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Title: AN INVESTIGATION INTO THE HUMORAL IMMUNE RESPONSE OF THE ASCIDIAN UROCHORDATE, STYELA MONTEREYENSIS

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Adaptive immune capabilities of the Urochordate S. montereyensis were studied. Animals repeatedly injected with bacteria were bled and the following properties were measured in vitro: agglutinin, bactericidin, bacteria-cell association. Both Gram (+) and Gram (-) bacteria were isolated from the gut of S. montereyensis and used as antigens at 10^8 bacteria ml^{-1}, 0.01 ml g^{-1}. Tests conducted over 25 days, with injections and samples made every two and five days respectively, failed to reveal a natural or induced bactericidin. Though increases in phagocytic rates and agglutinin titer were small under the aforementioned protocol, the groups injected with bacteria all showed positive trends with time following initial declines when compared to the saline-injected group. It was not clear whether these trends reflected a return to the basal level (with positive feedback) or whether they constituted

induced responses similar to the adaptive immune responses common in the vertebrates. The data do not indicate specificity with regards to the Gram (+) or Gram (-) antigen.

The health of the test animals was monitored by weighing each animal at the start of the experiment and again when the animals were sampled. No apparent correlation existed between the health of the animal and the values measured in the agglutinin and bacteria-cell association assays. The uninjected controls gained weight and the saline-injected group lost weight, yet both had similar assay values. In contrast to the saline-injected group, the bacteria-injected groups showed increasing trends while losing weight.

Laboratory specimens of S. montereyensis were maintained by supplying cultures of Pseudoisochrysis paradoxa.

The immunological study was preceded by experiments to select a food on which S. montereyensis was capable of weight-gain under laboratory conditions. Of three possible foods, S. montereyensis gained weight only when fed quantities greater than 22,000 cells animal^{-1} day^{-1} of P. paradoxa. The gain in wet weight was shown to be related to tissue growth.

An Investigation into the Humoral Immune Response of the Ascidian Urochordate, *Styela montereyensis*

by

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INTRODUCTION

The challenge of resolving the evolutionary origins of the immune system has stirred strong interest among both evolutionists and immunologists hoping to find a less complex precursor of the complicated and confusing mammalian immune system. The majority of studies have been on mammals and birds, these being most directly applicable to man. In the past two decades much attention has been directed toward immunity in the lower vertebrates. Thymic precursors have now been reported in all vertebrate classes, thus establishing the possibility of homologous cell-mediated immunity. Inducible immunoglobins (Ig) or immunoglobulin-like factors have been well established throughout the vertebrates with a couple of anomalies reported in the cyclostomes. Marchalonis and Edelman (1968a) induced the lamprey to produce a precipitin which was found to contain a subunit structure with the typical two heavy and two light chains, but the subunits were bound only by ionic forces and not by the disulfide bridges seen in the Igs of higher vertebrates. The more primitive hagfish has an inducible molecule the subunits of which resemble the human IgG light chain (SDS electrophoresis, De Ioannes and Hildemann, 1975). These results support the possibility that an ancestral light chain has been modified during vertebrate evolution to give the various heavy and light chains found today.

In nearly all animal and some plant phyla, functional analogues to the vertebrate Ig have been reported, but these have no structural similarity. In the vertebrates most of these analogues occur in the lower groups. The nurse shark has a precipitin which specifically binds with fructosans and has a molecular weight of 280,000 daltons with four 71,000 dalton subunits (Harisdangkul et al., 1972a, 1972b). An eel agglutinin to the H(O)-antigen of human red blood cells (RBCs) is a globulin molecule with a molecular weight of 123,000 daltons. It consists of three ionically bonded subunits of 40,000 daltons each of which is made up of four polypeptide chains of 10,000 daltons (Bezkorovainy et al., 1971; Springer and Desai, 1971). A most interesting finding by Litman et al. (1970) was the induction of a lamprey agglutinin specific for the human O RBC and having a molecular weight of 320,000 daltons with four identical 75,000 dalton subunits. This molecule is unique in that it was induced specifically and was structurally unlike Ig. Litman (1976) considered that the difference between the lamprey molecule of Marchalonis and that of his own group was possibly due to the different antigens used. The soluble antigen used by Marchalonis and Edelman induced the production of the Ig-like molecule; the particulate antigen introduced by Litman's group caused the production of the non-Ig molecule. In the same work, Litman suggested that the molecule found by Marchalonis and Edelman is the

primitive Ig molecule, whereas the molecule found by his own group is the most advanced form of the natural humoral factors (predominantly noninducible) of earlier phylogenetic development.

No one has yet demonstrated an Ig-like humoral factor in an invertebrate phylum. Though work has been done in all major groups, the economically more important molluscs and arthropods have seen more attention. The most intensively studied molluscan humoral factor is the oyster hemagglutinin (Tripp, 1966; Li and Flemming, 1967; Acton, 1970). This agglutinin has a molecular weight of 400,000 daltons and dissociates non-covalently into 20 subunits of about 20,000 daltons. Analysis of the amino acid composition of subunits shows no relationship with the light chain of mammalian IgG. Many other molluscan agglutinins have been reported (Boyd et al., 1966; Hammarstrom and Kabat, 1969; Jenkin and Rowley, 1970; Pauley et al., 1971; Uhlenbruck et al., 1972). The arthropod studies are even more extensive and abundant. The most thoroughly studied arthropod agglutinin is from the hemolymph of Limulus (Cohen et al., 1965; Marchalonis and Edelman, 1968b; Finstad et al., 1972). This molecule, like the oyster agglutinin, has a molecular weight of 400,000 daltons but consists of only six ionically bound subunits. Each subunit is composed of three polypeptide chains of 22,500 daltons each, joined by disulfide bonds. Many of the protostome agglutinins have been compared with vertebrate Ig. But the humoral factors of these phyla can be

considered only analogous with those of the vertebrates; unless strong conservation of primary structure is demonstrated throughout intermediate groups (lesser protostomes and lesser deuterostomes), evolutionary homologies are untenable. And again, the ancestral genes from which these molecules evolved have most likely disappeared, and the key to any relationship has gone with them.

There have been only a few studies on humoral factors of echinoderms. Only two of these have included structural analyses of the molecules. An agglutinin in the starfish Asterias forbesi was found to be a 6S complex consisting of a major subunit of 30,000 daltons and a minor subunit of 13,000 daltons (Finstad et al., 1972). The amino acid composition did not resemble that of any known Ig molecule. Interestingly, Carton (1974), using a statistical model devised by Marchalonis and Weltman (1971) and the data from Finstad et al., (1972), found that similarities did exist in the amino acid composition of the agglutinins of Limulus and Asterias; the similarity extended to the heavy and light chains of Ig. The contradictions of accepted phylogeny seen here emphasized the need to base such analyses on amino acid sequences rather than on gross composition.

Since the non-vertebrate chordates are the closest living relatives of the vertebrate line of evolution, immunological research on urochordates has become increasingly intense. Early on, Cantacuzene (1913) found a precipitin to rabbit serum in Phallusia mamillata.

Tyler (1946) noted agglutinin activity in the body fluids of Ciona intestinalis and Styela barnharti to spermatozoa from a wide range of invertebrates. Recently, preliminary work on the natural agglutinins of urochordates has begun. Wright and Cooper (1975) found an agglutinin to duck and human erythrocytes in C. intestinalis. This agglutinin was nonspecific, heat labile, and required both Ca^{2+} and Mg^{2+}. Halocynthia hilgendorfi and Styela picta contain agglutinins which were partially specific since they agglutinated most mammal and avian RBCs but did not agglutinate reptile, amphibian, fish, and certain other bird and mammal RBCs. This agglutinin was insensitive to low levels of trypsin and to temperatures up to 140°C, but was inactivated by periodate (Fuke and Sugai, 1972). The urochordate Halocynthia pyriformis contains an agglutinin against a large variety of avian and mammalian RBCs (Anderson and Good, 1975). However, there is only partial specificity since RBCs of different origin were agglutinated to different extents. The agglutinin was nondialyzable, heat sensitive at temperatures above 50°C but stable with repeated freezing and thawing. It was insensitive to low levels of trypsin but was inactivated by high levels. Anderson found the agglutinin required Ca^{2+} but not Mg^{2+}. He also found that the addition of sialic acid inhibited agglutination. Bretting and Renwrantz (1973) found that the plasma of P. mammillata, Halocynthia papillosa, and Microcosmos sulcatus agglutinated human RBCs.

There are few reports on other humoral factors of urochordates. Fuke and Sugai (1972) reported that S. picta does not have a natural opsonic factor, since blood cells in sea water phagocytozed as readily as did cells in the natural serum. This contrasts with the work of Fulton (1920) who reported that cells from Ascidia atra apparently required serum to phagocytoze carmine particles. Johnson and Chapman (1970) found a natural bactericidin to gram negative marine bacteria in C. intestinalis, but not to a terrestrial or gram positive marine pathogen.

All urochordate work has focussed on naturally occuring humoral factors. There are no reports on humoral responses of these animals to foreign substances over a prolonged period of time. This study was done to determine if the urochordate Styela montereyensis could alter the levels of selected innate factors in response to repeated injections of bacteria. Agglutination, phagocytosis, and bactericidal activities were determined in vitro with both gram negative and gram positive bacteria.

The genus Styela belongs to the family Styelidae. This family is considered to be advanced and therefore only distantly related to the most primitive family of living urochordates, the Cionidae (Berrill, 1955). Though Ciona would be more desireable from an evolutionary standpoint, Styela montereyensis was chosen for this study because it is much more hardy in the laboratory and because it is locally available. In nature, S. montereyensis attains a maximum length of about

30 cms, ranges from Southern California to British Columbia and is found from the low intertidal to subtidal depths of 26 meters (Abbott and Johnson, 1972).

Collection and Preparation of Animals

Specimens of Styela montereyensis were collected from Oregon coastal waters. The species is a permanent resident of subtidal rocks on the open coast, and was collected at 18 meter depths using SCUBA in May and September 1976, just offshore of Newport, Oregon. Whenever possible, collections were made within the quieter waters of Yaquina Bay, Newport. Specimens which settle as juveniles in the Bay in Spring and early Summer attain a size adequate for this work (3.5 g wet weight) by early Autumn, and then die, apparantly due to decreased salinity and increased silt, when the Winter rains begin. Collections were made from floating pontoons (Public Boat Dock) and submerged rocks (South Jetty) in Yaquina Bay in September 1974 and July and December 1976.

The animals were rinsed immediately and placed into an insulated five liter container half-filled with fresh sea water and transported to the laboratory in Corvallis (50 miles) where they were placed in holding tanks of a 6000 gallon recirculating sea water system at 12°C. After an initial holding period, specimens were prepared for the experimental tanks; the main epibionts were removed then the animals were weighed. All animals were weighed similarly in an effort to reduce variability due to retention of water in the pharyngeo-atrial

cavity. The animals were placed on paper towels to absorb excess water, were dabbed dry and placed in a pre-weighed Petri dish. The weight was recorded to the nearest 0.01 g.

Each specimen was then secured to a styrofoam cube about two cm^3 by means of ca. 10 cm of monofilament line tied around the lower stalk of the animal. An identification number was placed on the upper surface of the cube. This arrangement caused the animal to hang inverted in the water, in a relatively natural position. By having the animal on floats, individuals were easily identified and were better spaced in each tank than would have been possible by laying them on the bottom, or suspending them from the sides. When removing the floats from the surface, I rarely submerged my hands into the tanks, thus decreasing the possibility of introducing extraneous materials into the system.

Any morphological abnormalities were noted when preparing the animals for the experiments. Any remaining epifauna was recorded.

The experimental tanks were three 170-liter covered, white polyethylene tanks in a cold room at 10°C. The tanks contained a combination of filtered sea water obtained from the O. S. U. Marine Science Center and artificial sea water (Instant Ocean®). The water was maintained at a salinity range of 30.5 - 31.5‰ and was continuously filtered by a subsand system (Spotte, 1970).

The animals were fed Pseudoisochrysis paradoxa. Each day 3.5 liters of an actively growing culture was fed to each tank over a period of ten hours. Further details are given in the next section.

Selection of Diet for Maintenance of Styela montereyensis

Though S. montereyensis was capable of survival for over nine months in the laboratory without feeding, the animal lost weight continuously. Three possible foods were tested for their ability to support growth of S. montereyensis; these were the unicellular green alga P. paradoxa, the marine bacterium Vibrio anguillarum (heat killed), and corn starch.

Pseudoisochrysis paradoxa were grown in 2 liter flasks set into a white polyethylene tub to increase light exposure to the flasks. Light was provided by fluorescent growth lights placed 30 cm above the flasks. The cultures were maintained at room temperature (20-22°C), and were oxygenated and agitated by a continuous flow of compressed air humidified and cleansed by passing it through a small flask containing distilled water. The modified enrichment medium of Mathiessen and Toner (1966) was used to enhance the growth rate and density of the algae. Their recipe included sodium silicate since the medium was designed for diatoms; this ingredient was omitted during my study.

Vibrio anguillarum were grown in Brain Heart Infusion (BHI) broth at room temperature with occasional agitation for 36 hours. The bacteria were heat killed by placing the flasks in a 56°C water bath for 30 minutes. The bacteria were then centrifuged at 6000 rpm (4340 g) at 2°C for ten minutes. The bacteria were washed once in sterile sea water then the bacterial pellet was resuspended in sterile sea water.

Two liters of corn starch (Kingsford's ®) was prepared daily at a concentration of 1 gram liter^{-1}.

Fifteen healthy S. montereyensis were randomly selected for the experiment, five for each tank designated to receive one of the three food sources. A peristaltic pump served to distribute the food from the holding flasks to the test tanks over a period of ten hours. The food flasks were placed on magnetic stirrers which maintained an even suspension.

Two liters of each food source were used each day. During the feeding, the filtering system was disconnected.

An attempt was made to weigh each animal every day, starting 45 days prior to initiation of feeding of V. anguillarum, 36 days prior to feeding corn starch, and 33 days prior to feeding P. paradoxa. The weights were taken during the feeding regime until significant increases of weight were determined.

A second feeding experiment was designed to reaffirm that the weight gained in Group 2 of the first feeding experiment was due to the

feeding of P. paradoxa, and to define the conditions of weight gain (i.e., was the weight increase due to water retention alone or did tissue growth occur?). Four groups of 15 S. montereyensis were randomly selected. Groups 1 - 3 were weighed daily for 24 days. Feeding was started on the ninth day. Group 1, starved control, was "fed" three liters of artificial sea water daily. Groups 2 and 3 were fed daily three liters of P. paradoxa at stock concentrations of 5×10^5 and 5×10^6 cells ml^{-1} respectively. Group 4 was weighed four times on the first day of the experiment and oven dried at 90°C for 20 hours. Other conditions of the experiment were the same as those in the first experiment.

Following the final feeding on the 24th day, the animals were starved for 1.5 days, weighed (four times on the 26th day), and oven dried under the same conditions as Group 4. Weights were taken to the nearest 0.01 g.

Selection of Antigens

Two bacteria, one Gram (+) and the other Gram (-) (see Appendix I for characteristics) were isolated from the gut of S. montereyensis. The bacteria grow well on Tryptic Soy (TS) agar and in BHI broth at room temperature as well as at 10°C. Thus, the stock cultures were

maintained on TS agar slant tubes in an inclosed hood at room temperature. The cultures prepared for injections and assays were grown to stationary phase in BHI broth at room temperature.

A standard technique was used to prepare bacteria for experimental assays and injections. The Gram (+) bacterial culture was started one day prior to use. The Gram (-) bacteria were started two days prior to use due to a slower reproduction rate.

On the day of use, 20 ml of the bacterial culture were centrifuged at 8000 rpm (7710 g) for ten minutes at 4°C. The bacterial pellet was then washed with 10 ml of sterile filtered (Millipore®; 0.45 μm) artificial sea water and resuspended in 1.2 ml of the same.

A 0.1 or 0.2 ml aliquot of the bacterial suspension was added to a spectrophotometer tube and diluted to 5 ml with sterile artificial sea water. The absorbance was measured at 450 mμ, and converted to bacterial numbers from a standard plot for each bacterium (Appendix II). Microscopic counts with a hemacytometer and colony counts from pour plates of serial dilutions were used to construct the standard plot.

