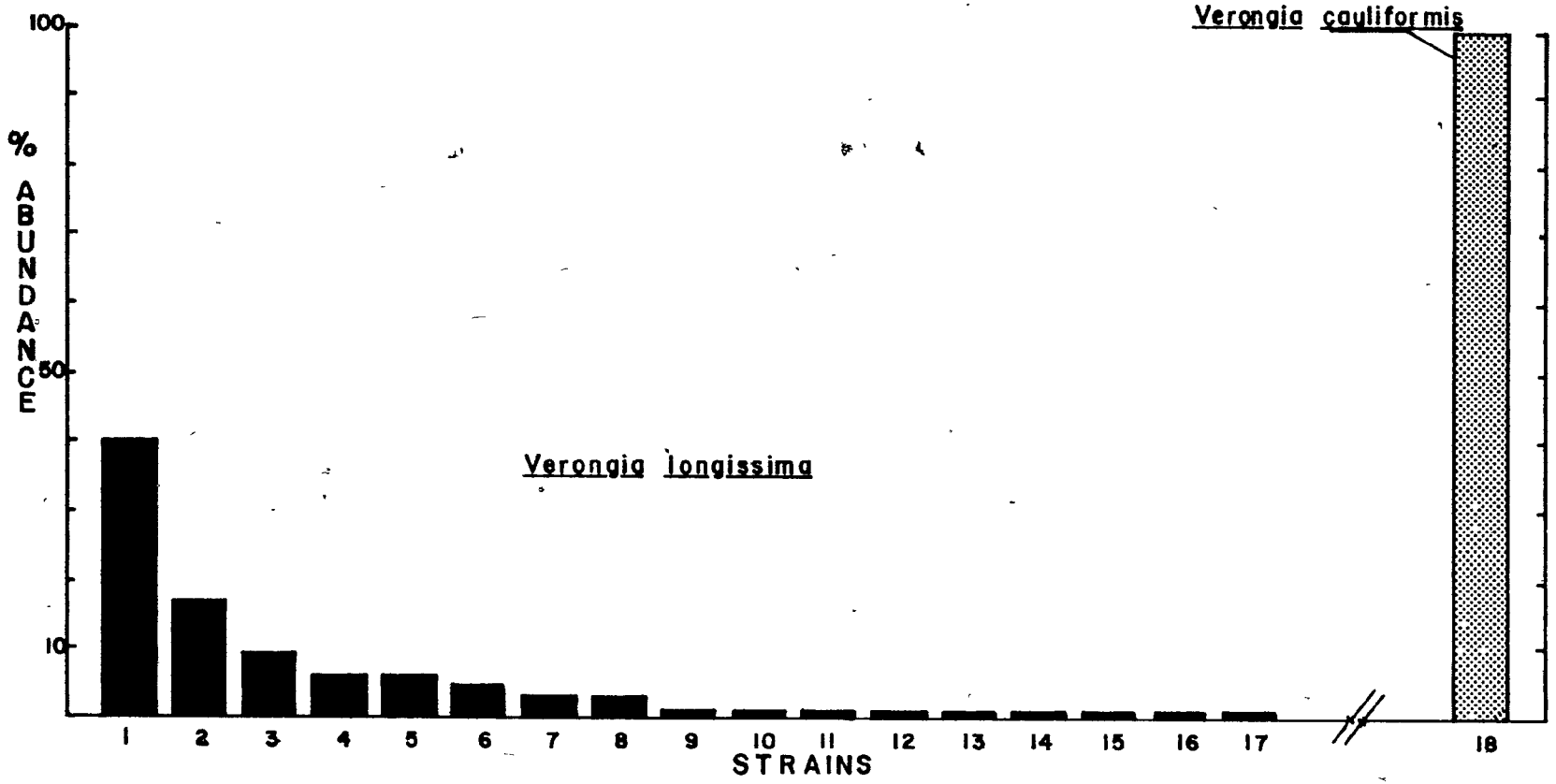


FIG. 4c: TOTAL % ABUNDANCE OF STRAINS FROM BOTH SITES

1. MEAN = $\frac{x_1 + x_2}{n}$

x_1 = number of strains in site A

x_2 = number of strains in site B

n = number of sites

Mean in this study = $\frac{11 + 9}{2}$

Mean = 10

2. VARIANCE = $\frac{(x_1^2 + x_2^2) - \frac{(x_1 + x_2)^2}{n}}{n - 1}$

Variance in this study = $\frac{11^2 + 9^2 - \frac{20^2}{2}}{2 - 1}$

Variance = 2

3. VARIANCE OF MEAN = $\frac{\text{variance}}{n}$

Variance of mean in this study = $\frac{2}{2}$

Variance of mean = 1

4. STATISTICAL ERROR = $\sqrt{\text{variance of the mean}}$

Statistical error in this study = $\sqrt{1}$

Statistical error = 1

In other sites we would expect to find 10 $\pm$ 1 strains, or in 68% of future sites sampled, one would expect to find between 9 and 11 strains.

It is understood that the statistical analyses presented in this study have used a minimum number of sample sites, and for more accurate statements of probability many more sample sites should be employed. Nevertheless, it is interesting to note the significance of strain occurrence in Verongia longissima, and the probability of finding a large number of strains within a single species of a marine sponge.

It was noted earlier (MATERIALS AND METHODS - GRAFTING EXPERIMENTS) that the results of all grafts were recorded 3 days after grafting, and again a month later. Grafts checked 6 months after the initial grafting were, in all cases, scored the same as they had been 3 days after grafting. During the initial observations of the grafts and subsequent observations it was noted that all grafts, whether accepted or rejected, were alive and in good health showing no signs of necrosis. This same observation was noted for all the host individuals concerned.

No distinctive features or characteristics readily discernable in nature could be used to distinguish between the strains in Verongia longissima. This species although typically an irridescent blue-violet colour, does occur in other forms. Its colours range from a deep blue-violet, to

a "tannish" blue-violet. These differences in colour could occur within one individual or between individuals of the same strain. Another common feature of this species that varied from individual to individual was the external surface relief. Some have a smooth, even surface whereas others are rough with many spinous protuberances but no consistent pattern related this feature to strain designation.

It should be noted that in the grafts that have been accepted by a host, the external and internal features of both donor and host eventually become continuous and matrices and skeletons are shared (Plate V-A, and V-B). In grafts that have been rejected by a host, the external and internal features of the original specimens remain separate and no intermingling or sharing occurs; after a time, a border or ridge forms on the two surfaces in contact (Plate V-C, and V-D). This same border forms on an individual in the zone of contact with the string used to tie the graft to the host (Plate V-A) and to other foreign (non-self) objects.

