RICE UNIVERSITY

RESPONSES OF THE FALSE LIMPET,
SIPHONARIA PECTINATA (GASTROPODA, PULMONATA)
TO OSMOTIC STRESS

by

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Thesis Director's signature: [Signature]

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Should the strongest arm endeavor
The limpet from its rock to sever,
'Tis seen its loved support to clasp
With such tenacity of grasp
We wonder that such strength should dwell
In such a small and simple shell

---Wordsworth
ABSTRACT

Investigations were conducted on the littoral euryhaline mollusc, *Siphonaria pectinata* Linnaeus, which inhabits the granite jetties near Galveston, Texas. Animals were subjected to a wide range of salinities, and the $\Delta t_1$ and sodium concentrations of the body fluids were measured under conditions of osmotic stress. Experiments were designed to investigate the possibility of some control mechanism being operative to explain this animal's tolerance to osmotic stress. Experimental evidence is presented that fluctuations in the osmotic concentrations of the body fluids are largely due to water movements. Evidence is further presented that fluctuations in the osmotic concentration of the hemolymph may be effectively reduced during stress for periods of up to 48 hours. This is done by physical exertion on the part of the animal, resulting in a tight binding of the mantle fringe and outer margin on the shell to the substrate. Such exertion effectively results in greatly reduced exposure of the soft parts of the animal to the stress environment. The end result is a resistance to swelling or shrinking in hypoosmotic and hyperosmotic situations, respectively.

The results suggest that dessication in air and exposure to hyperosmotic salinities of water yield similar end results in terms of deleterious effects on the body fluid concentration of the animal. A discussion is presented of the possible significance of the fact that the animals
seem more tolerant to hyperosmotic than to hypoosmotic conditions. The significance of the animal's apparent mechanical osmoregulatory ability is also discussed.
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INTRODUCTION

*Siphonaria pectinata* Linnaeus is a brackish water pulmonate mollusc which inhabits the intertidal zone on several of the man-made granite jetties along the Texas Gulf Coast. The animal has been referred to as the "false limpet." The word limpet, like the word "snail," has no scientific basis and is merely the name of a shape—a simple, conical shell which is found in those gastropods that habitually live in one place, clinging to the substratum. The assumption has often been made that the ancestral gastropod had a simple cap-shaped shell like that shown by the common limpets; however, in none of these forms is the shape of the shell primary (Fretter and Graham, 1962). The shell has been secondarily derived from a helicoid spiral shell which may still be seen in the young stages. As the animals mature, the whorl of the shell grows disproportionately large and provides accommodation for all the soft parts. In the process, the original spire is completely worn away. The mouth of the shell becomes round or oval, and the sole of the foot acquires more or less the same shape. Coupled with development of an existence on a rocky shore, the operculum has been lost, since the animal normally never lets go of the substrate to which it clings. Consequently, the columellar muscle becomes horseshoe-shaped, probably due to fusion of originally separate right and left shell muscles (Fretter and Graham, 1962).
Although limpets occur among all groups of gastropods, the majority of the most common species are prosobranchs. The prosobranch limpets, or "true limpets," are characterized by a well-developed head and cephalic tentacles, and a broad foot. The overgrowth of the mantle edge encloses a pallial groove which encircles the lateral margin of the foot and is continuous with the pallial cavity, or muchal cavity, anteriorly. The mantle margin is typically fringed with tentacles. The direction of the water currents through the muchal cavity and pallial grooves varies with the species.

Among the Patellacea (=Docoglossa) (Fretter and Graham, 1962) occur the best-known and most frequently studied genera of prosobranch limpets. Among these, Acmaea, a limpet occurring abundantly along the Pacific Coast of the U. S., is the only group which possess a ctenidium. It is a left one, and is elongated and held across the muchal cavity; the tip of the ctenidia may often be seen as the animal creeps, projecting from the right anterior margin of the shell. When the limpet comes to rest, the muscles of the ctenidial axis contract and withdraw the gill into the cavity.