Injection and Sampling

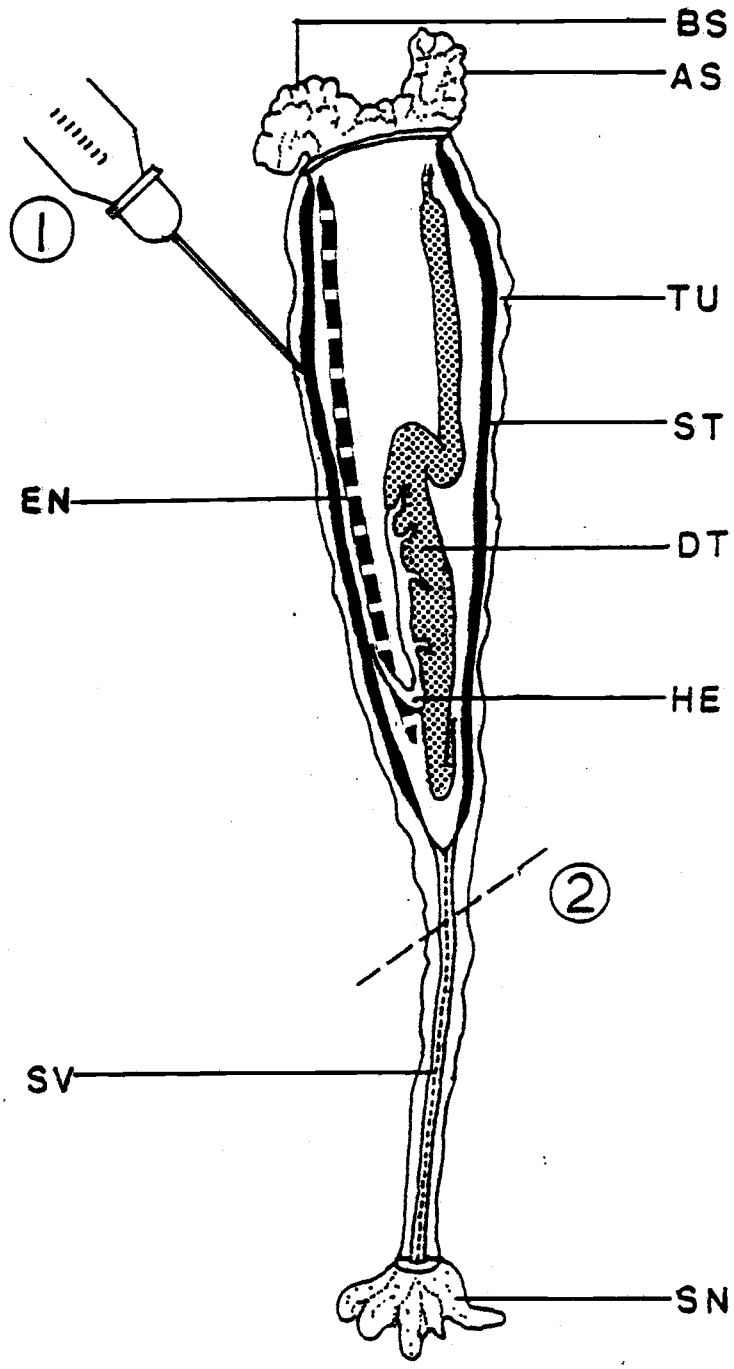
The schedule used was the same for all three assays--agglutinin, opsonin, and bactericidin. Animals were evenly distributed into four groups. One group served as the control and received no injections. A second group (shams) was injected with sterile saline. A third

group was injected with 1.0×10^8 bacteria per ml^{-1} of the Gram (+) bacteria. The fourth group was injected with a similar concentration of the Gram (-) bacteria. Each animal received 0.01 ml per $gram^{-1}$ wet weight. Injections were performed every other day for approximately one month. Samples were taken every fifth day starting at day zero which provided the initial control value. The length of the test period was based on the number of animals available. On the sample day the assay scheduled for that day was performed.

The injection site for each assay was the subtunic space around the midbody region (Fig. 1). The tunicates were removed from the tanks and wiped dry. The injections were made using a 1 ml syringe and 26 gauge disposable needle. The angle of injection was about 10^o to the surface of the animal. The contents were injected slowly and the needle was withdrawn slowly. The injection site was inspected for seepage. If no seepage or if only a trace of fluid appeared around the site, the injection was recorded as a number 1. If approximately 5 to 20% of the volume injected appeared at the wound site, the number 2 was recorded. If more than 20% of the injected volume was exuded, number 3 was recorded. This evaluation was strictly subjective.

The anatomy of S. montereyensis (Fig. 1) allows one a very simple, but lethal, method for obtaining blood. The stalk of the animal consists of blood channels and tunic only. Thus when the stalk is severed, the heart will pump blood out the exposed channel openings. Each

Figure 1. Styela montereyensis showing general anatomy and sites of injection and sampling. Included are the following: 1) injection and 2) bleeding sites; BS - buccal siphon; AS - atrial siphon; TU - tunic; ST - subtunic space; DT - digestive tract; HE - heart; SN - stolon; SV - stalk blood vessels; EN - endostyle.



sample animal was blotted dry and the siphons were covered with absorbant tissue paper. The stalk was cleaned of all debris, wiped with an alcohol-dampened swab and severed at an angle of 45° to the longitudinal axis using a fresh razor blade or scalpel. With the animal held above the chilled collection tube, the upper body region was gently squeezed to enhance blood flow; the fresh blood was quickly collected and prepared for assay.

Agglutinin Assay

The blood for the agglutinin assay was collected in 15 ml sterile glass centrifuge tubes. The cells were sedimented in a clinical table top centrifuge at 1000 rpm (107 g) for five minutes at 10°C. The cell-free plasma was removed by Pasteur pipette, placed in a clean vial, capped, and stored at 4°C until all samples were collected.

Since the blood yield of S. montereyensis is relatively small (e.g., 0.5 ml per 8.0 g of total wet weight) micro-titer plates (Linbro Chemical Co., Model IS MRC 96) were used. The plates contained eight rows of 12 wells each. The wells had round bottoms and held 0.3 ml total volume. Wells 1-11 of each row contained blood serially diluted by one-half with artificial sea water. The volume in each well was 50 µl. Well 12 was the control and contained 50 µl artificial sea water. Two such rows were prepared from each sampled S. montereyensis; the first was incubated with the Gram (+) bacteria and the second with

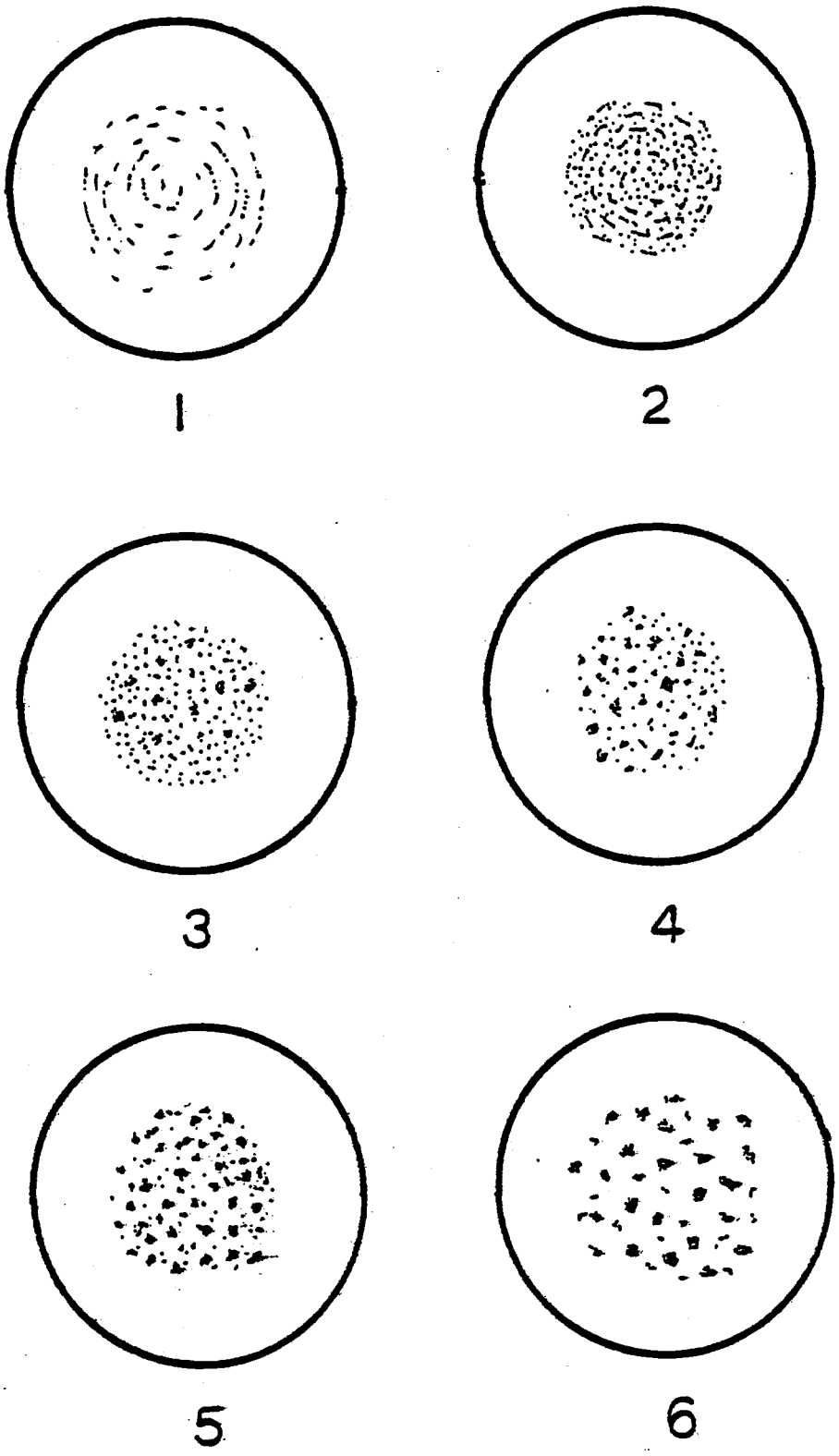
the Gram (-) bacteria. Each well received 5 x 10^6 bacteria in 50 µl. Each micro-titer plate was sealed by an adhering plastic cover, and placed on a rotary table set at 50 rpm at 22°C for 12 hours.

The plates were examined under a dissecting scope and the contents of each well was stirred with a small glass rod while viewing to judge the amount of agglutination. I judged the quality of agglutination by the following characteristics (Fig. 2):

- \- Settled bacteria swirl evenly and independently when disturbed by mixing.
- ± The majority of bacteria swirl evenly but some fine clumps are apparent.
- \+ The majority of the bacteria are agglutinated into small clumps.
- ++ The majority of clumps are larger and less numerous than + but some exist at the + size.
- +++ Most bacteria in large clumps with a few of ++ size.
- ++++ All clumps large and very few in number.

The agglutination titer was expressed as the reciprocal of the smallest serum dilution where a (+) rating was given.

Figure 2. Diagrams representing the quality of agglutination. 1 = -; 2 = ±; 3 = +; 4 = ++; 5 = +++; 6 = ++++.



Bacteria-Cell Association Assay

The blood of each animal was collected in separate siliconized 10 ml test tubes at 4°C. Using a micropipette, 50 µl of the blood was added to each of three siliconized petticups (Fisher Sci. ®) half filled with silicone oil (Aldrich Chemical Co. ®). Each petticup received 50 µl of one of the following: sterile artificial sea water, the Gram (+) bacteria at 10^8 cells ml^{-1}, or an equal concentration of the Gram (-) bacteria. In this system, the plasma forms a droplet in the silicone, and fuses with the 50 µl of added fluid. The petticups were capped with triple-0 neoprene stoppers and set in a cell culture roller apparatus. The cups were oriented such that the long axis of the cup was horizontal and at right angles to the plane of rotation of the apparatus. The roller apparatus was set at 1.3 rpm. The suspensions were allowed to incubate for two hours at 10°C.

Following the two hours of incubation the petticups were removed and the incubated cells were treated as soon as possible. The cells from each petticup were smeared over a slide, air dried and fixed in absolute methanol for five minutes, then stained with Giemsa according to Galigher and Kozloff (1971).

Each slide was scored in the following manner: for a total of 100 cells the number of bacteria per $cell^{-1}$ was noted. The 100 cells consisted of 20 cells from each of five different regions on the slide. The

four corners and center of the slide constituted these regions. The number of bacteria appearing within the perimeter of each cell was counted (Fig. 3). The total number of bacteria was divided by the total number of cells to obtain bacteria cell^{-1} values. The real value for the Gram (+) and Gram (-) incubated slide for each animal was calculated by the following formula:

$$AI = n_e - n_s$$

where, AI is the association index given in bacteria cell^{-1}.

n_e is the number of bacteria cell^{-1} from the bacteria-incubated slide of an individual.

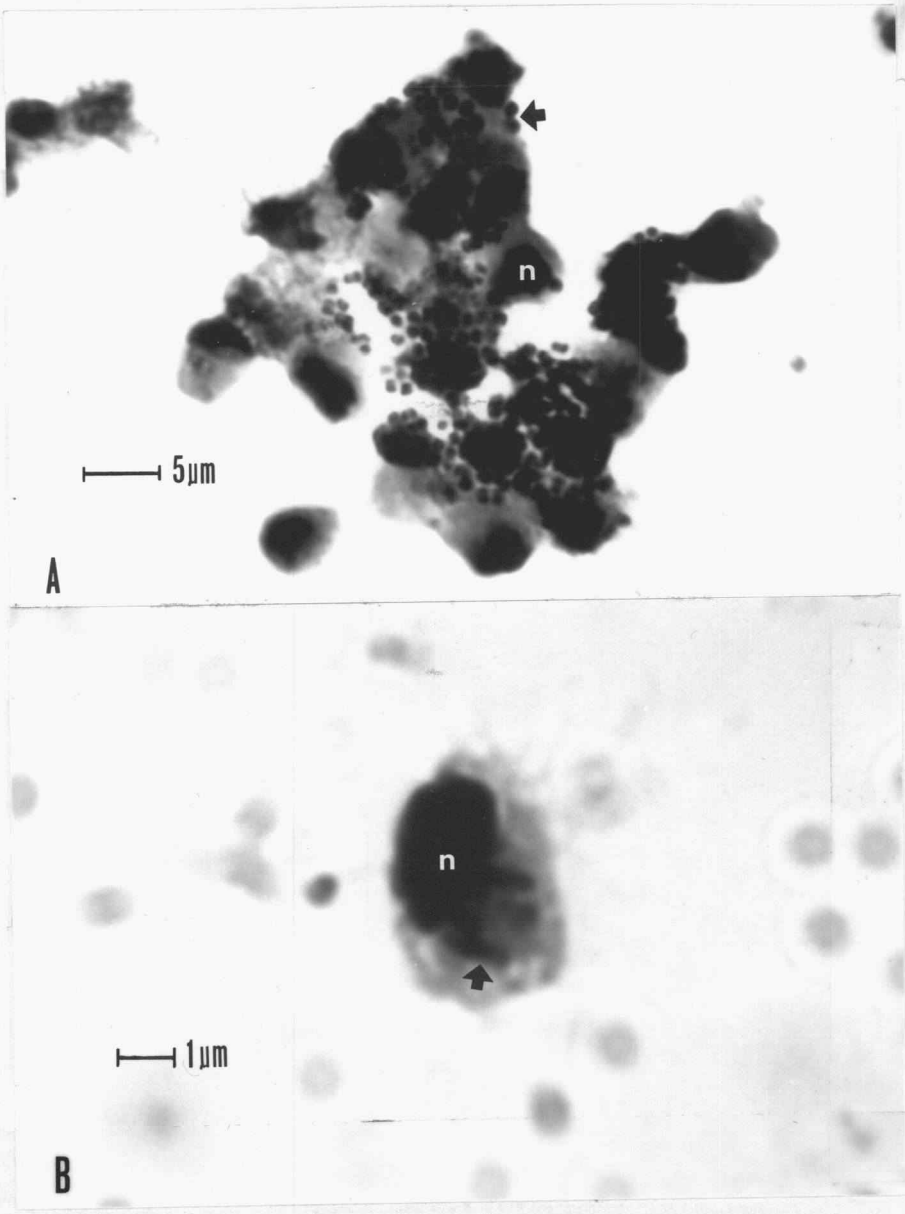
n_s is the number of bacteria cell^{-1} from the saline-incubated slide of the same individual.

Bactericidin Assay

The blood was collected and the cells were removed by centrifugation as described for the Agglutinin Assay. The plasma was sterilized by filtration through a 0.45 μm filter, and maintained at 4°C until incubated with the bacteria.

Pour plates containing 5×10^4 and 5×10^6 bacteria respectively were prepared for each animal sampled. Ten ml of TS agar containing bacteria were poured into 60 mm plastic Petri dishes. Into each plate four wells were created approximately half the distance from the center to the edge of the dish and equidistant from the two neighboring

Figure 3. "Typical" field with cells and bacteria from the Bacteria-Cell Association Experiment.
A. Cells associated with the Gram (+) bacteria.
B. A cell associated with the Gram (-) bacteria.
Arrow indicates the bacteria. n = Nucleus.



wells, such that a square was formed. The wells were made by using a heat-sterilized 4 mm diameter brass cork-borer attached to a vacuum aspirator. Into the two opposite wells, 20 μl of plasma was placed. Into the other wells, 20 μl of sterile artificial sea water was placed. The wells were labeled and the Petri dishes were placed at 10°C inside a covered plastic box. After two and one-half days, the distance from the edge of the well to the nearest bacterial colony was measured.