PLATE V

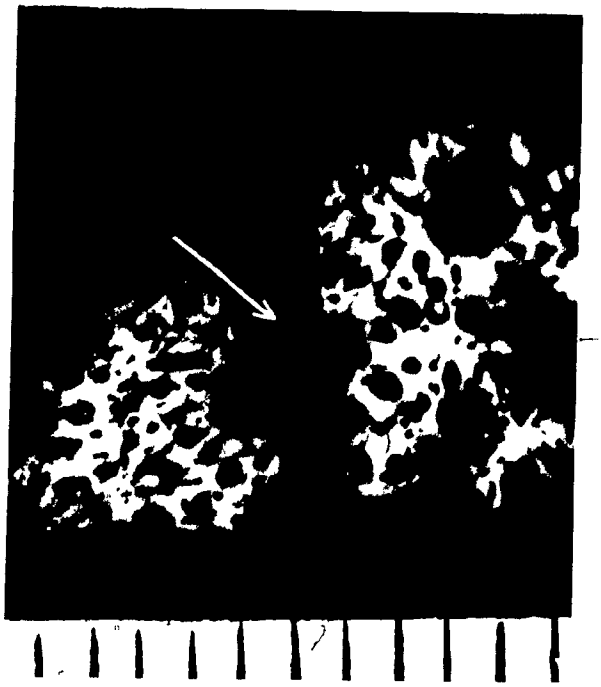
A. Close-up photograph of the external surface features of a typical acceptance. Note the zone of contact (indicated by the arrow) between host (h) and graft (g). Pinacoderms are joined and continuous. Pores (p) are open, and the two individuals are functioning as a unit. Notice in the area of the string (s) a border or ridge (r) is forming along the section of sponge in contact with the spring. 7.3X

B. Close-up photograph of the internal features (cross-section) of the zone of contact (indicated by arrow) of an acceptance. Note how the skeletons and matrices have become joined and continuous in the zone of contact. Scale is in millimetres.

C. Close-up photograph of the external surface features of a typical rejection. Note the zone of contact between the host (h) and graft (g). A border or ridge (r) has formed on the two surfaces in contact. The pinacoderms along the zone of contact remain distinct, and the pores in this region are closed. Each individual is functioning as a distinct unit. 7.3X

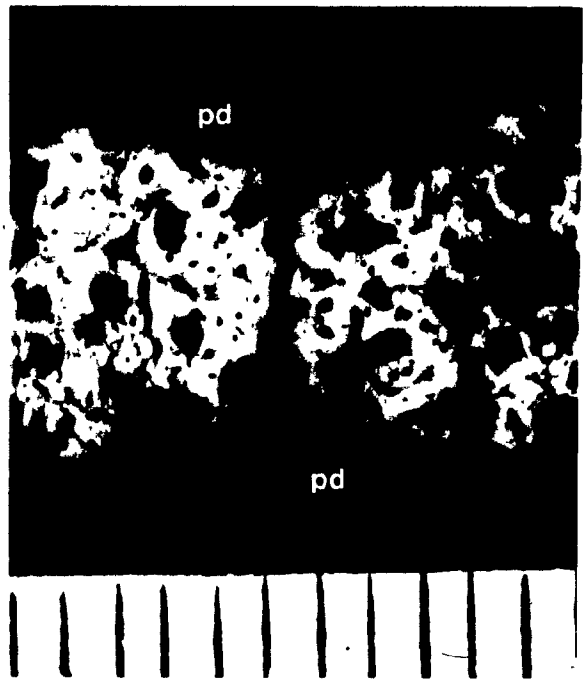
D. Close-up photograph of the internal features (cross-section) of the zone of contact of a rejection. The sponges remain separate and the pinacoderms (pd) along the border of each sponge remain distinct. Scale is in millimetres.

A



B

D



C

IMMUNOLOGICAL STUDIES:

In the grafting experiments the sponges showed recognition (fusion or rejection) at the levels of self (specimen), strains within a species, and species. This section of the study was performed to determine if these recognition differences could be demonstrated using immunological techniques. Rabbits were immunized against 6 different sponge individuals, and the antibodies produced were then tested for their ability to react with various other sponge individuals.

Antibodies specific for an antigen are hypothesized to possess a receptor site with a structural configuration complementary to that of the antigen, and are capable of reacting with the homologous (corresponding) antigen in a lock and key type complex. This antigen-antibody reaction will only occur when the antibody receptor site conforms closely enough to the determinant site of the antigen so that a union can occur (Carpenter, 1975). There are a number of techniques that can be employed to demonstrate this specificity of an antibody for its homologous antigen. In this study agglutination, cross-absorption, and immunofluorescence were the methods employed to exhibit this specific recognition system, and to substantiate further the occurrence of strain specificity in the marine sponge Verongia longissima.

When a small amount of antiserum was mixed with an

homologous antigen (sponge cell suspension) in the wells of a microtitre plate agglutination was observed. The cells clumped and gradually settled to the bottom of the fluid. Macroscopic examination revealed a cloudy liquid in the wells of the plate, compared to a definite pellet that formed in the control wells (Plate IV-A). High power microscopic examination revealed that the particles of antigen behaved as though they were sticky, and clung together in small clumps or aggregates compared to the generally even distribution of cells in the controls (Plate IV-B).

Quantitative analyses to determine the agglutinating antibody content of the antisera were made by serially diluting the antisera. The reciprocal of the last dilution of antiserum that showed agglutination is called the antiserum titre. The titres for the antisera tested in this study are presented in Table 1. As an example, antiserum 89 has a titre of 64 for antigen 89 and titres of 2 or less for the remaining antigens. In the case of antigen TA there is no titre recorded for antiserum 89 because agglutination was never observed when antiserum 89 was mixed with this antigen. It is assumed here that a high titre indicates a process of recognition while a low titre indicates that little recognition has occurred (for further discussion see page 40).

The results indicate that 5 of the 6 antisera (31, 2,

TABLE 1: SUMMARY OF AGGLUTINATION RESULTS

ANTIGEN	CELL COUNT ($\times 10^6$/ml)	ANTISERA					
		31	2	12	73	71	89
31	194.4	32	1	1	0	0	0
2	259.6	1	32	32	1	1	1
73	177.8	1	2	2	32	32	1
89	210.6	0	1	1	0	0	64
33	177.0	32	1	1	0	0	0
11	253.4	1	32	32	1	1	2
71	160.0	1	2	2	32	32	1
75	162.4	1	2	2	32	32	2
35	190.8	32	2	1	1	1	2
12	203.2	1	32	32	1	1	2
TA	281.8	1	—	—	—	—	—
20	168.2	2	2	1	0	0	0
23	147.4	1	2	2	32	32	1
19	164.4	1	2	1	1	1	0
67	149.8	0	1	1	0	0	0
77	213.2	0	0	0	0	0	0
44	161.2	1	1	1	1	1	1
87	215.4	2	2	2	2	2	2

Note: Numbers in antisera columns represent the highest dilution of that antiserum that would still cause an agglutination reaction to occur.
Antigens 31, 33 and 35 are from Verongia cauliformis.
Antigen TA is from Verongia fistularis.
All other antigens are from Verongia longissima.
Antiserum 31 is from Verongia cauliformis.
All other antisera are from Verongia longissima.

12, 73, and 71) appear to recognize more than one antigen, noted by the high titre values appearing in the antisera columns. Antiserum 31 recognizes antigens 31, 33, and 35. In the grafting experiments, it was noted that sponges 31, 33, and 35 were all of the same single strain of Verongia cauliformis. Antiserum 2 recognizes antigens 2, 11, and 12, and interestingly enough antiserum 12 also recognizes these same 3 antigens. Thus from these results antisera 2 and 12 recognize common antigens, and are therefore likely to have antibodies of similar specificity. Recalling the results of the grafting experiments again, it was shown that sponges 2, 11, and 12 were of the same strain type 2. Antisera 73 and 71 are also considered to have the same specificities each recognizing the same antigens, 73, 71, 75, and 23. The grafting results showed sponges 73, 71, 75, and 23 all to be of the same strain type 3.

Antiserum 89 appears to recognize only its homologous antigen, antigen 89. In the grafting results sponge 89 was the only sponge found to be of strain type 15.