The genera Patella and Patina, common British prosobranch limpets, have pallial gills set on the outer edge of the pallial groove. They hang down from the roof of the groove and encircle the head-foot in Patella, but in Patina the ring is incomplete anteriorly. In Patina, the exhalant current from the mantle cavity conveying all particulate matter leaves the body from the right side of
the nuchal cavity. The inhalant current is drawn into the
pallial groove in all regions where gills occur, by cilia
on the gills. Sediment entering with the current is entrapped
in mucus from a glandular region around the foot, and
passes anteriorly along the pallial groove with the water
flow, and through the nuchal cavity on the right.

Patella has a rather specialized arrangement of currents
in the mantle cavity and of disposal of material from the
body. A gentle inhalant current is drawn in all around the
margin of the mantle by the cilia on the gills, and a
weak current leaves the mantle cavity ventral to the
inhalant flow. Particulate material is directed from the
body by cilia on the sides of the foot and on the roof of
the nuchal cavity, which conduct it to a point midway along
the right pallial groove. Here it accumulates and is
expelled from time to time by sharp contractions of the
shell muscle. This forceful expulsion compensates for the
lack of the glandular region around the foot, which is
present in other prosobranch limpets.

Much of the preceding discussion of prosobranch
limpets is quoted from British Prosobranch Molluscs
(Fretter and Graham, 1962, chapter 19).

Due to their systematic position as pulmonates,
Ancylus, the fresh-water limpet, and Siphonaria, the group
under study herein, have been termed "false limpets."
Developmental distinctions found in Siphonaria include
the absence of a pallial groove or ctenidia; a considerable
space, usually filled with sea water, is present between
the margins of the foot, which are covered with mucus cells, and the shell. The mantle fringe is highly retractile and pulls back in response to the slightest tactile stimulation. The foot is oval and quite muscular; the cephalic region and foot have merged to the point that no clear line of demarcation of these two regions exists. Tentacles, both pallial and cephalic, are absent. There is a well-developed radula, and a rather typical pulmonate digestive tract. All species of Sinhorarta are hermaphroditic.

The pattern of water circulation in the animal is quite unique and is specifically localized, due to the fact that the animals are pulmonates. The mantle cavity is reduced, and is modified into a highly vascularized lung containing a secondarily derived gill, or pseudobranch. The opening to the environment appears at the pneumostome, on the left side of the mantle-foot junction. Both inhalant and exhalant currents pass through the pneumostome. The system is therefore much more "closed" than is the system described for prosobranch limpets, where water current entry and exit points are often some distance apart.

The systematic position of the genus Siphonaria, as well as the geographical distribution and distinguishing characteristics of the different species, is thoroughly discussed by Hubendick (1946). An excellent anatomical study of the organism is compiled Dieuzeide's monograph (1935).

In its natural habitat, the animal resides in the intertidal zone; populations reach their greatest density
along the jetties on those rocks which are covered by a thin film of microscopic algae. At the lower levels along the rocks, the populations are more sparse, and the individuals, whose average size is larger than the higher-level forms, live in close association with the macroscopic algal mats. This population of larger individuals, distinguishable by the rich macroscopic mat of green algae covering the shell, was observed at the South Jetty to be exposed only during periods of low tide. The higher-level population of smaller animals, distinguishable by the almost complete lack of visible algal growth on the shell, were observed to be exposed except during high tides. These animals were chosen for this study, due to their increased numbers and greater ease of collection over the lower level forms.

The animal may encounter considerable fluctuation in the environment in temperature, salinity, and length of exposure to air. Since the animal can breathe either air or water into the "lung," dessication presents little problem to Siphonaria pectinate, as long as it remains in a zone where it encounters sufficient surf or spray to maintain hydration of the soft parts. However, during the elevated temperatures encountered during the summer months, the rocky habitat may reach quite high temperatures during periods of low tide. In these cases, the animal is faced with environmental stress and presumably, any control mechanisms present which would aid the animal in retarding
dehydration during desiccation would be advantageous. Additionally, the environmental salinity at the South Jetty shows considerable variation (Appendix, Table II). Therefore, some mechanism to retard swelling or shrinking of the tissues during exposure to salinity extremes would also be desirable. This study was therefore directed toward elucidation of any possible control mechanisms possessed by the animal which would enable it to survive and flourish under such a wide range of environmental conditions.