Statistical Analysis of the Data

The daily mean wet weights (percent of initial weight) of each group in the first and second feeding experiments were analyzed by linear regression starting two days following the initiation of feeding. Regressions were run on time and $(\text{time})^2$ for best fit lines. Significance was determined by the F test and the Probability (p value) was reported.

The data for the second feeding experiment was analyzed further. Analysis of variance was used to determine if dry weight (percent of wet weight) values of any of the four groups were significantly different from the others. Linear Regression was used to fit the best line (straight or curved) to the effect of animal size on dry weight (percent of wet weight) and percent of wet weight gain analyses. The F test was used to determine significance.

The statistical analysis for both the Bacteria-Cell Association and Agglutination experiments was the same. Test group values were compared with saline group values (e.g., titer of test group minus titer of saline-injected group) over time. The best line was fit by linear regression and significance was determined by the F test.

The data were analyzed further, to test for variance caused by sampling alternately one and two days following the injection of S. montereyensis. Two lines were fit by linear regression, the first for the results obtained one day after injections and the second for results obtained two days following the injections. The significance was determined by the F test. The effect of animal size on the absolute values was regressed and tested for significance by the F test. The success of the injections was tested for each animal by regressing the mean injection value and the final injection value independently and testing for significance by the F test.

The health of the test animals were analyzed by regressing wet weight (percent of initial weight) with time and using the F test to determine significance.

The 95 percent confidence intervals of the agglutinin and bacteria-cell association time and health samples were calculated from the following formula:

$$95\ \%\ \text{Confidence Interval} = \bar{x} \pm t_{(\alpha/2)} \sqrt{s^2/n}$$

with n-1 degrees of freedom,

where, $\bar{x}$ = mean of the sample;
α = 0.05;
t = student t value determined from t tables;
s^2 = sample variance;
n = number of individuals sampled from each group.

RESULTS

Feeding

Feeding Experiment I (three potential foods supplied to groups of Styela montereyensis following a period of starvation) showed weight loss in all three groups during starvation (Fig. 4); in the group fed P. paradoxa a significant weight gain occurred after the initiation of feeding (F test; $p < 0.001$). The minimal weight gain observed in the group fed corn starch was insignificant (F test; $p > 0.10$). There was no significant weight change in the group fed Vibrio anguillarum (F test; $p > 0.10$). The statistical analyses were formed from data collected from two days after the initiation of feeding to the end of the test.

Feeding Experiment II was done to confirm that the weight gain of S. montereyensis was associated with the use of P. paradoxa for food. The three groups, starved control, fed 5×10^5 P. paradoxa cells ml^{-1}, and fed 5×10^6 P. paradoxa cells ml^{-1}, again showed a marked loss of weight during the starvation period (Fig. 5). The starved control group continued to lose weight significantly (F test; $p < 0.001$) during the "feeding" of filtered sea water. The group fed 5×10^6 P. paradoxa cells ml^{-1} gained weight (F test; $p < 0.001$) during the feeding period, reaffirming that weight gain in S. montereyensis is possible on a diet of P. paradoxa. The inconclusive weight change (F test; $p > 0.10$) exhibited by the group fed 5×10^5 P. paradoxa cells ml^{-1} indicates

Figure 4. Feeding Experiment I. Weight vs. Time. ↓ = initiation of feeding.

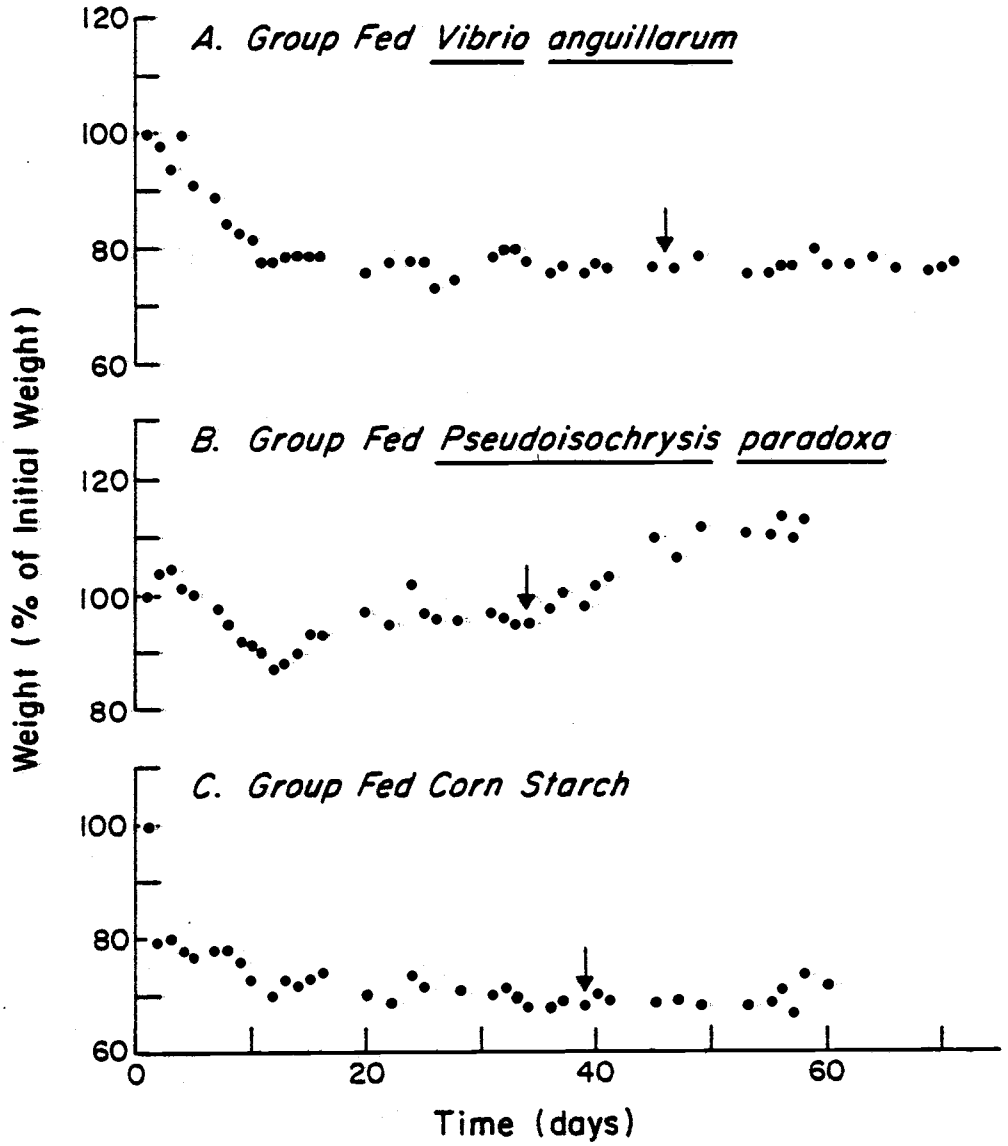
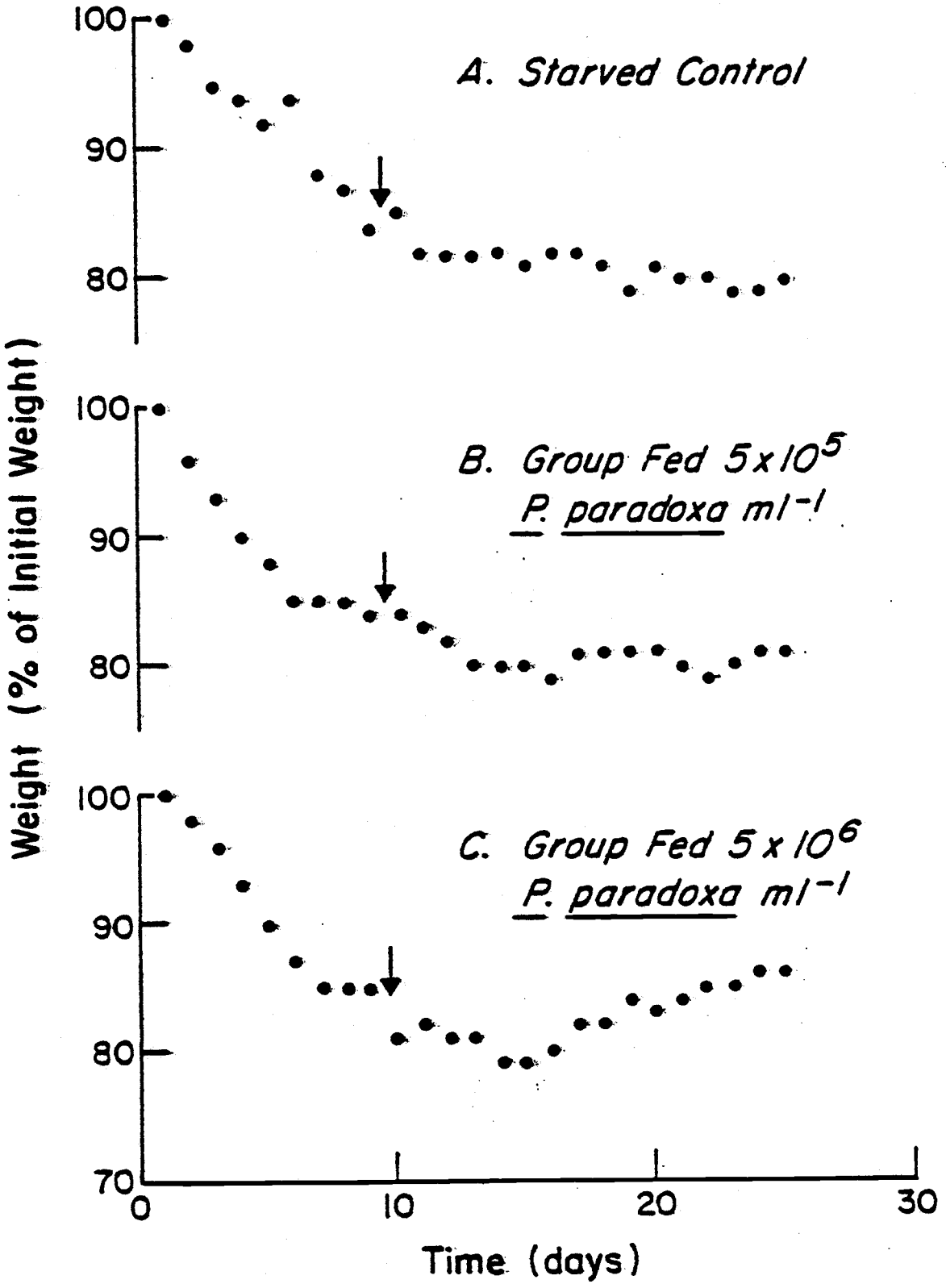


Figure 5. Feeding Experiment II. Weight vs. Time. ↓ = initiation of feeding.



that the maintenance ration for S. montereyensis under the conditions of the experiment was between 5 x 10^5 and 5 x 10^6 P. paradoxa at stock concentrations.

Dry weights were also taken for animals in Feeding Experiment II. This allowed me to determine whether growth potential may have been influenced by initial weights and to determine if increase in wet weight was due to a real growth or to fluid retention. Figure 6 shows the mean ($\bar{x}$) dry weight expressed as the percent of wet weight; there is no significant difference between the control group and the test groups (F test; $p > 0.10$). Thus the weight gain was not due to increased fluid retention alone. Statistical analysis of the dry weight expressed as a percent of wet weight compared to the animal's wet weight revealed significant correlation (F test; $p < 0.005$; Fig. 7). This indicates that the larger the animal the greater the proportion of water to tissue in S. montereyensis. The selection of the animals was random and the size distribution was essentially equal for this experiment. Also, the size range was not great enough to have any affect on the results reported above. To support this, the weight gain exhibited by the group fed 5 x 10^6 P. paradoxa cells ml^{-1} was insignificant as a function of initial wet weight (F test; $p > 0.10$; Appendix III).

Figure 6. Feeding Experiment II. Mean per cent dry weight for each group. Bars = ± 2 standard deviations; * - control was starved for the duration of the experiment; ** - control values obtained at the beginning of the experiment.

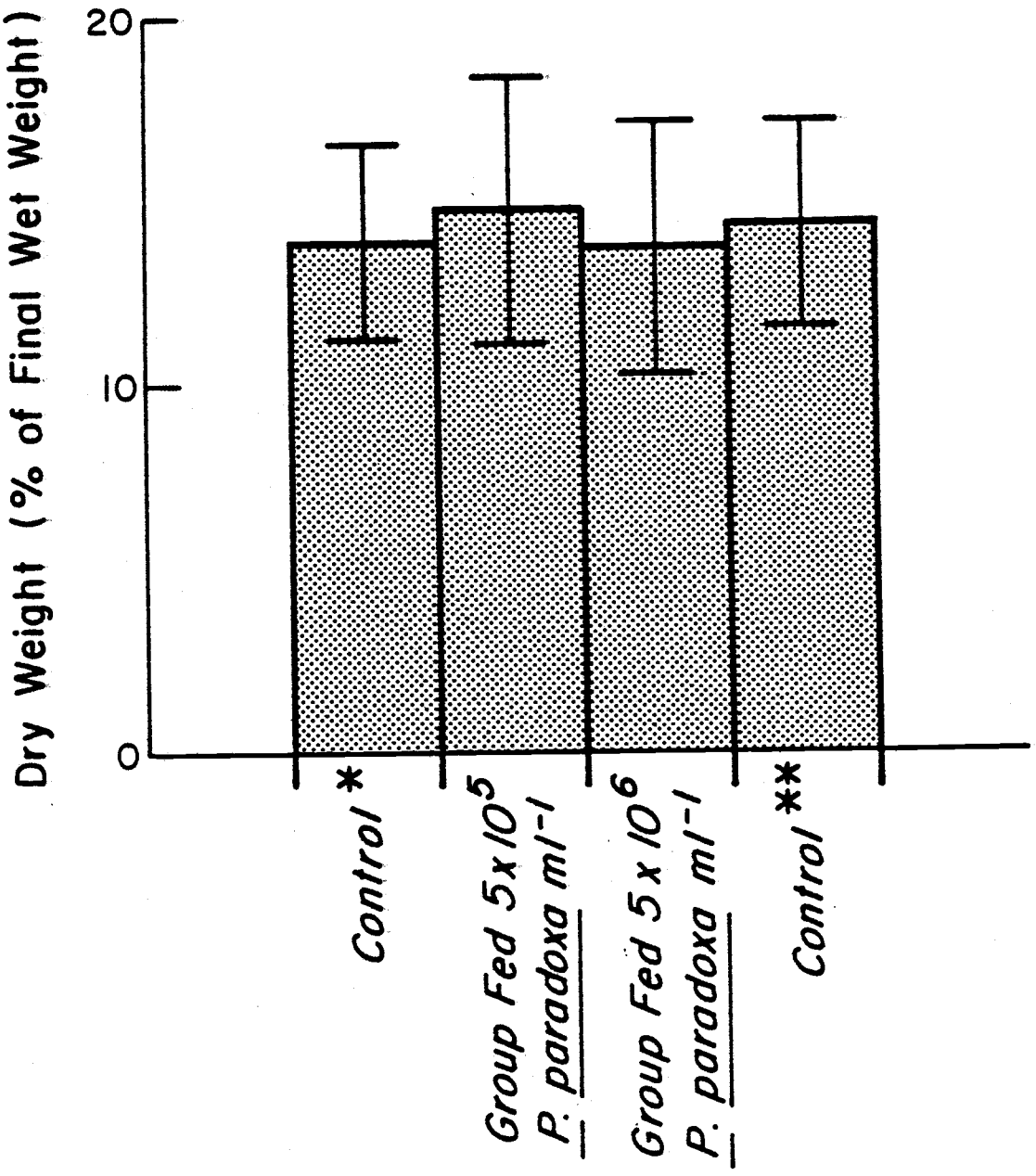
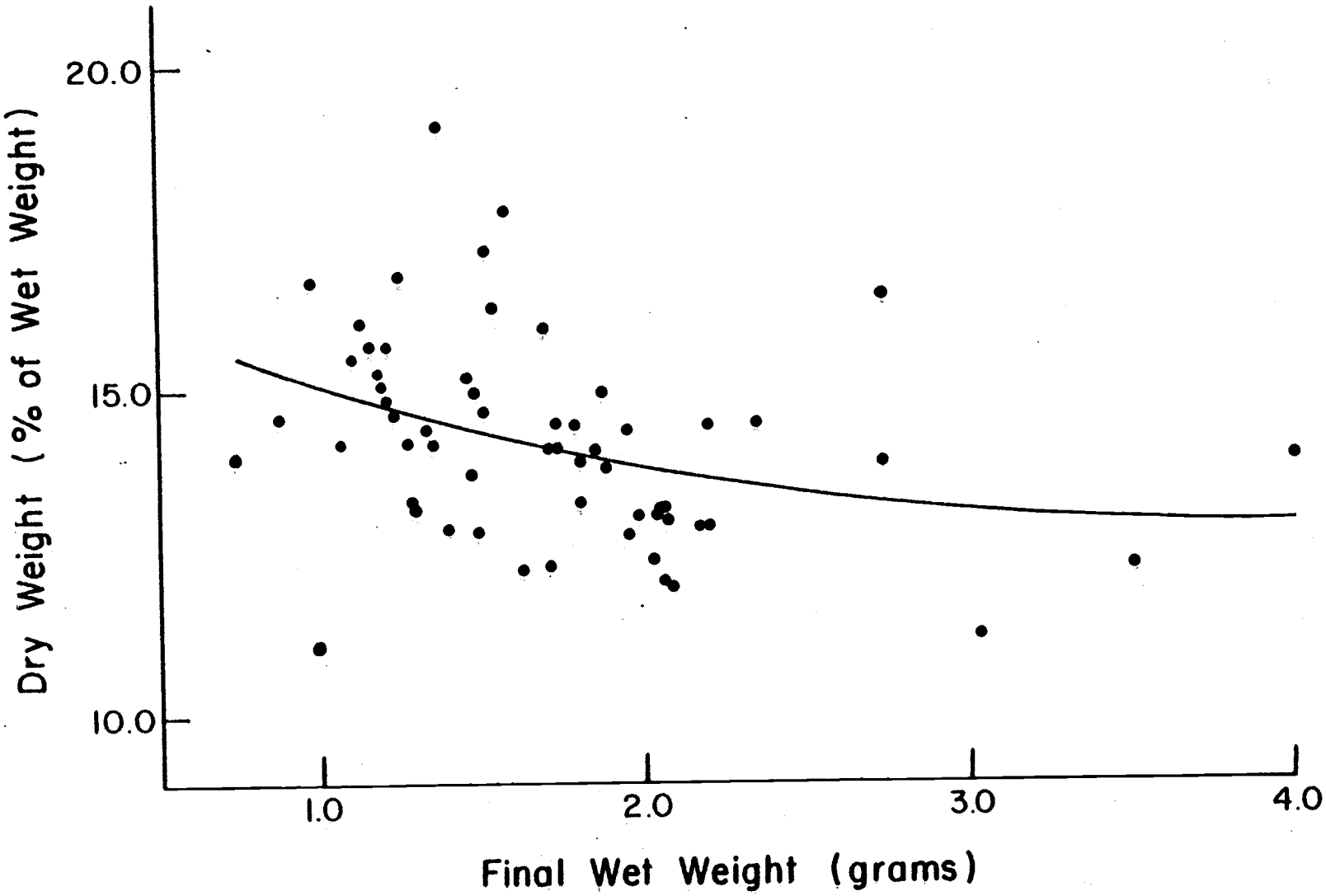