These data clearly demonstrate specificity in antibody formation. Knowing that the different antigens which were agglutinated in high titre with each antiserum are from the same strain and that sponges of the same strain elicit antisera which show identical recognition patterns it is clear that this specificity is directly related to strain designation based upon grafting results. There are 3 strain

types of Verongia longissima, and 1 single strain of Verongia cauliformis expressed in the agglutination results, and their expression of specificity concur precisely with the results of the grafting experiments (Figure 3c).

A high agglutination titre was observed when an antiserum was reacted with its homologous antigen from a sponge of the same strain. However a low titre was often observed with heterologous (unrelated) antigens from different sponge strains. Normally no agglutination would be expected if antibodies against those different sponge strains were not present. There are 3 conditions that could explain these low titre occurrences. First, the sponge cells could show antigenic cross-reactions, second, the cells have surface components common for all the strains but each strain having different levels of each antigen, and third, the antiserum could have antibodies against surface components for species and some for strain.

The agglutination test is somewhat insensitive, and a more detailed analysis is required to elucidate the type of recognition that is occurring at the low agglutination titres using other immunological techniques. In the cross-absorption test antibodies are removed by adherence to their specific antigens, and it was hoped that this test would reveal more precisely the type of recognition occurring at the low agglutinating titres of the antisera in this study.

The cross-absorption tests were performed on sponge antisera 71 and 12, and the results are presented in Figures 5a and 5b. These tests involved absorbing-out an antiserum by incubating various cell numbers of an antigen with the antiserum. If the antiserum was reacted with the antigen that had elicited the antiserum, an antigen-antibody reaction occurred and the antibodies united with the antigen cells and remained in the pellet after centrifugation. When the supernatant antiserum was tested again in an agglutination reaction with the antigen that had elicited the antiserum, there was either no agglutination or a low titre agglutination reaction, depending on the concentration of antigen cells originally incubated with the antiserum. If a high number of cells are incubated, a large portion of the antibodies will be absorbed from the antiserum, leaving the supernatant with only a few antibodies to react in the agglutination test (causing low titre agglutination); and if a small number of cells are incubated, more antibodies will remain in the supernatant to react in the agglutination test (causing high titre agglutination). Therefore when the antigen cell concentrations were plotted against the antiserum titres, an absorption curve was obtained for the antigen to which the antiserum was originally directed (Figure 5a, #71, and Figure 5b, #12). This curve was used to compare the efficiency of the other sponge cell suspensions to absorb the antibodies.

When the antisera were absorbed with other sponge cell

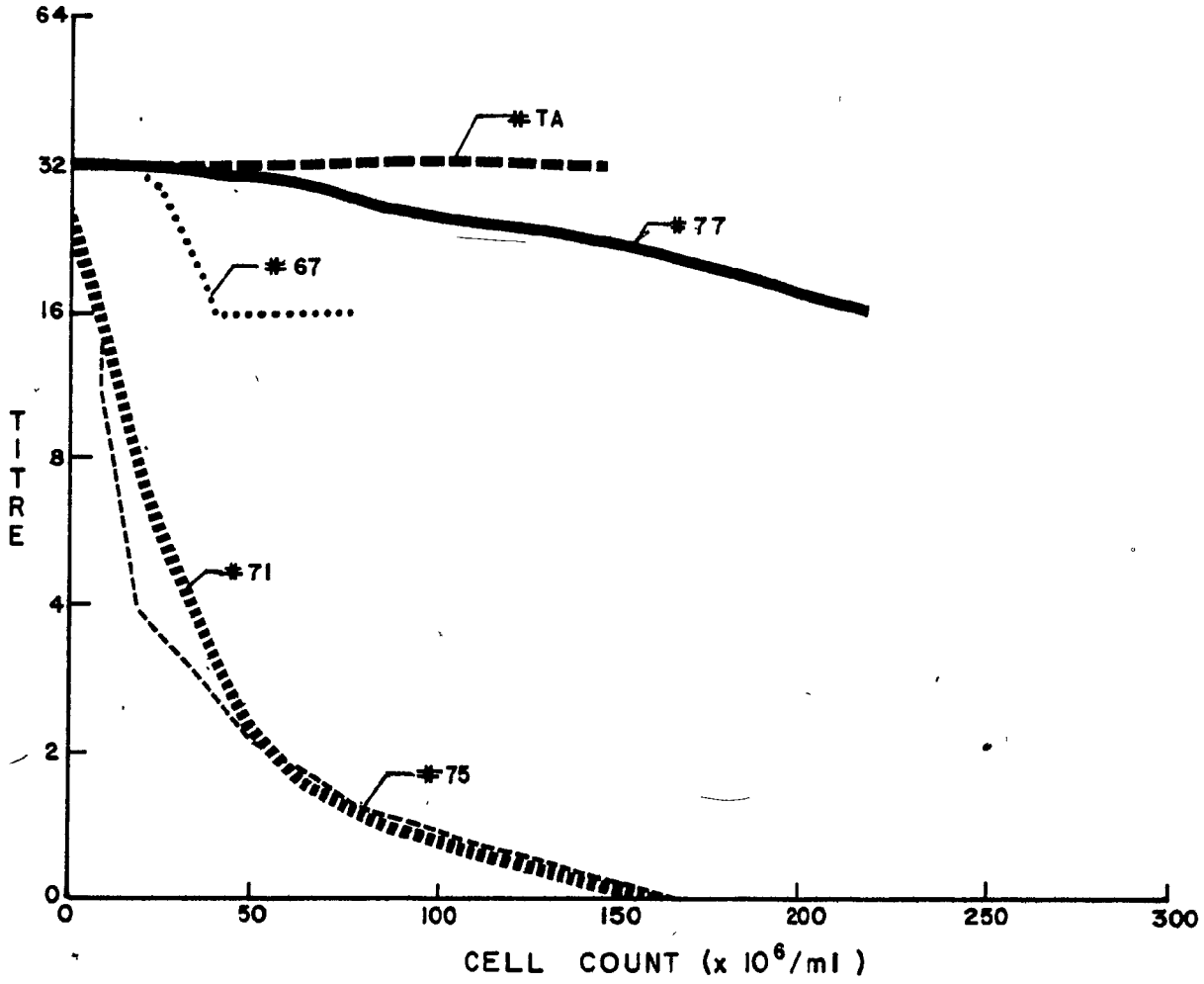


FIG. 5a: ABSORPTION OF ∝# 71 WITH VARIOUS SPONGE EXTRACTS

Note: Results of agglutination test after absorption of ∝# 71 by various sponge extracts. Graph shows the dilution (TITRE axis) and concentration of test extracts (CELL COUNT axis) causing an agglutination reaction to occur.
TA is Verongia fistularis.
67 and #77 are Verongia longissima.
71 and #75 are the same strain of Verongia longissima.