MECHANISMS OF IONIC AND OSMOTIC REGULATION

The ability to create and maintain ionic and/or osmotic differences between the tissues and/or body fluids of an animal and its environment is present in several groups of invertebrates. These concentration differences are the result of both passive and active factors, one or more of which may be involved in any example cited (Potts and Parry, 1964). The passive factors include the permeability of the animal's body wall to water and solutes, and the presence of protein in the body fluids, which may produce Donnan effects or may bind some ions selectively into indiffusible complexes. The available active mechanisms include the excretion of salts and water from the body, and the active uptake of salts and water, possibly, in the gut and at the body surface.

Among the invertebrates, there exist two major classifications of animals as regards their responses to osmotic concentration changes. One group, termed osmoconformers,
react passively to the medium by equilibration of the body fluid concentration with that of the medium; these animals are often termed polikilosmotic. Another group of animals are osmotically stable, and their body-fluid concentration remains constant and independent of medium concentration changes; these animals are termed homocirosmosmotic, and are thus osmoregulators. These two classifications represent ideal cases; in fact, there are all gradations between these two extremes. Some animals have the ability to conform osmotically in one concentration range and regulate in another. Other groups show varying degrees of volume regulation. Salt transfer usually occurs in these cases, enabling the animal to maintain a constant volume as the body fluid concentrations change with the environment.

Among the marine invertebrates, a large number are isosmotic with the external medium. The limitation to the distribution of all osmolabile animals is probably the dilution or concentration at which their cellular organization can still permit basal metabolic functions (Prosser and Brown, chapter 2)(1965). This implies that, among osmoconformers, each species has its own "normal range" of external concentrations in which it can successfully survive. The simplest and purest form of osmotic conformity is found in a few marine invertebrates which gain or lose water according to the concentration of the medium. These animals show no volume regulation; they are bounded by membranes permeable to water only (perfectly semipermeable)
and swell or shrink in proportion to the total solute concentration. However, this is again an ideal case and has few examples in nature. Assuming that most living cells show some degree of ionic exchange, it is not surprising that numerous marine invertebrates therefore show osmotic conformity, coupled with poor volume regulation. The term volume regulation is rather misleading; in this case, an active process is not implied. Volume regulation is due to a breakdown of the semipermeable nature of the limiting membranes, allowing salt exchange. One example of an organism which shows osmotic conformity with poor volume regulation is the sipunculid Golfingia (Adolph, 1937). The sipunculid Dendrostomum apparently has a more efficient method of volume regulation, using the gut and nephridio-pores for ion exchange as well as showing a limited ability to release osmotically active particles from its body wall into the blood (Gross, 1954).

A comprehensive survey of the various mechanisms demonstrated by invertebrates for limited ionic and osmotic regulation is beyond the scope of this study; the reader is referred to a number of excellent reviews on this subject (bibliography).

Among the molluscs, several species show limited abilities for volume regulation. The gastropod "sea hare" Anly sia shows an initial weight gain for 2-3 hours after transfer to 75% sea water, followed by a recovery of the initial weight. On transfer back to 100% sea water, weight loss
occurs, indicating that salt loss occurred during the recovery of the initial volume (Bethe, 1929, 1934).

The presence of an external shell or shells in molluscs lends to this group a unique potential for a rather specialized behavioral response during osmotic stress. This response consists of temporary exclusion of the environment by clamping or closing of the valves; it has been suggested to occur in several different species of molluscs as a mechanical method of osmoregulation. The principle involved here is quite similar to that shown by the eggs of marine and fresh-water fish. In this case, the eggs are protected against osmotic stress by the fact that they are impermeable to both water and salts. Thus a barrier against equilibration during periods of concentration differences is erected. Among the molluscs, the most notable examples of this phenomenon are cited.