Figure 7. Feeding Experiment II. Individual percent dry weight vs. wet weight.



In Vitro Assays

Bactericidin

Styela montereyensis plasma had no detectable innate bactericidal activity, and none was induced.

Bacteria-Cell Association

The bacteria-cell association was not clearly altered as a result of repeated injections. Each test group showed a trend of increased association when compared with the respective saline-injected controls (Figs. 8 B&C and 9 B&C); however, only the Gram (+)-injected--Gram (+)-incubated and the Gram (-)-injected--Gram (-)-incubated groups showed significant increases (F test; $p < 0.10$). Each animal was injected one or two days prior to sampling. Analysis showed that this variable (time elapsed between injection and bleeding) did not influence the bacteria-cell association, except in the Gram (-)-injected--Gram (+)-incubated group (F test; $p < 0.05$; Appendix IV). Figures 8 A and 9 A illustrate that no significant difference exists between the uninjected controls and the saline-injected group (F test; $p > 0.10$).

Animals in this experiment were weighed, since such data were considered indicative of the health of the specimens. The control group increased in weight (F test; $p < 0.005$) while the saline and Gram (-)-injected groups lost weight after initial weight gain (F test; $p < 0.005$

Figure 8. Bacteria-Cell Association Test: Index vs. Time. Results for Gram (+)-incubated groups. —— = Test Group - saline-injected group. --- = Saline-injected group - saline-injected group. Bar = 95% confidence interval.

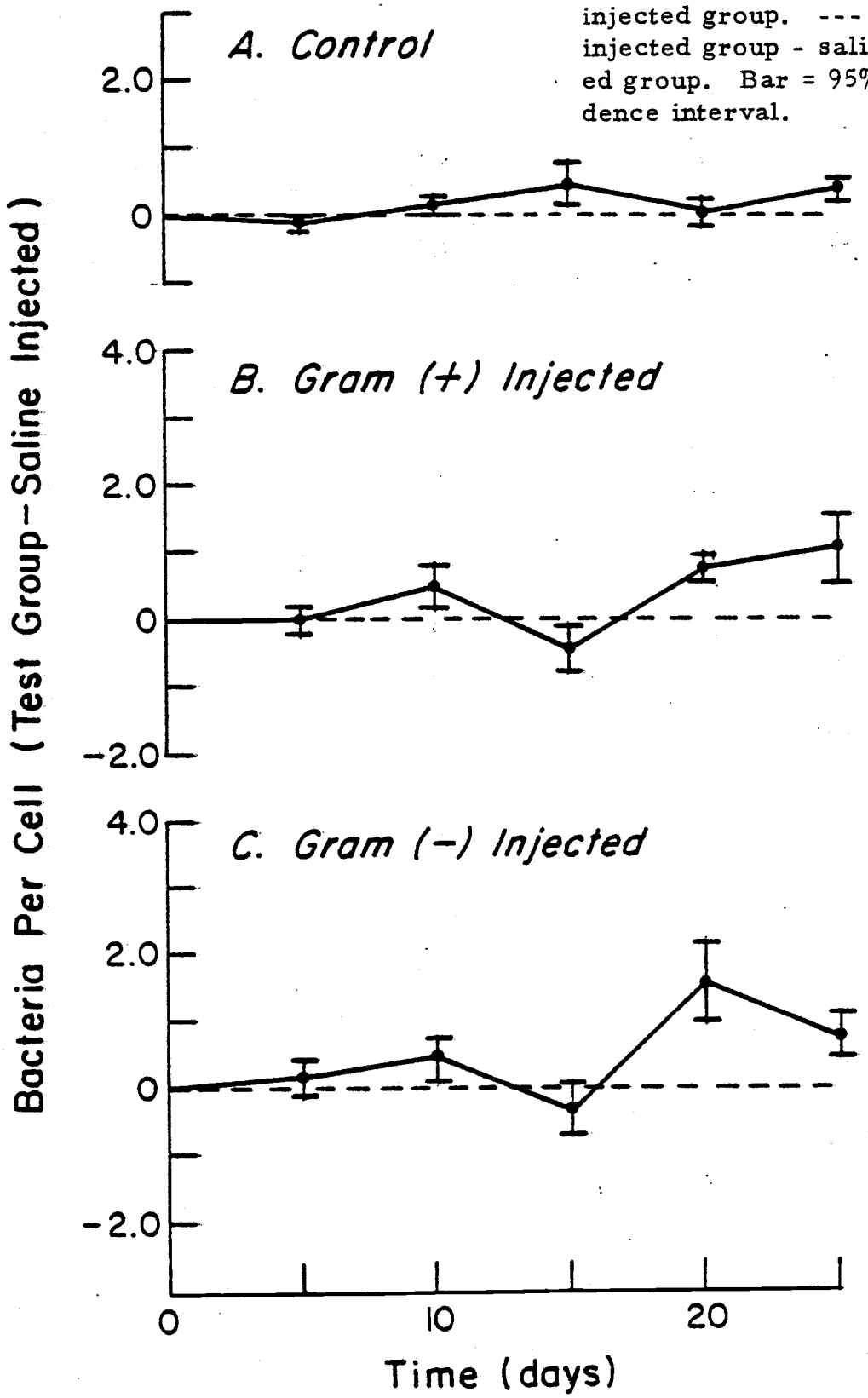
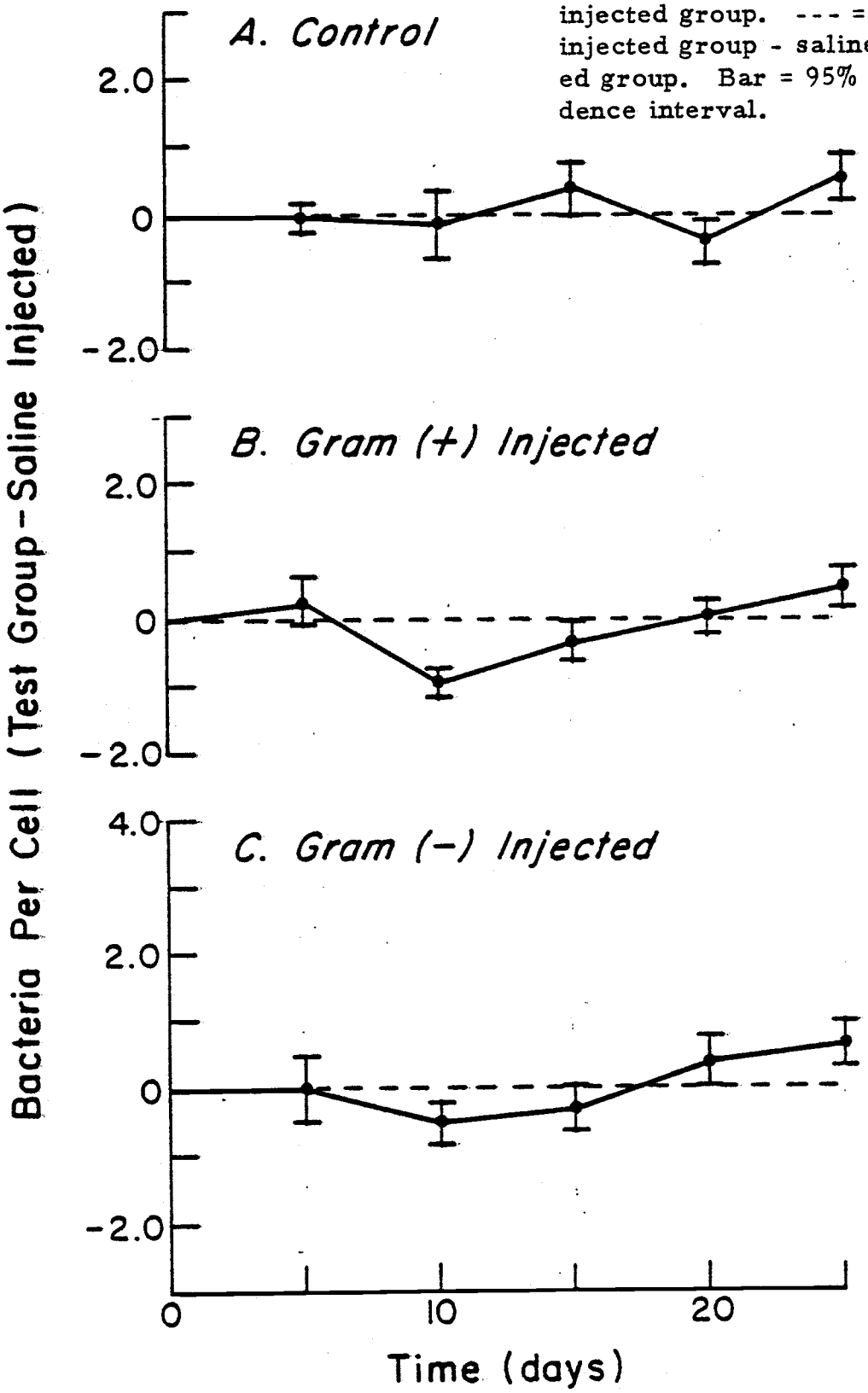


Figure 9. Bacteria-Cell Association Test: Index vs. Time. Results for Gram (-)-incubated groups. — = Test group - saline-injected group. --- = Saline-injected group - saline-injected group. Bar = 95% confidence interval.



and < 0.05, respectively; Fig. 10 A&C). The Gram (+)-injected group lost weight continually (F test; $p < 0.05$; Fig. 10 B).

In order to determine if animal "age" (weight) might affect the bacteria-cell association values, these two factors were subjected to regression analysis using the uninjected controls. Appendix V indicates that age did not affect the bacteria-cell association with either the Gram (+) or the Gram (-) bacteria (F test; $p > 0.10$). I found no correlation between the success of injections and the bacteria-cell association results (F test; $p > 0.10$ and $p > 0.10$; Appendix VI and VII). Such a correlation was sought in two ways: (1) the mean value of the success of injections (based on a scale of 1-3, with 1 being the most successful) was computed for each individual and compared to the association index for that individual, and (2) the success of the final injection prior to sampling was compared to the association values.

Agglutinin

Injections of Gram (+) and of Gram (-) bacteria both induced altered agglutinin titers. There were consistent trends for an early decrease (to the fifth day) and later increases in titer. Though titers were never significantly different from the values of the saline-injected animals, the trends were significantly different by regression analysis (F test; $p < 0.05$; Figs. 11 B&C and 12 B&C). The greatest increase, as well as the greatest fluctuations, were seen in the Gram (-)-injected

Figure 10. Bacteria-Cell Association Test: Weight vs. Time (a measure of health). Bar = 95% confidence interval.

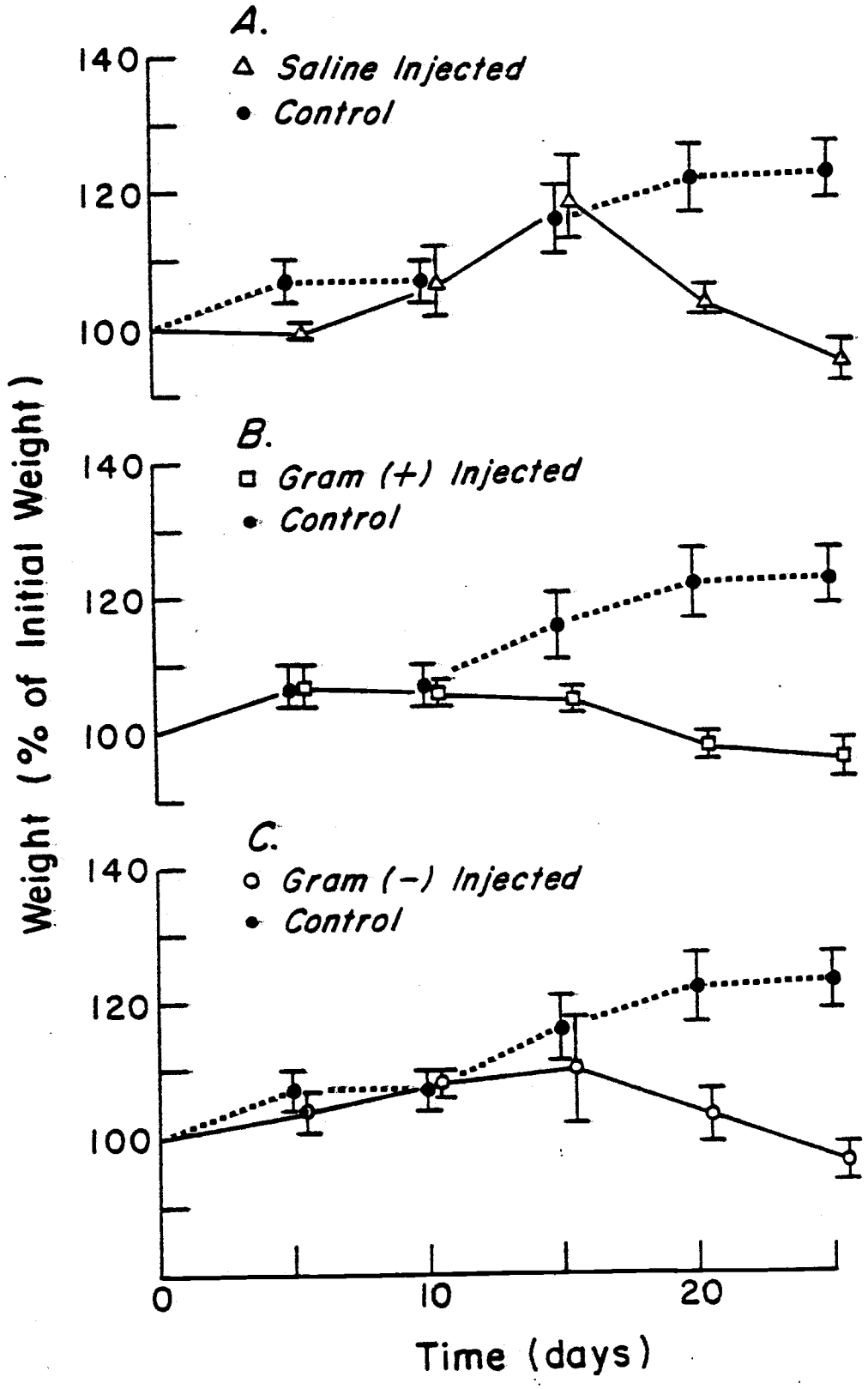


Figure 11. Agglutinin Experiment: Titer vs. Time. Results of incubations with Gram (+) bacteria. — = Test group - saline-injected group. --- = Saline-injected group - saline-injected group. Bar = 95% confidence interval.