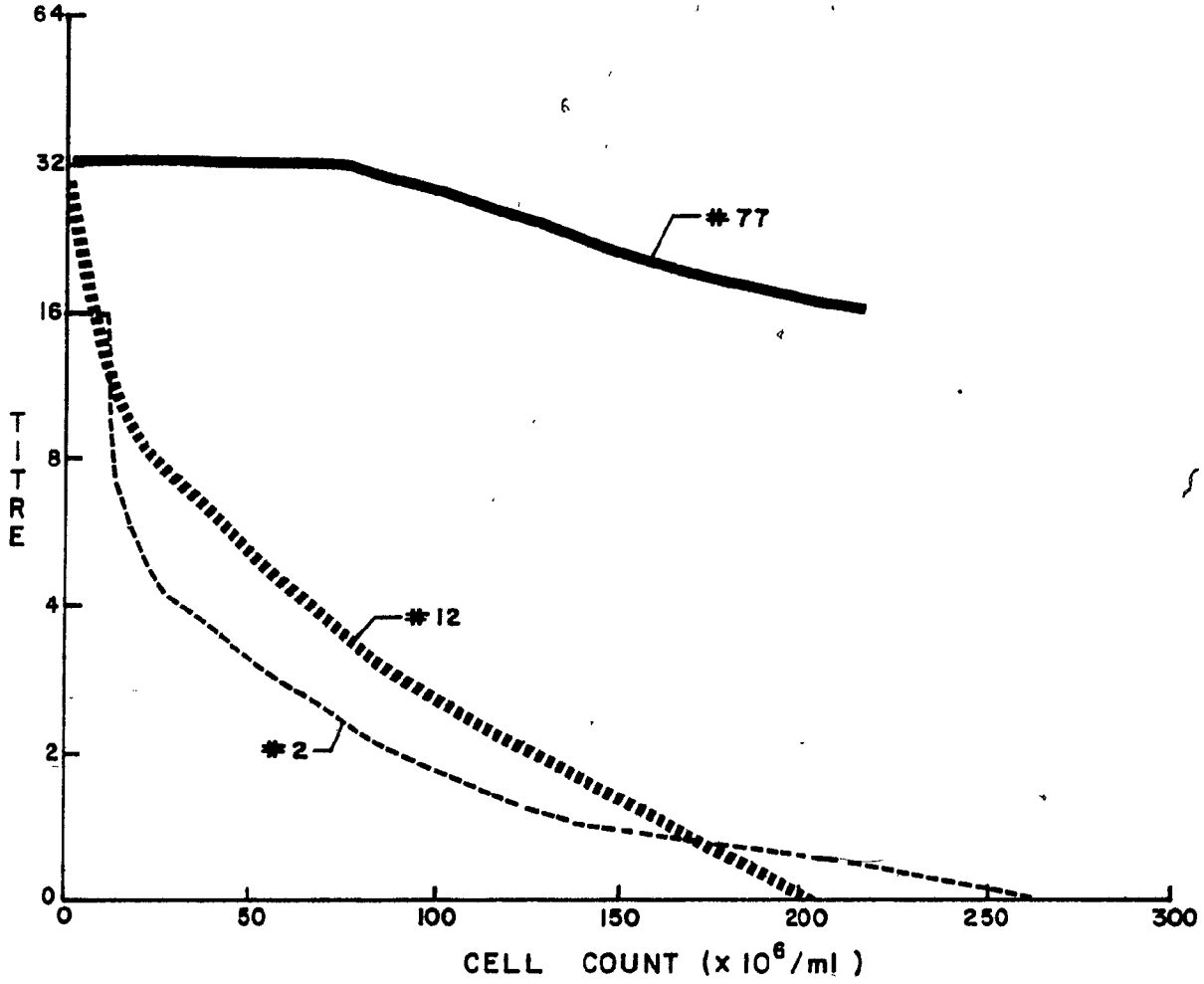


FIG. 5b: ABSORPTION OF ∝# 12 WITH VARIOUS SPONGE EXTRACTS

Note: Results of agglutination test after absorption of ∝# 12 by various sponge extracts. Graph shows the dilution (TITRE axis) and concentration of test extracts (CELL COUNT axis) causing an agglutination reaction to occur.
77 is Verongia longissima.
2 and #12 are the same strain of Verongia longissima.

suspensions, two types of absorption occurred. When sponge cells of the same strain as the antigen used in eliciting the antiserum were used, absorption curves similar to the control were seen. For example, sponge #75 absorbed-out the antibodies with the same efficiency as sponge #71 on antiserum 71 (Figure 5a). Similarly sponges #12 and #2 absorbed antiserum 12 in an identical manner (Figure 5b).

However when antiserum was reacted with heterologous antigens, even at high cell numbers, little antibody was absorbed-out as seen by the high agglutination titres still present. For example, sponge #TA did not reduce the agglutination titre antibodies in antiserum 71, and sponge #77 only gave a small reduction (Figure 5a). This indicates that regardless of cell concentrations there was little or no positive recognition as most of the antibodies remained behind in the supernatant and were available to unite with the antigens used in eliciting the antiserum.

The results of the cross-absorption tests for sponge antiserum 71 (Figure 5a) demonstrate that sponges 71 and 75 are similar. In the grafting experiments these two sponges were found to be of the same strain type 3. The results of the cross-absorption tests for sponge antiserum 12 (Figure 5b) demonstrate that sponges 2 and 12 are similar. Again this result agrees with those of the grafting experiment which found sponge 12 and 2 to be of the same strain type 2. These data, like those from the agglutination tests,

exhibit antigenic specificities, and they are directly related to strain designation based upon grafting results. These cross-absorption tests demonstrate the ability of same strain antigens to completely absorb the antiserum, same species antigens to absorb very little antiserum, and different species antigens to absorb none of the antiserum.

It is apparent from the cross-absorption tests that the antigens (sponge cell suspensions) have qualitative differences. In other words, the type of recognition that is occurring at the low agglutination titres is more likely to be due to a species recognition and not the result of cross reactivity or quantitative differences in the strains. More precisely, the antisera recognize the strain differences of the sponge cells. However the fact that a small amount of antibody was absorbed by heterologous sponge cells may indicate that some antibodies were formed against components common to the species. The strain surface components seem to play the dominant role in eliciting antibody formation in Verongia longissima.

A limited supply of sponge cell suspensions and antisera restricted the extent to which the agglutination reaction tests and the cross-absorption tests could be carried out. To allow for immunological results to be employed as conclusive evidence regarding strain occurrence in Verongia longissima the agglutination and cross-absorption tests should be carried out for all the sponges

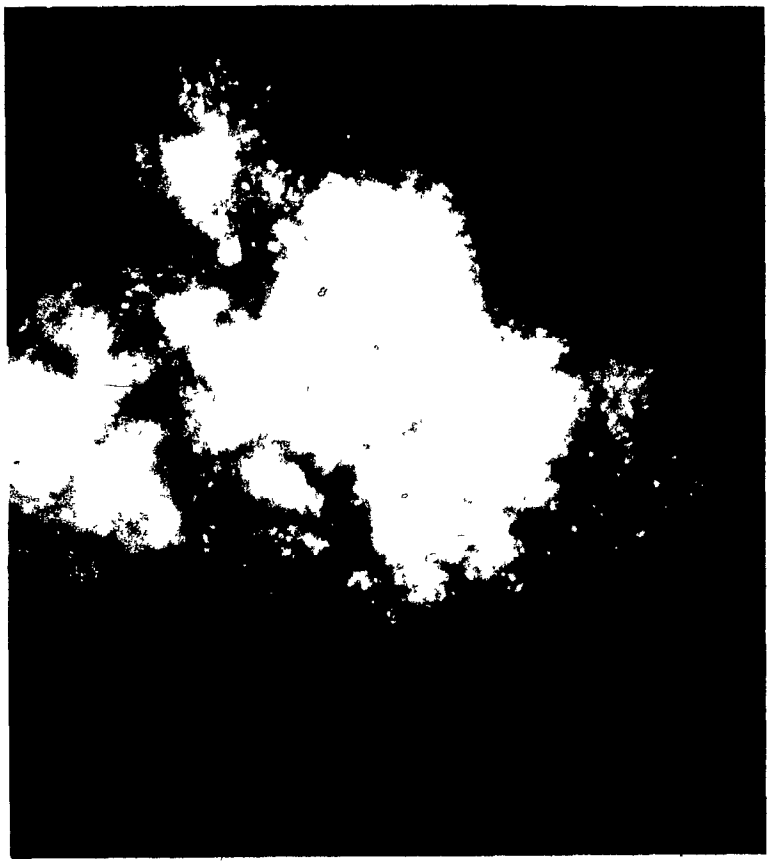
employed in the grafting experiments. However, sufficient data has been presented to demonstrate the possibilities of employing immunological methods to show the occurrence of strain specificity in Verongia longissima.