Krogh (1928) has demonstrated that the bivalves Mytilus, Crassostrea, and Modiolus respond for varying lengths of time to transfer from normal to dilute sea water by closing the valves. With the valves closed, these marine pelecypods could survive and maintain the hemolymph undiluted for several days. However, if the valves are forced open, the animal swells rapidly and little volume regulation is observed. Among these bivalves, Mytilus seems to show some degree of volume regulation, since Krogh (1928) found that the hemolymph and tissue chloride concentrations had
become reduced in dilute sea water. Further evidence that *Mytilus* is able to tolerate dilute sea water was furnished by Milne (1940), who found that, at low tide, the salinity inside the mantle cavity of *Mytilus* was 24%, while the salinity of the environment had fallen to 7%. Freeman and Rigler (1957) demonstrated that the estuarine lamellibranch *Scrobicularia plana* tolerates wide salinity variations by closure of the valves, seriously decreasing the rate of equilibration of the body fluids with the external milieu after environmental concentration changes.

Among the gastropods, Mayes (1960) found a gradual increase in weight in all the species of British *Littorina*, an operculate snail, upon transfer of the animals to 25% sea water. In this medium, all animals remained retracted until death; Mayes therefore concluded that the survival times of each species in this medium were correlated with the goodness of fit of the operculum. Avene and Sleigh (1965) have verified these findings using *Littorina saxatilis*. These workers concluded that hyperosmoticity of the body fluids of this animal in dilute solutions is a result of avoidance of deleterious conditions by retraction, rather than active hyperosmotic regulation, since active animals were always isosmotic with the medium. Thus, Avene and Sleigh conclude that the retraction must be of considerable importance in the survival of the animal under temporary adverse conditions.
Among the prosobranch limpets, Milne (1940) suggests that *Patella vulgata* may have the ability to exclude the environment during periods of osmotic stress. Arnold (1957, 1959) suggests that *Patella* does show such responses during dessication, and proposes that the cephalic tentacles and/or mantle fringe contain receptors for the perception of salinity. Segal (1956) and Segal and Dehnel (1962) give evidence that extravisceral water may serve an osmoregulatory function in *Acmaea limulata*.

The animal selected for this study, *Siphonaria pectinata*, has not been previously investigated as to its capacities for volume and/or ionic regulation, with the exception of a study done by Bucher and Harry (unpublished). These workers demonstrated that the chloride concentration of the hemolymph closely parallels that of the external milieu at all concentrations. Weight fluctuations were found to be less than those predicted for a perfect osmometer. It was concluded that this animal shows osmotic conformity coupled with limited volume regulation.

This study was designed to give definitive evidence that a clamping response, that is, active contraction of the foot and shell muscles to reduce equilibration with the adverse environment, is occurring in the false limpet. Experimental design was constructed in an effort to establish a normal salinity range for the animal, and experiments were conducted on both attached and unattached animals, in hopes of producing some measure of the efficiency
of the clamping response in reducing volume fluctuations in osmotic gradients. Although the false limpet has many features distinct from the prosobranch, or true limpet, it is hoped that this study will produce definitive numerical evidence in support of the observations of earlier workers, that limpets can indeed respond to osmotic stress by using the substratum as an "auxiliary valve," in a fashion similar to that demonstrated to be operative in pelecypods.
MATERIALS AND METHODS

COLLECTION AND MAINTENANCE OF ANIMALS

Animals were obtained along the South Jetty, Galveston, Texas, which is located at the southeastern tip of Galveston Island. The jetty lies due west of the mouth of the Houston Ship Channel. All animals were collected from the west, or Gulf, side of the jetty from a point approximately one-fourth mile from the beach. Animals were collected, whenever possible, at low tide.