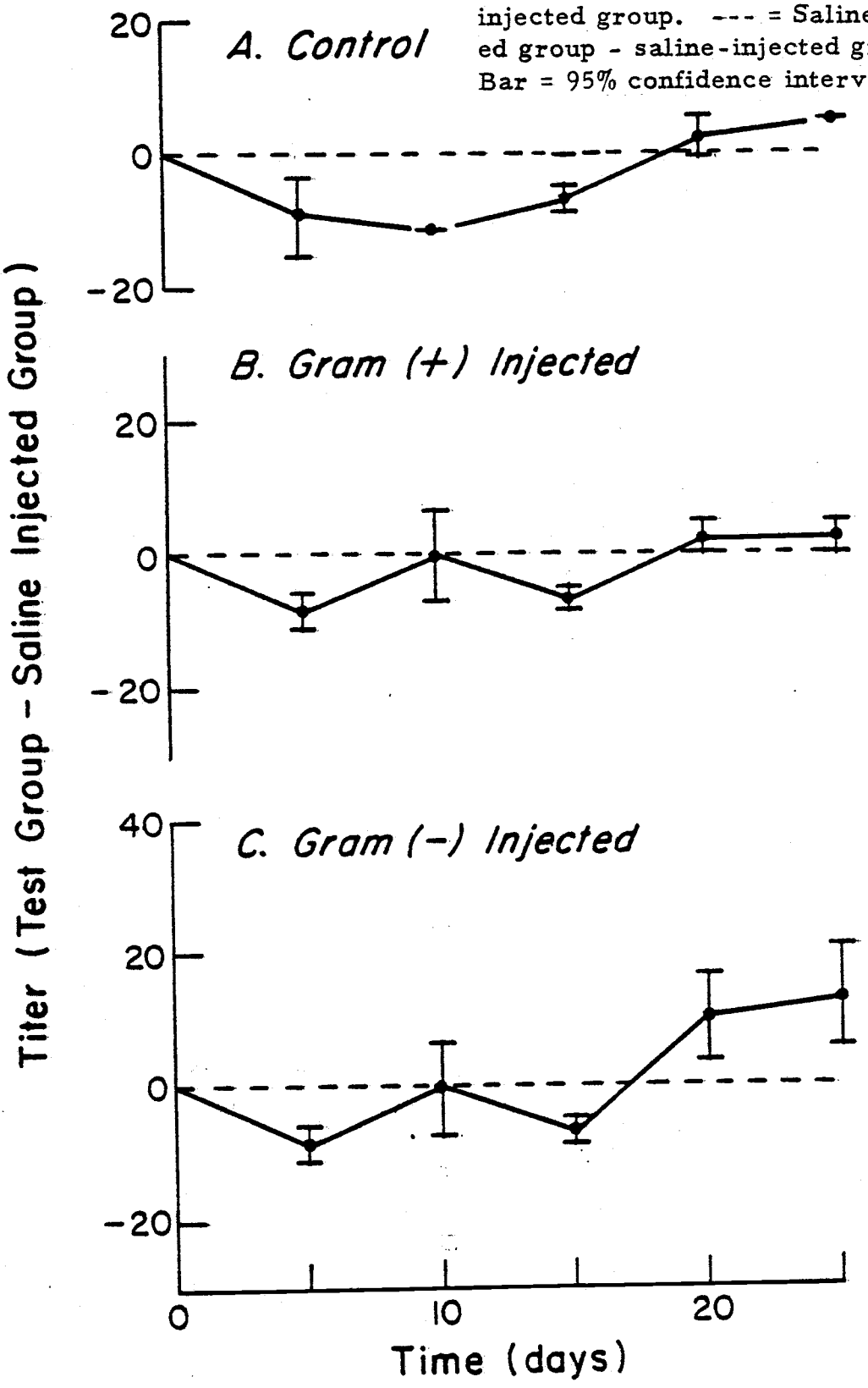
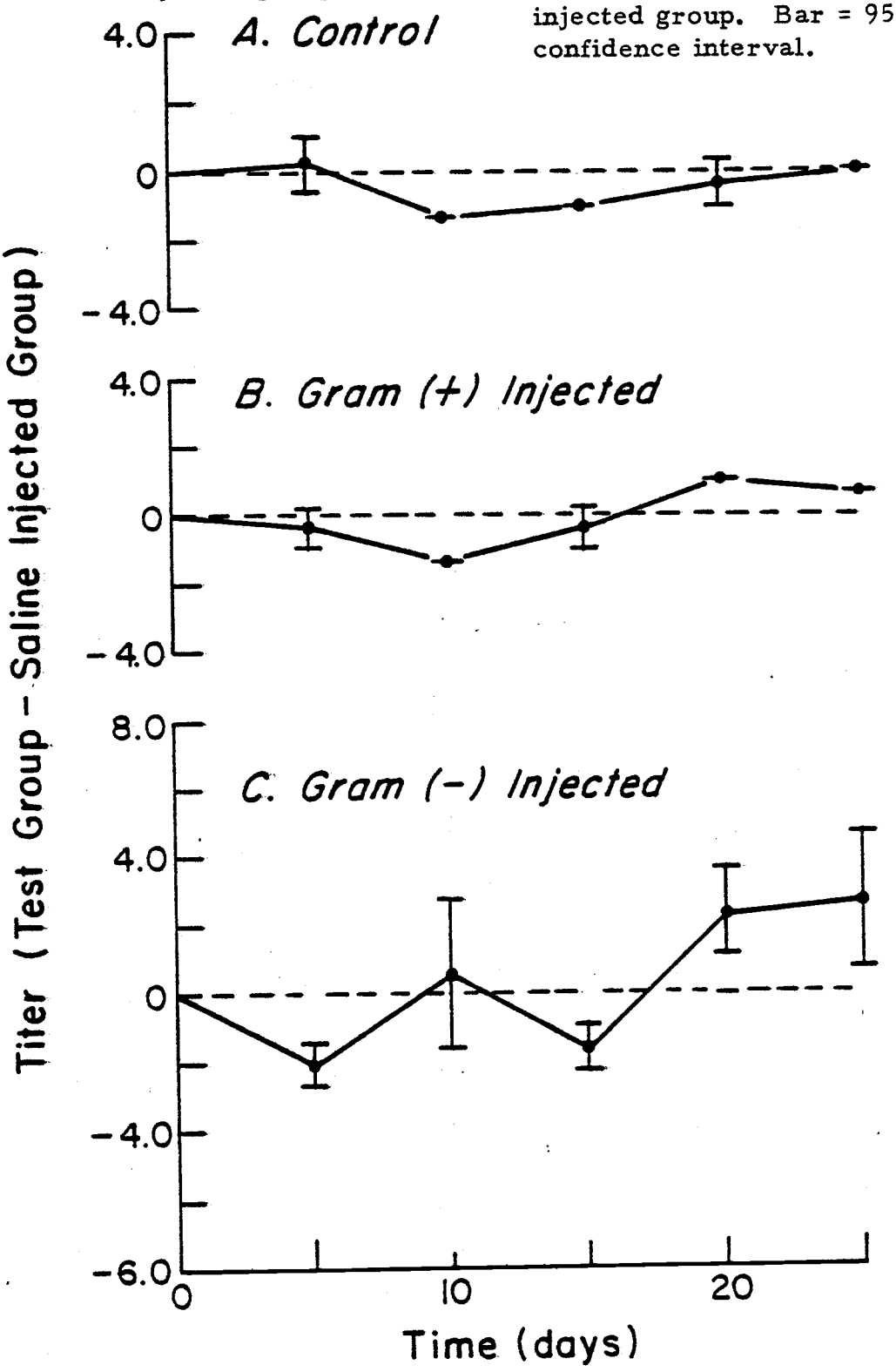


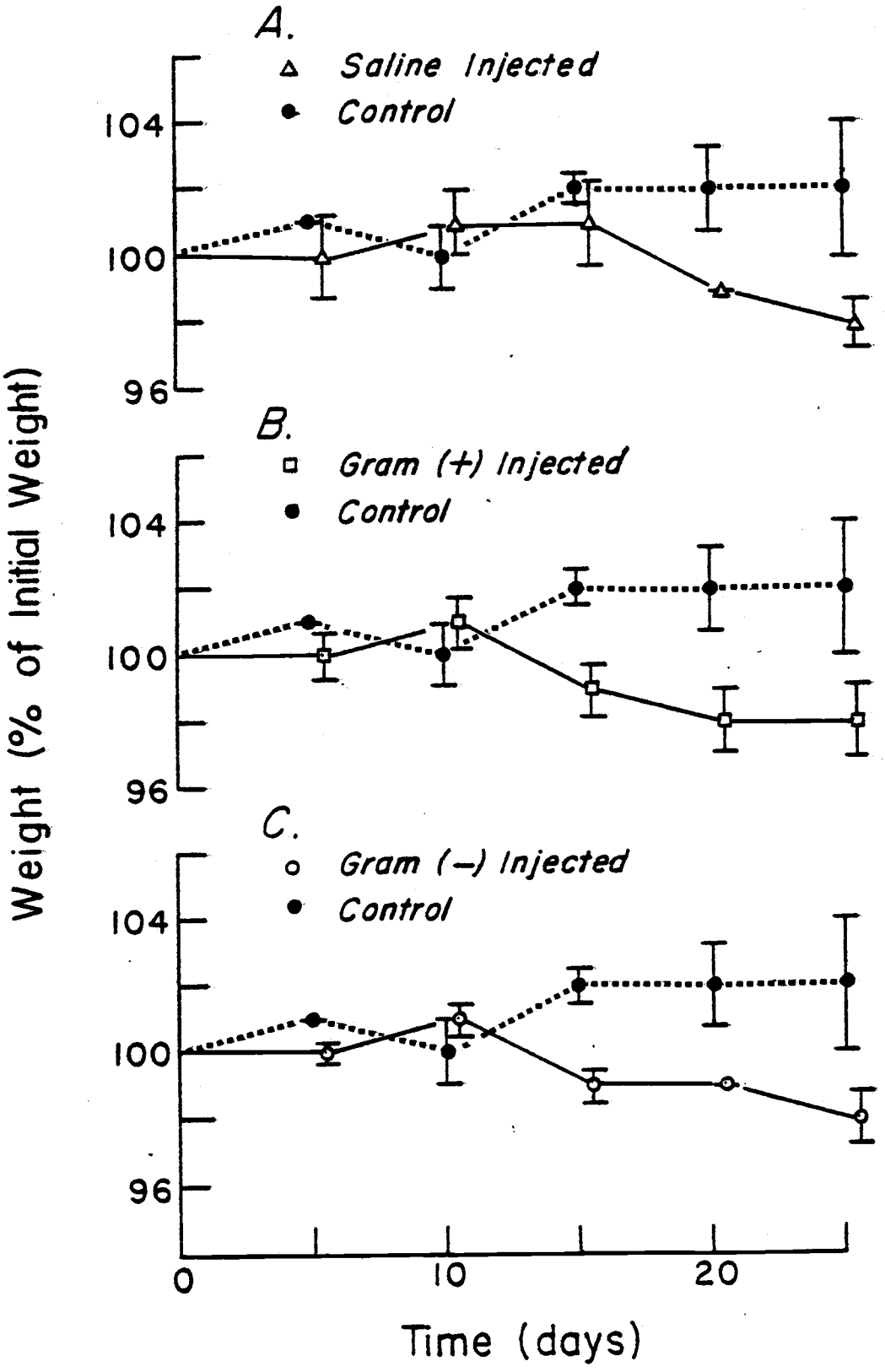
Figure 12. Agglutinin Experiment: Titer vs. Time. Results of incubations with Gram (-) bacteria. — = Test group - saline-injected group. --- = Saline-injected group - saline-injected group. Bar = 95% confidence interval.



animals and this held for incubation with Gram (+) and with Gram (-) bacteria. The control--Gram (+)-incubated group shows a significant increase (F test; $p < 0.005$; Fig. 11 A) while the control--Gram (-)-incubated group did not differ from the saline group (F test; $p > 0.10$; Fig. 12 A).

As in the bacteria-cell association assay the agglutinin data were also analyzed for possible correlations with the following factors: (1) the varying interval between the injection and bleeding, (2) the animals' weight, and (3) the mean and/or final injection success of each animal. Also, weights were followed as an indication of animal condition. The varying interval (last injection before bleeding) was without effect on agglutinin titer (F test; $p > 0.10$; Appendix VIII). Animals tended to lose weight in all injected groups while the uninjected control group did not appreciably change weight (Fig. 13). Animal weight had no significant positive or negative correlation with either Gram (+) or Gram (-)-incubated titers (F test; $p > 0.10$; Appendix IX). The success of injection was without significant affect on the agglutination titer (F test; $p > 0.10$; Appendix X and XI).

Figure 13. Agglutinin Experiment: Weight vs. Time. Bar = 95% confidence interval.



DISCUSSION

Feeding

The results from Feeding Experiment 1 indicate that Pseudoisochrysis paradoxa caused weight gain in Styela montereyensis. The corn starch and heat killed Vibrio anguillarum fed animals did not gain weight. Corn starch, as well as other artificial foods have been used in culturing bivalves (Gillespie et al., 1964; Haven, 1965; Castell and Trider, 1974). Corn starch caused weight gain in adult oysters but did not cause shell growth (Haven, 1965). I used slightly greater concentrations when feeding S. montereyensis than Haven used for feeding the oysters. However, the system I used may not have been as efficient for keeping the corn starch in suspension.

Studies on the food value of bacteria have been mostly with bivalves. These studies tested the growth of bivalves in the presence of bacteria and compared them to aseptic controls. Most often, inhibition of growth resulted (Davis, 1953; Guillard, 1959; Walne, 1963 and 1970). Hidu and Tubiash (1966) found that the use of some antibiotics inhibited pathogenic bacteria, allowing others to grow, providing some nutrition to the oysters. For this reason a heat killed bacterium such as V. anguillarum which has been washed free of toxic wastes was thought to be a possible food source for urochordates. The lack of growth in the

V. anguillarum fed group could be due to any combination of the following: (1) The bacteria did not carry nutritional value for S. montereyensis, (2) the concentration used was not great enough to elicit positive weight gain, and (3) the bacteria were not collected by the urochordate filtration apparatus. Ciona intestinalis has been shown to retain essentially 100% of the suspended particles ranging from 1 μm to 200 μm while feeding (Jorgensen, 1949 and 1952). Smaller particles are removed with much less efficiency. Since V. anguillarum (0.5 by 1.5 μm) is less than 1 μm wide, it is possible that S. montereyensis was not able to retain enough of the bacteria to feed effectively.

The use of flagellated algae with thin or no cell walls as food sources in bivalve culturing is well established (Walne, 1974; Ryther and Goldman, 1975). Croxall (1971) used unicellular algae while doing feeding studies on urochordates of New Zealand. Therefore, it came as no surprise that the S. montereyensis fed P. paradoxa gained weight. However, the concentrations used in Feeding Experiments 1 and 2 were considerably lower than reported optimal concentrations for bivalves. Walne (1974) found that for oysters the optimal food concentration was 50 cells μl^{-1} for unicellular algae in the size range of P. paradoxa. The maximum daily concentration of the total culture volume used for Group 2 of the second feeding experiment was only 8.6 cells μl^{-1}. Thus the optimal growth concentration for S. montereyensis when fed P. paradoxa may approach or exceed the optimal values

for bivalves. The maintenance ration for S. montereyensis under the conditions of this experiment appears to be 0.86 cells μl^{-1}. Since the calculations are based upon the total cells introduced to the tanks each day, and feeding occurred continuously, these values are higher than the actual concentration at any one time. Based on reported filtration rates (Goodbody, 1975), the volume of the test tanks, and total quantities of P. paradoxa supplied each day, the mean maintenance ration for S. montereyensis was maximally about 22,000 P. paradoxa cells $animal^{-1}$ day^{-1} with a mean animal wet weight of 1.56 grams.

Bactericidal Activity

The lack of bactericidal activity in S. montereyensis was surprising in light of the results of Johnson and Chapman (1970) on Ciona intestinalis. Plasma from C. intestinalis killed Gram (-) marine bacteria. If S. montereyensis had a similar bactericidin, Gram (-) bacterial growth should have been inhibited. Both the Gram (+) and Gram (-) bacteria grew equally well in the presence of S. montereyensis plasma. It is possible that the Gram (-) bacteria (and the Gram (+) bacteria) used in this study did not have cell surface characteristics which activated the bactericidal mechanism in S. montereyensis.