In attempts to employ the fluorescent-antibody label to demonstrate the specificity of a sponge antiserum for the antigen used to elicit the antiserum, it was discovered that cells of Verongia longissima are autofluorescent (Plate VI-A). It proved to be impossible to utilize the fluorescent-antibody labelling technique in this study since there is little difference in fluorescence when the label is added to the mixture of antigen and antiserum (Plate VI-B). The autofluorescence has an absorption peak in the range of 590 nm (orange-red region of the absorption spectrum).

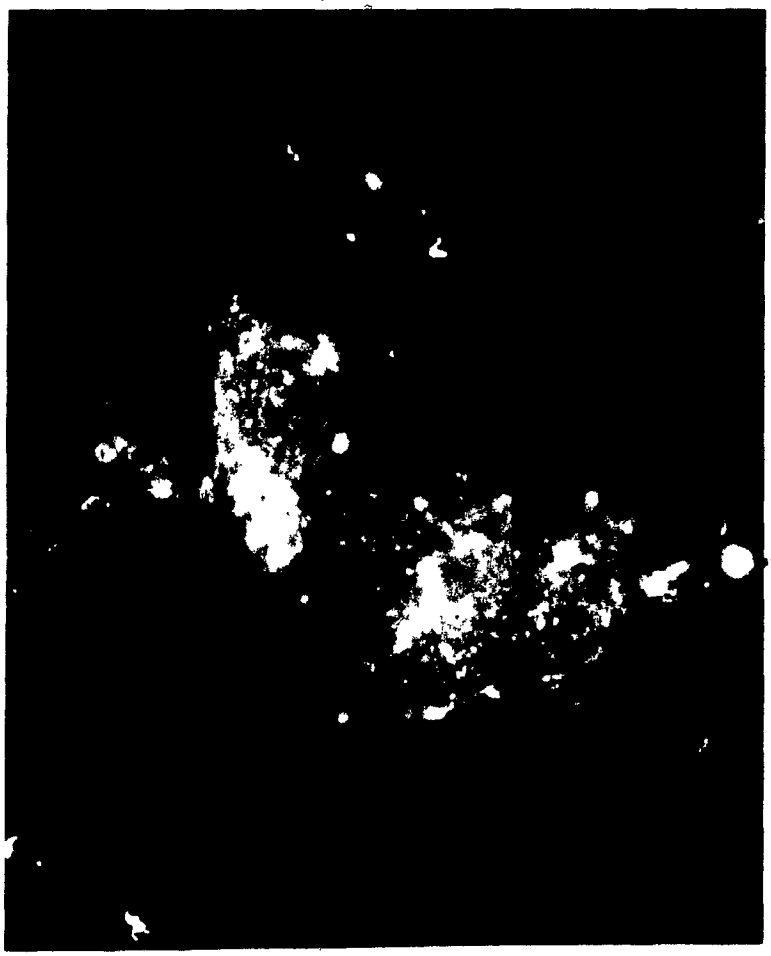
PLATE VI

A. Photomicrograph of autofluorescence of Verongia longissima cells clumped together. The fluorescence has an absorption peak in the range of 590 nm however other substances were also emitting a fluorescence so the absorption reading was not accurate. 320X

B. Photomicrograph of the fluorescence emitted when goat anti-rabbit immunoglobulin fluorescein (FITC) conjugated antiserum was added to a mixture of antiserum directed against the sponge cells and its homologous antigen. There is little difference between the fluorescence emitted by this aggregate of sponge cells and the autofluorescence of an aggregate of sponge cells. 320X



A



B

HISTOLOGICAL STUDY:

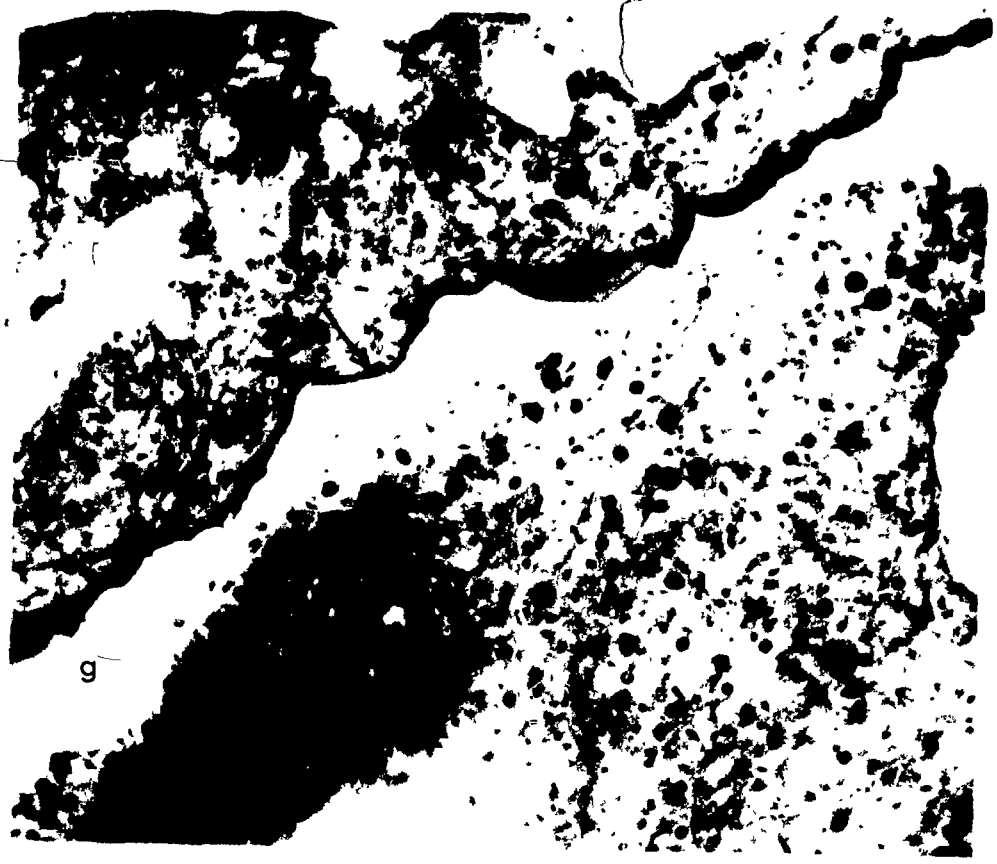
Histological sections through the zone of contact of rejected grafts revealed the presence of a cuticle between the contact surfaces of the two individuals of Verongia longissima (Plate VII-A). Tight junctions and small gaps could be observed along the rejected interface, but no fusion of tissues occurred along the contact surfaces. Distinct pinacoderms separated the two individuals. The cuticle was also found to be present around worm tubes found in sections of this sponge, and in zones of contact with the thread used for tieing the grafts to the hosts. It would appear from these results that a cuticle is laid down by Verongia longissima when any foreign substance, inanimate or animate comes into contact with any part of the sponge. This substantiates Vacelet's findings of the presence of a cuticle on the surface of Verongia aerophoba and Verongia cavernicola in contact with foreign substances, and extends it to include another member of the family Verongiidae.