The collection technique consisted of the introduction of a dull knife or scalpel under the shell and foot and exertion of a gentle prying force. Care was taken to prevent damage to the shell fringes or underlying soft parts; a mortality rate of approximately 20% was encountered due to injury after collection. A small amount of water was also collected in a plastic bottle, for salinity determination.

The animals were placed in an ice chest with a small amount of surrounding ice for transport back to the laboratory. Upon arrival, they were individually placed, foot down, in recovery aquaria (henceforth referred to as "tanks"), each of which contained 15 gallons of "Instant Ocean" (Aquarium Systems, Inc., Cleveland, Ohio) artificial sea water. Animals were distributed among the recovery tanks so that there were approximately 50-75 animals per tank. All tanks, both recovery (control) and experimental,
were aerated. To each tank was attached an external charcoal-glass wool filter, through which the water was circulated to facilitate removal of waste products. The salinity of the control tanks was maintained at 24%. All artificial sea water solutions, both experimental and control, were prepared by diluting a concentrated solution of the artificial sea water to the desired salinity. Salinity measurements were conducted using three hydrometers and sea water and conversion tables supplied by the Coast and Geodetic Survey, U. S. Department of Commerce. All experiments were conducted in an air conditioned laboratory, the temperature of which varied between 22° and 26° C.

Animals were acclimated in the recovery tanks for at least 48 hours after collection before an experiment. Average mortality during the first 24 hours was 19%. This was attributed to either (1) injury inflicted during collection or (2) shock inflicted either during transfer or after exposure to the artificial sea water.

After the 48-hour acclimation period, animals were maintained with little difficulty in the recovery tanks; however, in the latter stages of the study, some problems were encountered with bacterial growth in the tanks which in all cases led to 80-90% mortality in those tanks which had become contaminated. The bacterium was found to be a small Gram negative rod. The addition of 0.1 gram streptomycin sulfate (Lilly) per gallon of sea water was
found to prevent such bacterial growth and associated mortality.

COLLECTION OF HEMOLYMPH SAMPLES

For removal of body fluids, the animal was first blotted dry with absorbent paper, and the sole of the foot was then lightly lacerated with a razor blade around the lateral margins, thus puncturing the foot sinuses and ventral hemocoel. This method was only fairly successful, however, and an average of 40% of all animals tested yielded either no hemolymph at all or else a fluid of a brownish color. Only those animals which yielded a clear bluish fluid were used in the experiments. The difficulty of obtaining body fluid samples was most marked in animals acclimated to extreme hypoosmotic conditions. In these cases, only one animal in five yielded a measurable amount of hemolymph (see Discussion). Conversely, in hyperosmotic solutions, the animals yielded hemolymph quite readily.

Hemolymph samples were withdrawn after laceration using 25 microliter micropipettes. Since fluid volumes from individual animals varied between 5 μl and 300 μl, depending on the size of the animal and salinity of the water from which the animal was taken, pooled hemolymph samples from a minimum of three animals were used for all determinations. These pooled samples were stored in glass vials at -20°C, and the samples were taken from these vials for
the determination of $\Delta t_f$ and $\text{Na}^+$ concentration. A sample of the water from which each of the experimental groups was taken was also collected in vials and stored at $-20^\circ\text{C}$. Each time a quantity of the hemolymph was used in a determination, an equal amount of the sea water in which that group of animals was maintained was also measured.

**MEASUREMENT OF $\Delta t_f$**

The capillary technique used for the measurement of the freezing point depression, $\Delta t_f$, was a modification of that described by Gross (1954). Standard solutions of NaCl were prepared using reagent grade NaCl which had been heated in an oven for 24 hours at $100^\circ\text{C}$. Standards (0.1M, 0.25M, 0.5M, 0.75M, 1.0M, 1.25M, and distilled water blank) were run with each unknown determination. For the determination, 25 microliter disposable micropipettes (Kensington Scientific Co., San Francisco) were filled with appropriate solutions and sealed at each end with stopcock grease. These tubes were then glued to a plastic rack with rubber cement and quick-frozen in an ice chest containing dry ice. It was found that a maximum of twenty-five tubes could be run conveniently during a single determination. Therefore, seven NaCl standard tubes plus nine pairs of experimental samples were run in each determination. Each pair of experimental samples consisted of one tube containing hemolymph, and one tube containing the appropriate sea water.