Differences in the techniques employed by Johnson and Chapman, and myself may explain the conflicting results. C. intestinalis plasma was incubated with the bacteria from 0.5 to 72 hours before growing

the cultures on agar plates. I required the plasma to diffuse through an agar medium to act upon plated bacteria. The agar may have interfered with any bactericidal activity. Preliminary studies showed that incubation of bacteria with S. montereyensis plasma for two hours did not inhibit growth. C. intestinalis plasma inhibited growth within two hours incubation time. The results suggest that either no natural bactericidin is present in S. montereyensis plasma, or that such a substance is slow acting or requires an effective concentration or some factor missing from my assays.

Bacteria-Cell Association

A completely acceptable in vitro test for quantitatively measuring phagocytosis rates has yet to be devised. Many techniques have been employed; most involve the use of monolayers of cells on glass slides or chambers covered with suspensions of the antigen (Prowse and Tait, 1969; McKay and Jenkin, 1970; Anderson et al., 1973). These are incubated for a specified period, fixed, stained, and counted. Several objections to this method exist. First, the cells which have adhered to glass have changed their morphology and quite possibly their physiological nature. Also, part of the cell surface is not exposed to the antigen, possibly preventing some of the cells' binding sites from acting on the antigen. Second, the cells are not in circulation and chance of contact with the suspended antigen is not uniform. Third, the method

of counting the phagocytozed antigen by visual inspection is tenuous; some of the particles which appear to be phagocytozed may actually be only superimposed upon or bound to the outside of the cell. Also, cytoplasmic granules and other organelles may be mistakenly identified as bacteria. In an effort to avoid the problems caused by cell adherence to glass, a method in which the cell and antigen mixture was suspended in a droplet of non-soluble, non-toxic substance (Ratcliffe and Rowley, 1975) was adopted for this study. Objections of the third category have been handled differently by various researchers. In this study, a process of washing away unbound bacteria was logistically impossible. Bacterial association with blood cells was determined by counting the number of bacteria located within the perimeter of individual cells. Nonspecific chance occurrence of bacteria and blood cells should be a constant factor in this procedure and therefore not significantly influence specific binding and phagocytosis counts.

Injections of bacteria beneath the tunic in S. montereyensis affect the rate of bacterial binding by blood cells in vitro. The results of the experiments do not show dramatic changes. In fact, individual samples are not significantly different from the previous or later samples of each group. However, the trends seen in two of the injected groups are significantly different from the saline control; the other two appear to show similar trends but these were not significant. The trend of increasing values began following initial decreases in binding by the

injected groups. Two biologically sound explanations may account for these results: first, binding capacity initially increased in the saline-injected group; then, associated with a decline in health, there was a decline in bacterial binding by the blood cells. This seems unlikely since the results of the control group were not different from the saline-injected group, yet the weight of the controls increased during the experiment, indicating a healthy condition. Also, the handling and injection conditions of the bacteria-injected groups were identical with the saline-injected group except for the bacterial content. All three groups showed weight loss with time, yet the bacteria-injected groups showed increased binding rates with respect to the saline-injected groups. Second, binding rates actually increased in the bacteria-injected groups after initial decreases. Any of three mechanisms may be responsible for the observed changes in bacterial binding. First, the number of binding sites on the cell surface may have increased. Second, the number of "phagocytic" cells in the blood may have increased with respect to other cells. Third, an opsonin may be present in the plasma at varying titers. If only the first mechanism is responsible, then a reduction of the cell binding sites occurred early in the experiment. This seems unlikely unless the sites are consumed during bacterial phagocytosis and are not replaced for several days. The second mechanism is also probably not responsible for observed changes, since the cell types did not vary significantly between the

groups of any one sampling period (unreported personal observations). Most likely the observed results were due to the presence of a natural opsonin, which was reduced in titer early due to the invasion by bacteria. I envisage a delayed synthesis for the replacement of consumed opsonin. The higher values obtained with respect to the saline group may have been due to an overproduction while returning the level to normal, or to a "B"-cell-like primary response similar to vertebrate adaptive responses. A more definitive explanation may become possible if longer experiments are run. The concept of a natural opsonin is not supported by the results of Fuke and Sugai (1972). Their work revealed that a natural opsonin did not exist in S. plicata.

Due to innate problems of the technique used in this experiment, it now appears that the accuracy could have been improved further by the following: (1) the use of cell monolayers; these are more consistant and more easily handled than cell suspensions in oil droplets. Also, the cells can easily be washed free of extraneous bacteria; (2) the cell types should be studied until consistancy in identification is obtained; and (3) if the bacteria were radioactively or fluorescently labeled, a more objective quantitative value could be obtained.

Agglutination

The agglutination test used in this study is an established technique for quantitatively measuring the clumping of particulate material

caused by animal serum (Carpenter, 1975). Preliminary tests showed that S. montereyensis plasma contained a low titer agglutinin for both the Gram (+) and Gram (-) bacteria used in this experiment. Though this is the first report of a bacterial agglutinin in urochordates, the presence of such an agglutinin is not surprising since urochordate blood agglutinates a variety of other particles as reported earlier.

The time studies on the agglutinins indicate trends of increased titers in all injected groups. As in the bacteria-cell association study, no one sample value is significantly different from another. The high increase in titers seen in the vertebrates when exposed to certain antigens were not seen in this study. The low initial titers and the high titers at the 20 and 25 day sample periods may indicate only a reestablishment of the normal level of the agglutinin following the initial lowering of titer by early injections. However, some of the titers of individuals in the bacteria-injected groups were higher than any observed in the uninjected control or saline-injected individuals. Thus, some individuals from the bacteria-injected groups may not have responded to bacterial injections while others did, increasing the variance within each group at each sampling. This wide variance may have resulted from unobserved differences in the success of individual injections. Though statistical evaluation showed no correlation between titer and injection success, it may be that the criterion for a successful injection was faulty.

Interestingly, the titers show an up and down effect for three of the four groups. This effect corresponds with the time between injection and sampling. The higher values were observed when the injections of bacteria were done two days before sampling. The lower values were observed one day after injections. Thus, it appears that the bacteria reduce the titer upon injection and it is not replenished until 24-48 hours later. Wright (1974) found that Ciona intestinalis' agglutinin titer was decreased in the presence of duck and human A RBCs but returned to normal levels within 24 hours. Since injections were continually made one or two days prior to sampling, it may be possible that large increases in titer may have occurred and injections been suspended several days prior to sampling.

Statistically, trends are indicated for the injected groups. No explanation can be given for the Gram (+)-incubated control groups' equally significant increasing trend in titer. Caution must be observed when considering the agglutinin results. The possible values obtainable were limited (e.g., 2, 4, 8, 16 and not 1, 3, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, etc.). Thus, in some cases differences between two animals may not have been observed as the titers were not different enough. Also, high variances could be caused by placing an animal's titer in a higher category from a nearly equivalent titer of another animal. The sample size of each group for each sample was small.

Specificity is not indicated by these results. The two bacteria-injected groups showed similar responses when incubated with either bacterium. This may result from agglutinin-binding with a receptor common to both bacteria; such a binding ligand may or may not be universally present in the surface membranes of microorganisms.

The Antigens

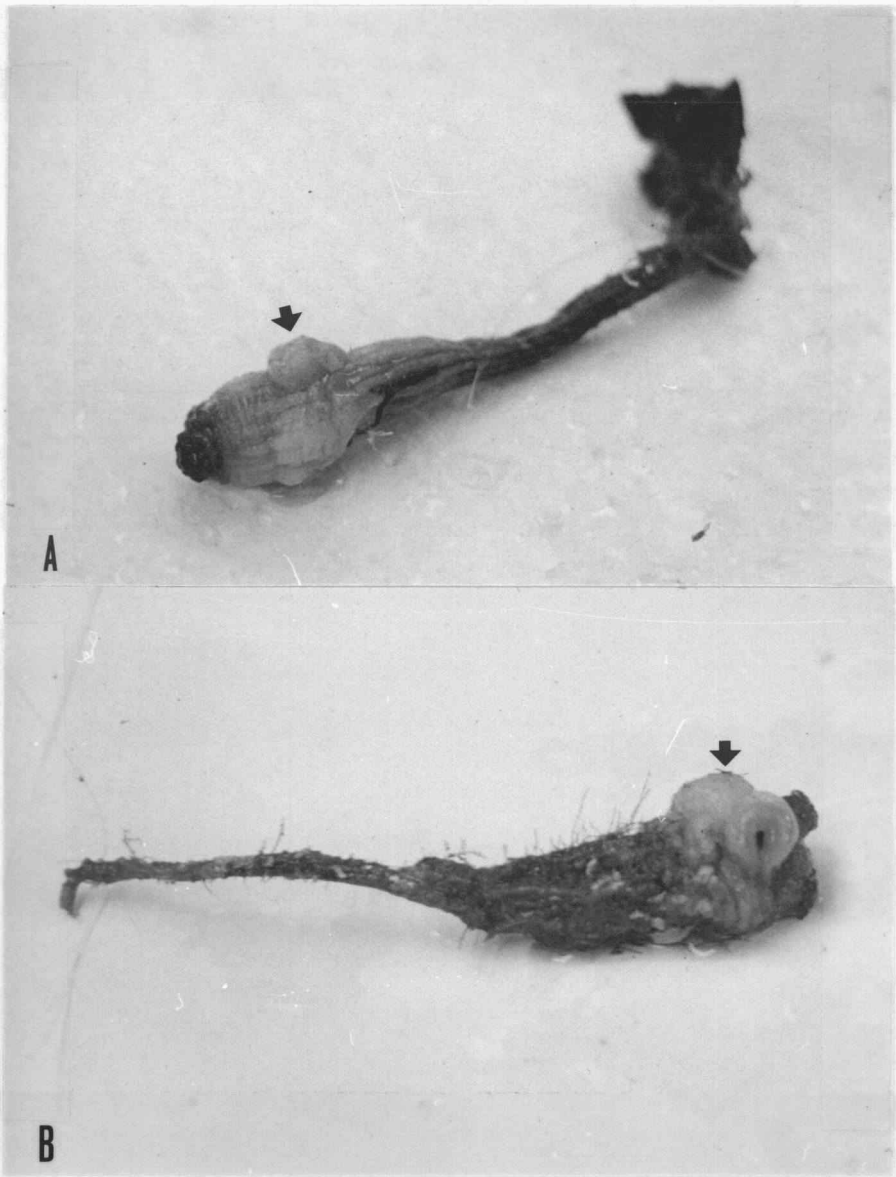
The two marine bacteria were chosen to serve as antigens for several reasons. Since the blood of S. montereyensis is very nearly isotonic with sea water, bacteria were needed which would not be affected by the high salt concentration. Chapman and Johnson (1970) felt that an animal may be more likely to develop a protective system against substances in which it is most often in contact. They supported this with results from C. intestinalis plasma indicating inhibition of growth for marine Gram (-) bacteria but not for the terrestrial Gram (-) bacterium Serratia marcescens.

Some characteristics of the two bacteria are found in Appendix I. The Gram (+) bacterium has characteristics of Staphylococcus aureus, a salt loving pathogen of many organisms. It is quite possible this bacterium was isolated as a contaminant since I have not been able to isolate a bacterium with similar characteristics from another S. montereyensis. The Gram (-) bacterium is much more difficult to classify but has many characteristics of the heterogeneous genus Flavobacterium.

Other General Conclusions

Injections caused the formation of nodules on the tunic of S. montereyensis (Fig. 14). The nodules generally formed by the next day

Figure 14. Photographs illustrating the presence of nodules on the tunic. The nodules were caused by the injections.
A. Styela with nodule one week old (arrow).
B. Styela with nodule two months old (arrow).



and persisted for the duration of the experiment. Thus animals injected every two days for 25 days contained 12 nodules. The nodules were apparently caused by the mechanical damage of insertion of the needle or by irritation of the injection media, since the salin-injected animals also developed nodules. Thomas (1931a, 1931b, and 1932) reported that injections of Bacterium tumefaciens and various chemicals into Ascidia mentula caused inflammation and nodule formation. Anderson et al., (1977) reported tunic lesions and inflammation in Halocynthia pyriformis probably caused by foreign material in the tunic. The tunic in these areas was heavily infiltrated with hemocytes and basophilic cells.

Healthy S. montereyensis can be maintained in the laboratory using basic culture conditions, and by feeding the tunicates the unicellular naked alga Pseudoisochrysis paradoxa at concentrations of at least 9.9×10^3 cells ml^{-1} for at least ten hours a day. The health of the animal does not seem to be correlated with the rate of bacterial binding by blood cells nor with the agglutinin titer.

The plasma of S. montereyensis does not contain a bactericidin which is active under the conditions used in this study. The plasma does have an agglutinin which is affected by the presence of bacteria in the blood. The trends reported in this study suggest that the titer may increase with time when stimulated by repeated injections of bacteria. Similar results were obtained with the binding rate of bacteria by blood

cells. The results indicate that the changes are caused by altering titers of an opsonin, though changes in cell surface binding sites cannot be discounted. Larger sample sizes, longer testing periods and fewer injections are needed to substantiate these results.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abbott, D. P. and J. V. Johnson. 1972. The ascidians Styela barnharti, S. plicata, S. clava, and S. montereyensis in California waters. So. Calif. Acad. Sci. Bull. 71(2):95-105.

Acton, R. T. 1970. Immunobiological and immunochemical studies of the oyster Crassostrea virginica. MSS Information Corporation, N. Y. pp. 1-134.

Anderson, R. S. and R. A. Good. 1975. Naturally-occurring hemagglutinin in a tunicate Halocynthia pyriformis. Biol. Bull. mar. biol. Lab., Woods Hole. 148:357-369.

_____. 1976. Opsonic involvement in phagocytosis by mollusk hemocytes. J. Invertebr. Pathol. 27:57-64.

Anderson, R. S., L. A. Jordan, and J. C. Harshbarger. 1977. Tunic abnormalities of the urochordate Halocynthia pyriformis. J. Invertebr. Pathol. 30:160-168.

Berrill, N. J. 1955. The origin of vertebrates. 257 pp. Oxford University Press, New York.

Bezkorovainy, A., G. F. Springer, and P. R. Desai. 1971. Physicochemical properties of the eel anti-human blood-group H(0) antibody. Biochemistry. 10:3761-3764.

Boyd, W. C., R. Brown, and L. G. Boyd. 1966. Agglutinins for human erythrocytes in mollusks. J. Immunol. 96:301-303.

Bretting, H. and L. Renwrantz. 1973. Untersuchungen von invertebraten des mittlemeeres aufihren gehalt an hamagglutinierenden substanzen. Z. immun.-Forsch. Bd. 145:242-249.

Cantacuzene, J. 1913. Recherches sur la production experimentale d'anticorps chez quelques invertebres marins. C. R. Soc. Biol. 74:111-112.

Carpenter, P. L. 1975. Immunology and Serology. pp. 146. 3rd Ed. W. B. Saunders Company, Philadelphia.

Carton, Y. 1974. Parentes entre les hemagglutinines naturelles d'echinoderms et les chaines des immunoglobulines de vertebres. Ann. Immunol. (Inst. Pasteur). 125C:731-745.

Castell, J.D. and D.J. Trider. 1974. Preliminary feeding trials using artificial diets to study the nutritional requirements of oysters (Crassostrea virginica). J. Fish. Res. Bd. Can. 31(1):95-99.

Cohen, E., A.W. Rowe, and F.C. Wissler. 1965. Heteroagglutinins of the horseshoe crab Limulus polyphemus. Life Sci. 4:2009-2016.

Croxall, J.P. 1971. The Digestive System, Feeding and Ecology of some New Zealand Ascidians. Ph.D. Thesis, Aukland, New Zealand. From, the physiology of ascidians. By I. Goodbody. 1975. In Advances in Marine Biology. 12:1-149. Academic Press, N.Y.

Davis, H.C. 1953. On food and feeding of larvae of the American oyster Crassostrea virginica. Biol. Bull. mar. biol. Lab., Woods Hole. 104:334-350.

DeIoannes, A.E. and W.H. Hildemann. 1975. Preliminary structural characterization of Pacific hagfish immunoglobulin. In Immunologic Phylogeny, Advances in Experimental Medicine and Biology. 64:151-160.

Finstad, C.L., G.W. Litman, J. Finstad, and R.A. Good. 1972. The evolution of the immune response. XIII. The characterization of purified erythrocyte agglutinins from two invertebrate species. J. Immunol. 108:1704-1711.

Fuke, M.T. and T. Sugai. 1972. Studies on the naturally occurring hemagglutinin in the coelomic fluid of an ascidian. Biol. Bull. mar. biol. Lab., Woods Hole. 143:140-149.

Fulton, J.F. 1920. The blood of Ascidia atra (Lesueur); with special reference to pigmentation and phagocytosis. Acta. Zoolog. 1:381-433.

Galigher, A.E. and E.N. Kozloff. 1971. Essentials of Practical Microtechnique. 2nd Ed. Lea and Febiger, Philadelphia.