Histological sections through the zone of contact of accepted grafts revealed fusion of the tissues of the two individuals (Plate VII-B). The zone of fusion was marked by a region of new tissue which still showed signs of immaturity. There were few flagellated choanocyte chambers (regions of sponge responsible for water circulation and pumping) and the bacteria population in this region was very

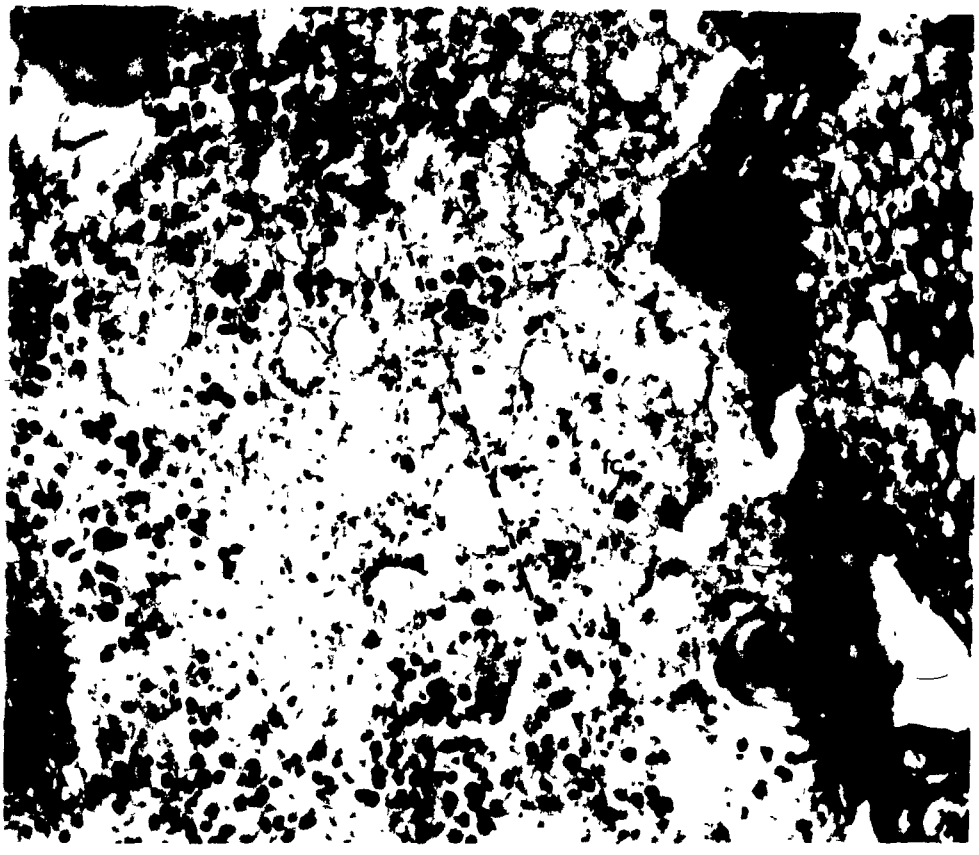
PLATE VII

A. Photomicrograph of a cross-section through the zone of contact of a rejected graft. Note the presence of a cuticle (indicated by the arrow) and the gap (g) between the host and graft individuals of Verongia longissima. The skeletons and internal mileu remain separate and distinct. 260X

B. Photomicrograph of a cross-section through the zone of contact of an accepted graft. No cuticle is present and the skeleton and internal mileu have fused together and are shared by both individuals of Verongia longissima. Note the presence of flagellated chambers (fc) in the zone of fusion. The dotted line indicates the original zone of contact. 260X



A



B

reduced compared to that of older tissues. No distinct features could be seen to mark the original point of contact; the two individuals had grown together and were sharing a common internal mileu.

RADIOAUTOGRAPHIC RESULTS:

The radioautography experiments were performed to demonstrate the supposed collagenous nature of the cuticle (Vacelet, 1971) formed at rejection surfaces of Verongia longissima and to identify the source and rate of incorporation of cuticle precursors. It was presumed that the cells along the contact surfaces would incorporate the ^{3}H-proline directly from solution which would then be utilized in the formation of a collagenous cuticle in the zone of contact between the two individuals. This would be evidenced by the high concentration of tritium labels in the cells along the contact surfaces. Depending on the length of time the rejecting sponges were in contact in situ, and the length of time in the chase solution one would expect to find an increase in the number of labels in the cells of the surfaces in contact over a period of time, and once the amino acid had been incorporated, and the collagen synthesized there would be a decrease in the number of labels in the cells.

The results of these experiments show many unexplainable inconsistencies in the controls of the six hour experiment and in the 48 hour time interval of the nine hour experiment. These are most likely due to contamination and experimental error during the incorporation of the labelled proline. There were also inconsistencies between the paraffin and epon sections of

all of the time intervals. The most likely source of error here would be in the tissue transfer from paraffin to epoxy. For this reason the results include only observations of the paraffin sections.

Labelled proline is taken up and incorporated into cells of the free non-contact surfaces of the grafted sponges and then slowly becomes lightly distributed throughout the cells in the centre of the sponge. Grain counts made on internal choanoderm sponge tissue show an increase in grain density over the time intervals of both six and nine hour experiments (Figure 6). The grain counts of the tissue have been corrected for the background activity of the emulsion by subtracting the average number of silver grains occurring on areas of the slide free from tissue sections (0.5 - 0.8 grains/100 μm^2). The controls for each of the time intervals in the nine hour experiment all showed grain counts in the same range as that of the background radioactivity. As mentioned earlier the controls of the six hour experiment had to be discarded due to contamination.

It appears from these results and general observations of the labelled tissues that proline can be taken up at the free surfaces of the sponge but cannot be taken up by cells at the graft contact surfaces. This may simply be due to the physically tight contact of these artificially joined surfaces acting as a barrier to fluid transport in this