Gillespie, L., R.M. Ingle, and W.K. Havens. 1964. Glucose nutrition and longevity in oysters. Quarterly Journal of the Florida Academy of Science. 27(4):279-288.

Goodbody, I. 1975. The physiology of ascidians. In Advances in Marine Biology. 12:1-149. Academic Press, N. Y.

Guillard, R. R. L. 1959. Further evidence of the destruction of bivalve larvae by bacteria. Biol. Bull. mar. biol. Lab., Woods Hole. 117:258-266.

Hammarstrom, S., and E. A. Kabat. 1969. Purification and characterization of a blood-group A reactive hemagglutinin from the snail Helix pomatia and a study of its combining site. Biochemistry. 8:2696-2705.

Harisdangkul, V., E. A. Kabat, R. J. McDonough, and M. M. Sigel. 1972a. A protein in normal nurse shark serum which reacts specifically with fructosans. I. Purification and immunochemical characterization. J. Immunol. 108:1244-1258.

_____. 1972b. A protein in normal nurse shark serum which reacts specifically with fructosans. II. Physicochemical studies. J. Immunol. 108:1259-1270.

Haven, D. S. 1965. Supplemental feeding of oysters with starch. Chesapeake Science. 6:43-51.

Hidu, H. and H. S. Tubiash. 1966. A bacterial basis for the growth of antibiotic-treated bivalve larvae. Proc. Nat. Shellfish. Ass. 54:25-40.

Jenkin, C. R. and D. Rowley. 1970. Immunity in invertebrates. The purification of a haemagglutinin to rat and rabbit erythrocytes from the haemolymph of the murray mussel (Velesunio ambiguus). Aust. J. exp. Biol. med. Sci. 48:129-137.

Johnson, P. T. and F. A. Chapman. 1970. Comparative studies on the in vitro response of bacteria to invertebrate body fluids. II. Aplysia californica (Sea hare) and Ciona intestinalis (Tunicate). J. Invertebr. Pathol. 16:259-267.

Jorgensen, C. B. 1949. Feeding rates of sponges, lamellibranchs and ascidians. Nature, Lond. 163:912.

_____. 1952. On the relation between water transport and food requirements in some marine filter feeding invertebrates. Biol. Bull. mar. biol. Lab., Woods Hole. 103:356-363.

Li, M.F. and C. Flemming. 1967. Hemagglutinins from oyster hemolymph. Can. J. Zool. 45:1225-1234.

Litman, G.W. 1976. Physical properties of immunoglobulins of lower species: A comparison with immunoglobulins of mammals. pp. 239-275. In Comparative Immunology. Ed. J.J. Marchalonis. Halsted Press, a division of John Wiley & Sons, N.Y.

Litman, G.W., D. Frommel, J. Finstad, J. Howell, B.A. Pollara, and R.A. Good. 1970. The evolution of the immune response. VIII. Structural studies of the lamprey immunoglobulin. J. Immunol. 105:1278-1285.

Marchalonis, J.J. and G.M. Edelman. 1968a. Phylogenetic origins of antibody structure. III. Antibodies in the primary immune response of the sea lamprey (Petromyzon marinus). J. exp. Med. 127:891-914.

_____. 1968b. Isolation and characterization of a hemagglutinin from Limulus polyphemus. J. Mol. Biol. 32:453-465.

Marchalonis, J.J. and J.K. Weltman. 1971. Relatedness among proteins: A new method of estimation and its application to immunoglobulins. Comp. Biochem. Physiol. 38(B):609-625.

Mathiessen, G.C. and R.C. Toner. 1966. Possible methods of improving the shellfish industry of Martha's Vineyard, Duke's County, Massachusetts. Edgartown, A publication by the Marine Research Foundation Inc. Part I, sec. 1, p. 5-6.

McKay, D. and C.R. Jenkin. 1970. Immunity in the invertebrates. The role of serum factors in phagocytosis of erythrocytes by hemocytes of the freshwater crayfish (Parachaerups bicarinatus). Aust. J. exp. Biol. med. Sci. 48:139-150.

Pauley, G.B., G.A. Granger, and S.M. Krassner. 1971. Characterization of a natural agglutinin present in the hemolymph of the California sea hare, Aplysia californica. J. Invertebr. Pathol. 18:207-218.

Prowse, R.H. and N.N. Tait. 1969. In vitro phagocytosis by amoebocytes from the hemolymph of Helix aspersa (Muller). I. Evidence for opsonic factor(s) in serum. Immunol. 17:437-443.

Ratcliffe, N.A. and A.F. Rowley. 1975. Cellular defense reactions of insect hemocytes in vitro: phagocytosis in a new suspension culture system. J. Invertebr. Pathol. 26:225-233.

Ryther, J.H. and J.C. Goldman. 1975. Microbes as food in mariculture. Annu. Rev. Microbiol. 29:429-443.

Spotte, S.H. 1970. Fish and invertebrate culture: Water management in closed systems. 145 pp. Wiley-Interscience, a division of John Wiley & Sons, Inc. N.Y.

Springer, G.F. and P.R. Desai. 1971. Monosaccharides as specific precipitinogens of eel anti-human blood-group H(O) antibody. Biochemistry. 10:3749-3761.

Thomas, J.A. 1931a. Sur les reactions de la tunique d'Ascidia mentula Mull. a l'inoculation de Bacterium tumefaciens Sm. C.R. Soc. Biol. (Paris). 108:694-696.

_____. 1931b. Reactions de deux invertebres: Ascidia mentula Mull. et Nereis diversicolor O.F.M. a l'inoculation de substances a properietes cancerigenes. C.R. Soc. Biol. (Paris). 108:667-669.

_____. 1932. Contribution a l'etude des reactions de quelques invertebres a l'inoculation de substances a properietes cancerigenes et de Bacterium tumefaciens Sm. et Town. Ann. Inst. Pasteur (Paris). 49:234-274.

Tripp, M.R. 1966. Hemagglutinin in the blood of the oyster (Crassostrea virginica). J. Invertebr. Pathol. 8:478-484.

Tyler, A. 1946. Natural hemagglutinins in the body fluids and seminal fluids of various invertebrates. Biol. Bull. mar. biol. Lab., Woods Hole. 90:213-219.

Uhlenbruck, G., G.I. Pardoe, O. Prokop, and I. Ishiyama. 1972. The serological specificity of snail agglutinins (protectins). Anim. Blood Grps. Biochem. Genet. 3:125-139.

Walne, P.R. 1963. Observations on the food value of seven species of algae to the larvae of Ostrea edulis. I. Feeding experiments. J. mar. biol. Ass. U.K. 43:767-784.

Walne, P.R. 1970. Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera Ostrea, Crassostrea, Merceneria, and Mytilus. Fishery Invest. Ser. II. 26:1-62.

_____. 1974. Culture of bivalve molluscs: 50 years' experience at Conwy. 132 pp. West Byfleet: Fishing News (For the Buckland Foundation).

Wright, R.K. 1974. Protochordate immunity. I. Primary immune response of the tunicate Ciona intestinalis to vertebrate erythrocytes. J. Invertebr. Pathol. 24:29-36.

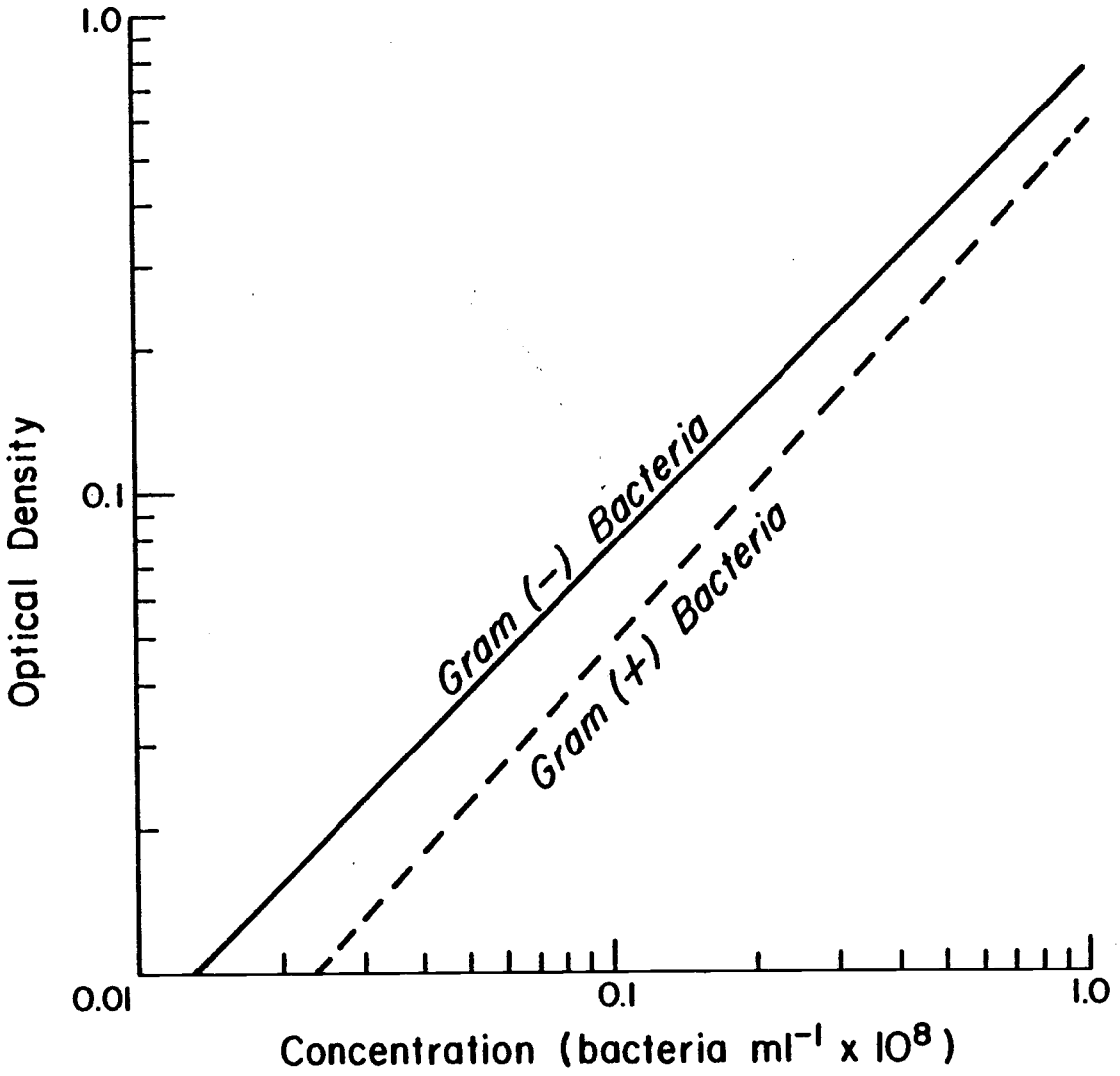
Wright, R.K. and E.L. Cooper. 1975. Immunological maturation in the tunicate Ciona intestinalis. Amer. Zool. 15:21-27.

APPENDICES

Appendix I. Characteristics of the Gram(+) and Gram(-) bacteria used as antigens in the bactericidin, agglutinin, and bacteria-cell association studies.
* - Gram(+) temperature range tested; ** - Gram(-) not tested below 10°C.

Test or characteristic	Gram(+) bacterium	Gram (-) bacterium
Colonies:		
Color	orange on BHI ivory on TSA	off white to yellow
Appearance	circular, entire, convex	smooth, continuous
Incubation temp.	10-37° C *	10-25° C **
Texture	sticky, elastic	
Gram stain	+	-
Form	coccus	rod
Acid fast stain	-	-
Spore stain	-	-
Capsule stain		-
Catalase test	+	+
Fermentation on Hugh-Liefson medium	+	+
Indophenol oxidase test		-
Indole test	-	-
Methyl red test		-
Voges-Proskauer test		-
Citrate-utilization test		-
Nitrate reduction		-
Lactose		-
Sucrose		-
Manitol aerobic	+ with gas	-
Manitol anaerobic	+ with gas	-

Appendix II. The standard plot for turbidimetrically measuring the concentration of the Gram(+) and Gram(-) bacteria.



Appendix III. Feeding Experiment II. The percent weight gained by the group fed 5×10^6 *P. paradoxa* cells ml^{-1}.

Animal rank by W_I	Initial weight (W_I) in grams[a]	Final weight (W_F) in grams	Weight gain (g) $W_F - W_I$	Percent weight gained[a] $\frac{\text{Weight gain}}{\text{Initial weight}} \times 100$
1	1.30	1.29	-0.01	-0.77
2	1.34	1.34	0.00	0.00
3	1.37	1.38	0.01	0.73
4	1.38	1.67	0.29	20.10
5	1.57	1.60	0.03	1.91
6	1.64	2.02	0.38	23.20
7	1.67	1.93	0.26	15.60
8	1.71	2.25	0.54	31.60
9	1.72	1.82	0.10	5.81
10	1.81	1.91	0.10	5.52
11	2.10	2.01	-0.09	-4.29
12	2.11	2.14	0.03	1.42
13	2.16	2.48	0.32	14.80
14	2.25	2.50	0.25	11.10
15	2.74	3.09	0.35	12.70

F test = 0.6047 with 1, 13 degrees of freedom

[a]Statistical analysis (linear regression) based on percent weight gained vs. initial weight.

Appendix IV. Bacteria-cell association experiment. Change in bacteria cell^{-1} association with time. Linear regression tested by F test for an interval effect (1 or 2 days between injection and sampling) and no interval effect.

Group	Incub. bac. + or -	Bacteria cell^{-1} index (test group - saline group) Time in days (interval time - injection to sampling) 5 (1)	10 (2)	15 (1)	20 (2)	25 (1)	F test, degrees of freedom ($\frac{\text{No interval effect}}{\text{Interval effect}}$)
Control	(+)	-0.06	0.00	-0.44	0.07	0.30	
		0.07	-0.06	1.06	-0.23	-0.28	
		0.23	-0.03	0.54	-0.39	0.53	$\frac{1.9635\ (1,\ 27)}{0.7131\ (1,\ 26)}$
		-0.27	0.72	-0.14	0.66	0.73	
		-0.39	0.45	0.65	0.77	0.80	
		-0.16	-0.02	1.51	-0.40		
$\bar{x} \pm$ sd		-0.10	0.18	0.53	0.08	0.42	
		$\pm$.226	$\pm$.328	$\pm$.727	$\pm$.522	$\pm$.435	
Control	(-)	-0.84	-0.07	1.00	0.55	0.03	
		0.55	-1.01	1.19	-0.03	-0.13	
		-0.24	0.45	-0.28	-0.08	0.43	$\frac{0.5930\ (1,\ 27)}{1.249\ (1,\ 26)}$
		-0.24	-2.06	0.48	-1.48	1.91	
		-0.23	1.85	-1.46	-1.15	0.89	
		0.74	0.25	1.49	0.22		
$\bar{x} \pm$ sd		-0.04	-0.10	0.40	-0.33	0.63	
		$\pm$.585	$\pm$1.33	$\pm$1.10	$\pm$.803	$\pm$.819	

(Continued on next page)

Appendix IV. (continued)

Group	Incub. bac. + or -	Bacteria cell^{-1} index (test group - saline group) Time in days (interval time - injection to sampling) 5 (1)	10 (2)	15 (1)	20 (2)	25 (1)	F test, degrees of freedom (No interval effect / Interval effect)
(+) Injected	(+)	0.49	1.57	0.66	0.64	2.05	
		0.56	0.49	0.14	0.37	3.31	
		0.08	0.29	-0.49	0.62	0.10	4.1625 (1, 27)
		-0.18	1.33	-1.65	1.73	-0.22	1.4364 (1, 26)
		-0.76	-0.44	-0.70	0.70	1.36	
		-0.09	-0.26	-0.35		-0.10	
$\bar{x} \pm$ sd		0.003 ±.484	0.50 ±.817	-0.39 ±.785	0.81 ±.528	1.08 ±1.42	
(+) Injected	(-)	0.56	-1.06	-0.40	0.14	0.60	
		-0.26	-0.79	0.91	-0.69	0.15	
		1.84	-0.93	0.20	-0.21	0.24	5.4005 (1, 27)
		-1.12	0.77	-1.27	1.02	1.64	0.9830 (1, 26)
		0.61	-0.06	-0.62	0.10	1.14	
		-0.09	-1.76	-0.57		-0.60	
$\bar{x} \pm$ sd		0.29 ±.991	-0.90 ±.548	-0.29 ±.754	0.07 ±.625	0.53 ±.789	

(Continued on next page)

Appendix IV. (continued)

Group	Incub. bac. + or -	Bacteria cell^{-1} index (test group - saline group) Time in days (interval time - injection to sampling) 5 (1)	10 (2)	15 (1)	20 (2)	25 (1)	F test, degrees of freedom ($\frac{\text{No interval effect}}{\text{Interval effect}}$)
(-) Injected	(+)	0.02	0.36	1.02	0.46	1.64	
		1.21	1.60	0.80	3.90	1.09	
		-0.02	0.83	-0.49	2.82	1.77	$\frac{2.3897\ (1, 25)}{4.3230\ (1, 24)}$
		-0.39	-0.54	-1.12	0.45	-0.28	
			0.11	-0.74	1.68	0.75	
				-1.26	0.18	-0.03	
$\bar{x} \pm$ sd		0.21	0.47	-0.30	1.58	0.82	
		$\pm$.695	$\pm$.801	$\pm$.977	$\pm$1.511	$\pm$.847	
(-) Injected	(-)	-0.50	0.24	-0.34	0.84	1.26	
		1.52	-1.52	0.53	0.94	1.94	
		-1.23	-0.96	-0.01	1.44	0.41	$\frac{3.3675\ (1, 22)}{0.3794\ (1, 21)}$
		0.59	-0.45	-1.30	-0.56	0.01	
			0.03		-0.64	-0.14	
						0.48	
$\bar{x} \pm$ sd		0.01	-0.53	-0.28	0.40	0.66	
		$\pm$1.21	$\pm$.721	$\pm$.769	$\pm$.945	$\pm$.794	

Appendix V. Bacteria-Cell Association Experiment. Index vs. wet weight of the control (uninjected) group.

Individual weight (g)	Bacteria cell^{-1} ($\bar{x}$)	
	Gram(+) incubation	Gram(-) incubation
3.64	1.74	0.92
3.78	1.43	1.73
3.92	0.72	2.07
3.93	1.34	2.06
4.05	0.70	3.65
4.05	0.64	2.71
4.05	1.20	1.33
4.13	1.55	3.37
4.16	1.78	2.19
4.21	0.62	1.17
4.38	0.68	3.97
4.40	1.18	2.85
4.48	0.84	2.06
4.54	0.95	3.04
4.63	2.53	2.09
4.86	0.68	2.62
4.93	1.15	2.95
4.97	1.85	1.25
4.99	1.63	3.21
5.21	1.15	5.57
5.30	3.05	3.56
5.30	0.69	2.32
5.33	2.64	0.91
5.85	1.42	1.66
6.04	1.05	1.46
6.55	1.85	2.85
7.21	0.85	2.37
7.48	3.50	3.86
12.05	0.67	4.17
F test	0.1609	1.368
Degrees of freedom	1, 27	1, 27

APPENDIX VI

BACTERIA-CELL ASSOCIATION EXPERIMENT

Appendix VIa. Bacteria cell^{-1} vs. the success of the injections (mean value) for the saline-injected individuals.

Injection success (mean value)	Bacteria cell^{-1} ($\bar{x}$) Gram(+) incubation	Gram(-) incubation
1.00	0.28	2.57
1.00	1.12	-
1.00	0.35	2.10
1.00	0.46	3.83
1.00	0.22	4.01
1.20	1.48	3.54
1.25	2.63	3.05
1.33	3.59	3.19
1.38	-	2.05
1.50	0.55	1.59
1.54	1.41	0.91
1.54	0.38	1.28
1.60	-	3.00
1.60	1.59	2.72
1.63	1.39	2.54
1.67	0.71	2.48
1.67	0.69	2.15
1.69	0.37	1.17
1.67	0.16	0.72
1.75	1.29	2.32
1.75	2.64	2.08
1.77	1.56	1.72
1.80	0.39	5.41
1.80	0.95	2.77
1.85	1.55	1.99
2.00	1.68	2.47
2.13	2.17	2.19
2.20	0.66	1.88
2.20	0.33	2.61
2.40	1.29	2.40
F test	0.2768	0.1885
Degrees of freedom	1, 25	1, 26

Appendix VIb. Bacteria cell^{-1} vs. the success of the injection (mean value) for the Gram(+)-injected individuals.

Injection success (mean value)	Bacteria cell^{-1} ($\bar{x}$)	
	Gram(+) incubation	Gram(-) incubation
1.00	1.02	2.21
1.00	0.44	1.96
1.00	2.13	3.28
1.10	1.78	2.50
1.20	2.27	2.66
1.20	0.99	2.79
1.20	1.72	2.54
1.20	1.74	-
1.20	2.81	3.42
1.25	1.50	2.17
1.30	1.70	2.19
1.31	2.95	1.90
1.40	0.80	0.70
1.50	1.64	1.80
1.54	2.26	2.44
1.62	0.68	2.94
1.63	1.29	1.75
1.67	1.60	2.86
1.67	0.93	1.18
1.69	4.21	1.45
1.69	1.00	1.54
1.80	0.26	3.66
1.88	2.65	1.97
2.00	1.67	2.04
2.00	1.19	2.93
2.00	2.03	4.49
2.00	0.34	1.10
2.33	1.19	4.14
2.33	0.35	2.91
2.40	1.45	1.71
F test	0.2692	0.0281
Degrees of freedom	1, 27	1, 26

Appendix VIc. Bacteria cell^{-1} vs. the success of the injection (mean value) for the Gram(-)-injected individuals.

Injection success (mean value)	Bacteria cell^{-1} ($\bar{x}$) Gram(+) incubation	Gram(-) incubation
1.00	1.13	1.80
1.00	2.32	3.82
1.00	0.81	3.75
1.38	0.73	1.07
1.38	1.54	-
1.40	1.53	2.76
1.40	1.36	2.14
1.46	1.99	3.24
1.46	0.62	1.31
1.46	0.87	1.78
1.50	4.80	2.24
1.50	1.53	1.84
1.54	2.67	1.71
1.60	1.06	3.96
1.60	2.30	2.30
1.60	0.16	3.27
1.60	2.76	1.76
1.60	1.26	0.93
1.63	2.79	2.90
1.67	1.09	1.07
1.69	2.54	2.56
1.77	1.65	1.16
1.88	3.01	2.03
1.88	1.25	2.36
2.00	3.22	2.74
2.13	0.87	-
2.33	0.72	2.89
F test	0.0194	0.1526
Degrees of freedom	1, 24	1, 22

APPENDIX VII

BACTERIA-CELL ASSOCIATION EXPERIMENT

Appendix VIIa. Bacteria cell^{-1} vs. the success of the final injection for the saline-injected individuals.

Injection success (final injection)	Bacteria cell^{-1} ($\bar{x}$) Gram(+) incubation	Gram(-) incubation
1.00	0.28	2.57
1.00	0.71	2.48
1.00	3.59	3.19
1.00	0.69	2.15
1.00	1.12	-
1.00	0.35	2.10
1.00	0.39	5.41
1.00	0.46	3.83
1.00	1.48	3.54
1.00	0.22	4.01
1.00	1.29	2.32
1.00	-	2.05
1.00	1.39	2.54
1.00	2.64	2.08
1.00	-	3.00
1.00	1.59	2.72
1.00	0.55	1.59
1.00	0.16	0.72
1.00	1.55	1.99
1.00	0.38	1.28
1.00	0.37	1.17
2.00	1.29	2.40
2.00	2.17	2.19
2.00	0.66	1.88
3.00	0.33	2.61
3.00	2.63	3.05
3.00	0.95	2.77
3.00	1.68	2.47
3.00	1.56	1.72
3.00	1.40	0.91
F test	1.3805	0.0139
Degrees of freedom	1, 25	1, 26

Appendix VIIb. Bacteria cell^{-1} vs. the success of the final injection for the Gram(+)-injected individuals.

Injection success (final injection)	Bacteria cell^{-1} ($\bar{x}$) Gram(+) incubation	Gram(-) incubation
1.00	0.93	1.18
1.00	1.02	2.21
1.00	0.99	2.79
1.00	0.26	3.66
1.00	0.44	1.96
1.00	2.13	3.28
1.00	1.50	2.17
1.00	0.34	1.10
1.00	1.64	1.80
1.00	1.72	2.54
1.00	1.45	1.71
1.00	1.70	2.19
1.00	2.81	3.42
1.00	1.78	2.50
1.00	2.95	1.90
1.00	1.00	1.54
1.00	0.68	2.94
1.00	2.26	2.44
1.00	0.80	0.70
2.00	1.67	2.04
2.00	1.74	-
2.00	4.21	1.45
3.00	1.60	2.86
3.00	1.19	4.14
3.00	0.35	2.91
3.00	2.27	2.66
3.00	1.19	2.93
3.00	2.03	4.49
3.00	2.65	1.97
3.00	1.29	1.75
F test	4.9672	1.4581
Degrees of freedom	1, 27	1, 26

Appendix VIIc. Bacteria cell^{-1} vs. the success of the final injection for the Gram(-)-injected individuals.

Injection success (final injection)	Bacteria cell^{-1} ($\bar{x}$)	
	Gram(+) incubation	Gram(-) incubation
1.00	1.13	1.80
1.00	2.32	3.82
1.00	1.09	1.07
1.00	0.72	2.89
1.00	1.06	3.96
1.00	1.53	2.76
1.00	0.16	3.27
1.00	0.81	3.75
1.00	2.79	2.90
1.00	0.73	1.07
1.00	1.36	2.14
1.00	4.80	2.24
1.00	2.54	2.56
1.00	1.99	3.24
1.00	2.67	1.71
1.00	0.62	1.31
2.00	2.30	2.20
2.00	1.25	2.36
2.00	1.53	1.84
2.00	2.76	1.76
2.00	1.65	1.16
2.00	0.87	1.78
3.00	3.01	2.03
3.00	1.54	0.87
3.00	3.22	2.74
3.00	1.26	0.93
F test	0.0979	1.5010
Degrees of freedom	1, 23	1, 23

Appendix VIII. Agglutinin Experiment. Change in agglutinin titer with time. Linear regression tested by F test for an interval effect (1 or 2 days between injection and sampling) and no interval effect.

Group	Incub. bac. + or -	Agglutination titer (test animal - $\bar{x}$ for the saline-injected group) Time in days (interval time - injection to sampling) 5 (1)	10 (2)	15 (1)	20 (2)	25 (1)	F test, degrees of freedom ($\frac{\text{No interval effect}}{\text{Interval effect}}$)
Control	(+)	-14.67	-10.67	- 8.00	8.00	5.33	
		- 2.67	-10.67	- 4.00	0.00	5.33	$\frac{18.1044\ (1,\ 9)}{0.0189\ (1,\ 8)}$
					0.00		
$\bar{x} \pm$ sd		- 8.67	-10.67	- 6.00	2.67	5.33	
		± 8.49	± 0	± 2.83	± 4.62	± 0	
Control	(-)	- 0.67	- 1.33	- 1.00	1.00	- 1.33	
		1.33	- 1.33	- 1.00	- 1.00	0.67	$\frac{2.7283\ (1,\ 10)}{0.7266\ (1,\ 9)}$
					- 1.00	0.67	
$\bar{x} \pm$ sd		0.33	- 1.33	- 1.00	- 0.33	0.003	
		± 1.41	± 0	± 0	± 1.15	± 1.15	
+ Injected	(+)	-10.67	13.33	- 8.00	0.00	5.33	
		-10.67	-10.67	- 8.00	8.00	5.33	$\frac{5.0447\ (1,\ 13)}{2.1565\ (1,\ 12)}$
		- 2.67	- 2.67	- 4.00	0.00	- 2.67	
$\bar{x} \pm$ sd		- 8.00	- 0.003	- 6.67	2.67	2.67	
		± 4.62	±12.22	± 2.31	± 4.62	± 4.62	

(Continued on next page)

Appendix VIII. (continued)

Group	Incub. bac. + or -	Agglutination titer (test animal - $\bar{x}$ for the saline-injected group) Time in days (interval time - injection to sampling) 5 (1)	10 (2)	15 (1)	20 (2)	25 (1)	F test, degrees of freedom (No interval effect / Interval effect)
+ Injected	(-)	1.33	- 1.33	1.00	1.00	0.67	
		- 0.67	- 1.33	- 1.00	1.00	0.67	4.6801 (1, 13) / 0.2997 (1, 12)
		- 0.67	- 1.33	- 1.00	1.00	0.67	
$\bar{x} \pm$ sd		- 0.33	- 1.33	- 0.33	1.00	0.67	
		± 1.15	± 0	± 1.15	± 0	± 0	
- Injected	(+)	-10.67	- 2.67	- 8.00	8.00	- 2.67	
		-10.67	13.33	- 8.00	0.00	21.33	8.3484 (1, 13) / 1.1933 (1, 12)
		- 2.67	-10.67	- 4.00	24.00	21.33	
$\bar{x} \pm$ sd		- 8.00	- 0.003	- 6.67	10.67	13.33	
		± 4.62	±12.22	± 2.31	±12.22	±13.86	
- Injected	(-)	- 2.67	0.67	- 3.00	1.00	- 1.33	
		- 2.67	4.67	- 1.00	1.00	4.67	5.1017 (1, 13) / 1.8060 (1, 12)
		- 0.67	- 3.33	- 1.00	5.00	4.67	
$\bar{x} \pm$ sd		- 2.00	0.67	- 1.67	2.33	2.67	
		± 1.15	± 4.00	± 1.15	± 2.31	± 3.46	

Appendix IX. Agglutinin Experiment. Agglutination titer vs. the wet weight of the control individuals.

Weight (g)	Agglutinin titer	
	Gram (+) incubation	Gram(-) incubation
4.26	-	4
5.10	8	2
5.31	8	2
6.49	4	2
8.03	8	2
8.12	16	4
9.30	8	2
10.52	4	2
10.75	16	4
11.19	16	2
12.20	16	4
13.53	8	2
F test	0.0059	1.4698
Degrees of freedom	1, 9	1, 10

APPENDIX X

AGGLUTININ EXPERIMENT

Appendix Xa. Agglutination titer vs. the success of the mean injection value for the saline-injected individuals.

Injection success (mean value)	Agglutination titer	
	Gram(+) incubation	Gram(-) incubation
1.00	32	4
1.00	8	2
1.15	8	4
1.20	8	2
1.50	8	4
1.60	8	2
1.63	8	4
1.67	8	2
1.67	16	2
1.80	32	8
1.85	8	2
1.92	16	4
2.00	16	4
F test	0.1021	0.5083
Degrees of freedom	1, 10	1, 10

Appendix Xb. Agglutination titer vs. the success of the mean injection value for the Gram(+)-injected individuals.

Injection success (mean value)	Agglutination titer	
	Gram(+) incubation	Gram(-) incubation
1.00	8	4
1.00	8	2
1.00	16	2
1.25	4	4
1.25	4	2
1.33	16	2
1.38	16	4
1.40	32	2
1.50	8	2
1.50	16	4
1.50	8	4
1.62	16	4
1.80	8	2
1.83	8	4
2.10	8	4
F test	0.0127	0.1935
Degrees of freedom	1, 12	1, 12

Appendix Xc. Agglutination titer vs. the success of the mean injection value for the Gram(-)-injected individuals.

Injection success (mean value)	Agglutination titer	
	Gram(+) incubation	Gram(-) incubation
1.00	8	0
1.00	8	0
1.00	16	2
1.00	32	8
1.23	32	8
1.25	4	0
1.50	8	2
1.54	8	2
1.54	32	8
1.60	32	8
1.80	16	4
1.80	8	4
2.20	16	4
2.20	8	0
2.13	4	2
F test	1.0895	0.1847
Degrees of freedom	1, 12	1, 12

APPENDIX XI

AGGLUTINATION EXPERIMENT

Appendix XIa. Agglutination titer vs. the success of the final injection value for the saline-injected individuals.

Injection success (mean value)	Agglutination titer	
	Gram(+) incubation	Gram(-) incubation
1.00	8	2
1.00	16	2
1.00	32	4
1.00	8	2
1.00	16	4
1.00	32	8
1.00	8	4
1.00	8	2
1.00	8	2
1.00	8	4
1.00	8	4
1.00	16	4
2.00	8	2
F test	0.0006	0.7090
Degrees of freedom	1, 10	1, 10

Appendix XIb. Agglutination titer vs. the success of the final injection value for the Gram(+)-injected individuals.

Injection success (final injection)	Agglutination titer	
	Gram(+) incubation	Gram(-) incubation
1.00	8	4
1.00	8	2
1.00	32	2
1.00	16	2
1.00	4	4
1.00	4	2
1.00	8	4
1.00	16	4
2.00	16	2
2.00	8	4
2.00	16	4
2.00	16	4
3.00	8	2
3.00	8	2
3.00	8	4
F test	0.2933	1.1409
Degrees of freedom	1, 12	1, 12

Appendix XIc. Agglutination titer vs. the success of the final injection value for the Gram(-)-injected individuals.

Injection success (final injection)	Agglutination titer	
	Gram(+) incubation	Gram(-) incubation
1.00	8	0
1.00	8	0
1.00	16	2
1.00	4	0
1.00	8	2
1.00	32	8
1.00	8	2
1.00	32	8
2.00	32	8
2.00	4	2
2.00	8	4
2.00	32	8
3.00	16	4
3.00	8	0
3.00	16	4
F test	0.0015	0.1498
Degrees of freedom	1, 12	1, 12