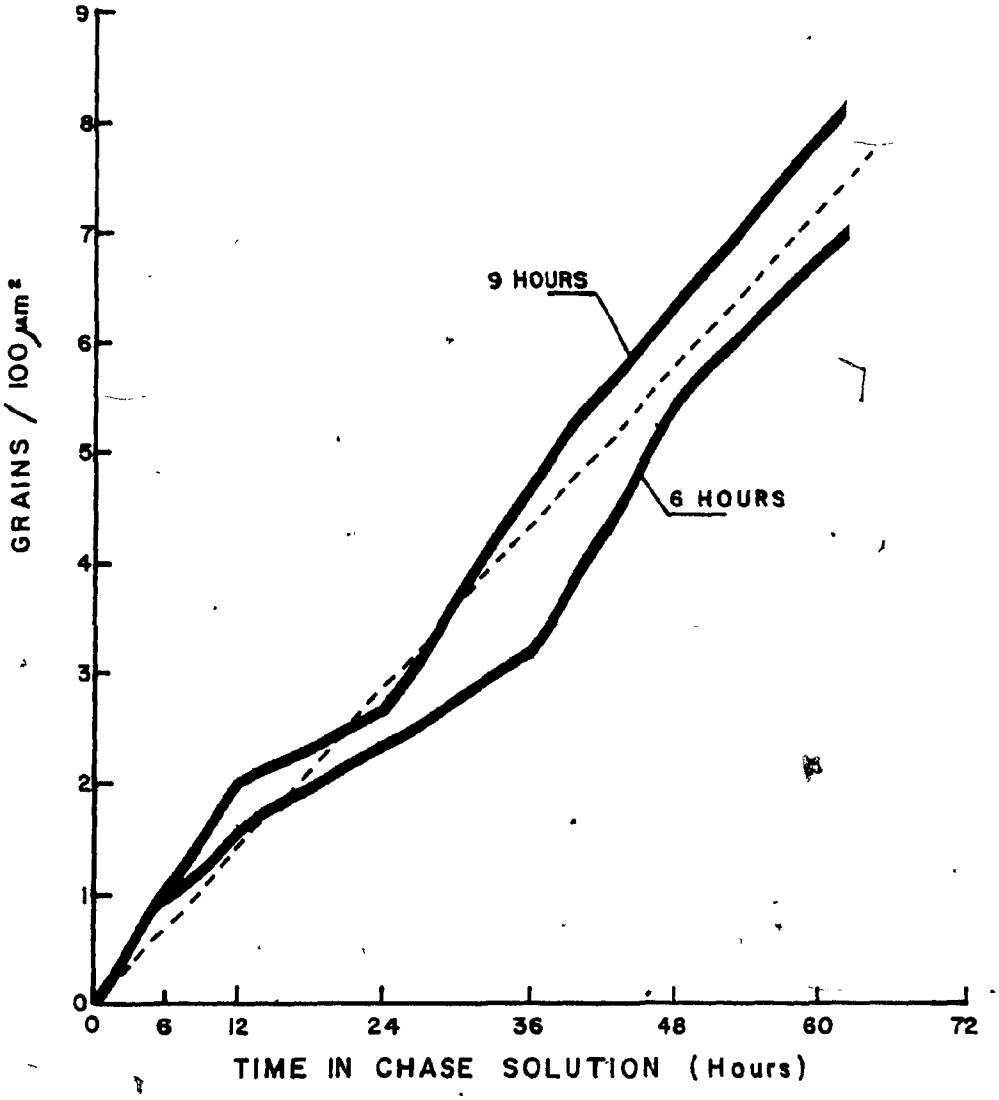


FIG. 6: RADIOAUTOGRAPHY RESULTS

Note: Six hour and nine hour lines represent the time the sponges spent tied *in situ* before being placed in ^{3}H-proline
The dotted line indicates a linear increase in in silver grains over time.

region. The experimental sponges did however show development of a thin cuticle 46 - 49 hours after initial contact (36 hours in chase + 6 or 9 hours *in situ* + 4 hours in ^{3}H-proline), and this remained unlabelled. Since the labelled proline was not incorporated, the cuticle may be either non-collagenous, or, if collagenous, formed from an internal pool of proline or preformed collagen. As can be seen in Figure 6, the label in the control sponge tissue was still increasing at this time (36 hours) in both six and nine hour experiments, and observations of these tissue sections showed that none of the label had been incorporated into the cells of the contact edges of the sponges but was still largely concentrated in the free non-contact surfaces.

Restricted laboratory conditions and the small pieces of specimens used in the experiments almost certainly adversely affected the activities of the water pumping system of the sponges. Under natural conditions this highly active pumping system is responsible for the fast and efficient transport of materials across the surface of the sponge. When the activities of the pumping system are reduced, the rates of transporting material across the surface of the sponge are also reduced. In the present study the reduced pumping rates possibly resulted in lower uptake of the labelled proline than expected. This may have contributed to the relatively long time required for the movement of the label into the inner tissue of the sponge.

The lack of silver grains in the cells of the contact surfaces 62 hours after exposure to ^{3}H-proline can be accounted for by the long time required to move the label from the cells of the free surfaces to the cells in the inner tissue of the sponge, and by the fact that none of the label is taken up by the cells of the surfaces in contact.

If the experiments had been performed under ideal conditions and the pumping activities of the sponges were operating at their normal efficiency, it is highly probable that the cells of the contact surfaces would still have been unable to take up precursor material directly due to the physical limits at the contact surfaces. These cells would still be expected to mobilize internal "pools" of collagen or precursors in these contact zones and deposit new collagen in the form of a cuticle between the two surfaces in contact. Further investigations into the physical, chemical, and structural nature of this cuticle, and the processes involved in its formation and deposition are required to confirm this hypothesis.

DISCUSSION

Sponges comprise a major portion of the animal biomass of many benthic marine habitats. In spite of the importance of species of Porifera in these communities their interactions among themselves and with other community members and the precise ecological significance of these interactions remain undefined.

When one looks at the results of related areas of recent sponge research such as cell reaggregation (Burger, et al, 1978; Humphreys, 1970; MacLennan and Dodd, 1967; and McClay, 1974), spatial competition (Jackson and Buss, 1975; and Sarà, 1970), grafting (Curtis, 1978(a); Hildemann, Johnson, and Jokiel, 1979; and Van de Vyver, 1970), and immunological responses (Kuhns, et al, 1974) patterns of inter- and intraspecific interactions begin to emerge. The mechanisms involved in these patterns of specific interactions seems to be directed by the specificity of a "self/non-self" recognition process which appears to be inherent in these superficially simple organisms. What is the mechanism operating in these specific interactions that allow for sponges to co-habit, over long periods at high diversity with other sponges and benthic organisms in a space-limited habitat like a coral reef community? To answer this question one must first understand the patterns of specific interactions involved and then try to interpret them from an ecological viewpoint.

Sponges are constantly interacting among themselves and with other organisms in their habitat. These interactions can be viewed from three basic levels, based upon the participants involved: between individuals of the same species, between individuals of different sponge species, and between individuals of different taxonomic groups (sponges and non-sponge organisms). Once an interaction commences, usually resulting from growth bringing the two into physical contact, the individuals react in one of two ways: actively, employing either an aggressive strategy, or a defensive strategy, or, passively showing no visible signs of reaction. The type of strategy employed by a given sponge species or individual in such contacts may or may not vary with different interactants.

In the work by Jackson and Buss (1975) a pattern of interaction at the level of different taxonomic groups is exhibited by certain cryptic encrusting sponges possessing allelochemicals which are toxic to specific ectoproct species and harmless to others. In this specific interaction an active response occurs to the susceptible ectoproct species and the sponge effectively executes an aggressive strategy. This strategy has apparently proven to be successful for the encrusting sponges in cryptic, discontinuous habitats by eliminating specific spatial competitors and allowing them to gain access to substrate previously occupied by other organisms in a portion of the isolated communities.

The interactions that occur in complex epizoic relationships involve primarily the levels of different sponge species and different taxonomic groups. These interactions encompass the highly specific symbiotic relationships known to exist between sponges and other organisms such as gastropods, bivalves, crabs, shrimps, worms, zoanthids, bacteria, and blue-green algae. These symbiotic relationships are not completely understood and in most cases the role of the sponge remains undefined. Generally the sponge is considered to be passive, or a non-reactant in epizoic assocations. In these interactions the sponge may live attached to another individual, or act as a host for other organisms which thereby avoid competition for space by such strategy. Further investigations into symbiotic relationships and the role that sponges play in these associations may reveal an active reaction hitherto unknown.

The grafting experiments of this study and those of other workers (Curtis, 1978(a); Hildemann, Johnson, and Jokiel, 1979; and Van de Vyver, 1970) provide excellent evidence of the range of specificity of the recognition system operating at the two levels of interaction within and between different species of sponges, and the strategies that have been evolved by sponges to deal with problems of space utilization in benthic marine habitats. The incidence of patterns in allograft acceptance and rejection, and xenograft rejection discovered in these studies suggests

that these interactions are compatible with the concept of strain and species specific recognition systems in sponges. The patterns of interactions range from extremes of consistent allograft acceptance and xenograft rejection to consistent rejection of both allografts and xenografts. In other words, sponges may fuse with each individual of its own species and reject all individuals of other species, or, fuse with some individuals of its own species and reject all other individuals of its own species and all individuals of other species, or, reject all individuals of its own and other species.

Interactions resulting in rejections are likewise ultimately expressed in a variety of ways. In the present study it was noted that in Verongia longissima, rejection was accompanied by the deposition of a cuticle (Vacelet, 1971) between the two surfaces of individuals in contact. The cuticle may serve to effectively isolate tissues of each member of the graft, essentially removing the living tissues from direct interaction through development of an impermeable defensive barrier. Tissue damage is prevented and space utilization by the species is maximized by such strategy. It may be a result of the mechanisms involved in the specific recognition system and the strategy employed that has resulted in the expression of strain specificity in Verongia longissima. In the studies by Curtis (1978a) and Hildemann, Johnson, and Jokiel (1979) rejection of sponge grafts involved the eventual death and destruction of the

graft individual shortly after the initial contact. Van de Vyver (1970) observed a line or zone of non-coalescence representing physical retreat between rejected individuals of the freshwater sponge Ephydatia fluviatilis. In these interactions the evolution of aggressive or defensive (withdrawal) strategies demonstrate a specific recognition system.

Studies on sponge cell reaggregation (Burger, et al, 1978; Humphreys, 1970; MacLennan and Dodd, 1967; and McClay, 1974) have helped elucidate the biochemical nature of some strain- or species-specific diffuseable surface-active molecules which operate to promote or prevent cell-cell adhesion. The specific "self/non-self" recognition system which leads to fusion or non-fusion in the grafting experiments is almost certainly modulated by the action of aggregation factors of the type identified in cell reaggregation studies. It seems likely that this type of system is at the basis of the natural sponge-sponge interactions occurring in all benthic communities including a coral reef system.

From an ecological viewpoint, the various patterns of interactions and the strategies that have evolved in various sponge species appear to be a means of coping with the basic problem of spatial competition by first gaining substrate, and then defending it. In a space-limited habitat like a coral reef community this is of utmost importance if high

diversity is to be maintained. However other niche diversification factors such as the flexibility in growth form and growth rate, reproductive pattern, etc. must be partially effective as mechanisms of establishing and maintaining high diversity of sponge populations in a space-limited habitat.

The immunological techniques employed in this study have been focused on the strain specific recognition system of Verongia longissima in hopes of providing some insight into the complexity of this specific interaction. Why do we find rejection occurring between individuals of the same species, and what is involved at this level of interaction? Perhaps it is a form of the adaptive immunity response to foreign tissue well known among the vertebrates. Hildemann, Johnson, and Jokiel (1979) have described it as an immuno-recognition at the allogeneic level previously thought to occur only in higher phylogenetic levels, namely the vertebrates. These authors also expressed the idea that it could represent the origins of the major histocompatibility complex and cell-mediated immunity found in higher vertebrates.

There are three fundamental features of an immunological response: memory, specificity, and the recognition of "non-self" (Roitt, 1977). These features embody the ultimate sophistication of an exquisite immunorecognition system. The results of the study by Hildemann, Johnson, and

Jokiel (1979) have finally demonstrated that sponges possess the third fundamental feature, that of memory. However, the present study did not examine this feature and so there is no evidence here to support those findings. Thus it appears from the results of this study and those of other workers that sponges do possess an immunorecognition at the allogeneic level and a form of adaptive immunity. Whether this might possibly represent the origins of the major histocompatibility complex and cell-mediated immunity found in higher vertebrates suggested by Hildemann, Johnson, and Jokiel (1979) remains to be seen. However, these pioneering studies surely stimulate and invite further confirmations and extensions to provide insight into the mechanisms operating in the specific interactions occurring between sponges in their habitat.

Most of our present knowledge on strain specificity in sponges has come from the extensive investigations on the freshwater sponges (Van de Vyver, 1970; 1971(b); 1975; Curtis and Van de Vyver, 1971; and Van de Vyver and Willenz, 1975). As mentioned earlier (INTRODUCTION) Van de Vyver noted the existence of only a few different strains of Ephydatia fluviatilis in a relatively large habitat space. Recent investigations on marine sponges by Curtis (1978a) on Hymeniacidon (sp.) in England, and Hildemann, Johnson, and Jokiel (1979) on Callyspongia diffusa in Hawaii noted the existence of large numbers of different strains of these species in a relatively small habitat space. These limited

results seem to imply that the marine system offers conditions more favourable to the evolution of a higher number of strains within a species. This is substantiated in the present study by the occurrence of 17 different strains of a single marine species, Verongia longissima, in a relatively small area. When the results were analyzed statistically it was shown that in 68% of future sites, similar to those of the present study, one would expect to find between 9 and 11 different strains of Verongia longissima. It is understood, as previously stated, that the analyses were based on a minimum number of sample sites and therefore little emphasis can be placed on these results.

However, in contrast to these patterns of strain diversification, only one single strain of Verongia cauliformis was observed in the present study. This would seem to contradict the earlier implication of the rapid evolution and maintenance of strains in marine species. Verongia cauliformis is, however, similar to Verongia longissima in all morphological characters except pigmentation. It is possible that Verongia cauliformis may prove to be merely a visually distinctive strain of Verongia longissima and not a separate species. Investigation of reproductive relationships between these two groups, presently recognized as separate species, will be required to resolve this problem.

In conclusion, this study has demonstrated the presence of a complex strain specific recognition system in the marine Demosponge, Verongia longissima. Insight into the basis of this specificity has been provided through the successful application of immunological techniques, and the role that it plays in the patterns of specific interactions and strategies occurring in a benthic marine community have been discussed. Further investigations of the complex patterns of interactions that occur between sponges and other organisms in a benthic habitat may help to define the precise ecological role that sponges play in the marine ecosystem and the major selective pressures which have been operative in the evolution of relatively sophisticated immunorecognition systems in these primitive organisms. Such information may also provide greater insight into the general evolutionary history of the exquisite immunorecognition systems of higher metazoans.

SUMMARY

1. Extensive grafting experiments were performed in the field, by means of SCUBA, to investigate the occurrence of strain specificity in the marine Demosponge, Verongia longissima. Less extensive grafting experiments were performed on Verongia cauliformis. Seventeen strains of Verongia longissima and one single strain of Verongia cauliformis were noted. No distinctive features or characteristics discernable in nature could be used to distinguish between the strains. All grafts and hosts employed in the grafting experiments were healthy and alive six months after the initial grafting had been performed.

2. Immunological studies employing rabbit antisera and sponge antigens supported the strain designations based on the grafting experiments, and revealed the extensive antigenic polymorphism among strains of Verongia longissima. These studies demonstrated an immuno-recognition at the allogeneic level, similar to the immunological response of higher organisms.

3. Verongia longissima cells were found to possess an auto-fluorescence hitherto unknown.

4. Histological preparations of graft rejections showed the deposition of a cuticle between the surfaces of the two sponges in contact. No such cuticle was present in

preparations of graft acceptances. It appears that the formation of a collagenous cuticle is a common response by Verongia species to a foreign solid surface.

5. It has been hypothesized from radioautography results that preformed collagen is probably mobilized from "pools" within the sponge tissue and deposited between the contact surfaces of two individuals that reject one another. The first signs of a cuticle were observed 46 hours after two individuals of different strains of Verongia longissima had been brought into contact.

6. Inter- and intraspecific acceptance and rejection, noted in sponges, appear to be one of several patterns of interactions that occur between individuals in a space-limited habitat. The ecological significance of this specificity can be seen when one considers the abundance and diversity of the Porifera in benthic marine communities.

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