When frozen, the rack of tubes was quickly placed
into an insulated sealed chamber containing 25% ethanol which had been previously cooled to -4°C. A small electric stirrer was built into the insulated ethanol bath to allow gentle agitation and thus an approximately linear rise in temperature in the bath. The stirrer was controlled by a constant-voltage power supply constructed of a battery charger connected to a variable transformer. The tubes were viewed from above through a window in the insulated chamber. Crossed sheets of polaroid film were introduced between the light source and the observer, in order to make the frozen crystals appear birefringent. The progress of melting in each tube was monitored using a dissecting microscope with 10X magnification. A stopwatch was used to time the melting of the crystals in each tube.

Melting of the last crystal as an end-point criterion was found to be unsatisfactory; the entire length of the tube could not be viewed when using the microscope. Therefore, the arbitrary end-point adopted by the writer was the time of melting of the last crystal in a small specified region of the tube, delineated by the inner margins of the two plastic supporting racks.

The stopwatch was started when the end-point was reached in the standard of highest concentration, i.e. 1.25M NaCl. The end-points of melting of the other standards and the experimental solutions were recorded as minutes and seconds after the zero time standard. After the last standard had reached the end-point, the bath
was again cooled to -4°C, the tubes were again frozen in dry ice, and the determination was repeated with the position of the rack of tubes reversed. This was done to nullify any discrepancies in readings due to the tubes on the end of the rack nearest to the stirrer responding more rapidly to the gradual temperature rise and thus giving a non-linear standard curve. All values reported for $\Delta t_f$ are thus a result of the average melting time of two successive measurements.

After each $\Delta t_f$ determination, the melting times of the standards were plotted against time, yielding a linear curve (Table 1). Standard slopes obtained using this method were found to be reproducible ±1%. From the standard curve, the experimental values of the melting times of the hemolymph and corresponding external milieu could be superimposed and values of equivalent molarity NaCl could be determined. From these values for the experimental solutions, the $\Delta t_f$ values for all solutions measured were calculated from tables of standard values for NaCl solutions from the *Handbook of Chemistry and Physics* (46th Ed., p. D-158). Pertinent formulae used in such calculations are summarized in Table 1a.

The rationale behind running a double set of standards, i.e. reagent grade NaCl solutions of known molarity and sea water solutions of known salinity may best be explained in reference to the formulae in Table 1a. Formulae (1) and (2) depend for their accuracy on a constant percentage of
### Table I—Standard values measured for $\Delta t_F$

<table>
<thead>
<tr>
<th>Molar NaCl</th>
<th>Melting time (range)</th>
<th>$\Delta t_F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>31'45&quot; (22'20&quot;-38'40&quot;)</td>
<td>0.86</td>
</tr>
<tr>
<td>0.50</td>
<td>24'40&quot; (18'30&quot;-30'15&quot;)</td>
<td>1.74</td>
</tr>
<tr>
<td>0.75</td>
<td>15'30&quot; (11'45&quot;-19'20&quot;)</td>
<td>2.57</td>
</tr>
<tr>
<td>1.00</td>
<td>8'45&quot; (6'10&quot;-11'15&quot;)</td>
<td>3.44</td>
</tr>
<tr>
<td>1.25</td>
<td></td>
<td>4.35</td>
</tr>
<tr>
<td>H2O blank</td>
<td>39'50&quot; (30'45&quot;-50'10&quot;)</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Above values are averages of 56 determinations
Average slope of standard curve = $-3.33 \pm 0.034$ (S.D., N=56)

### Table Ia—Formulae used in $\Delta t_F$ calculations

1. Salinity ($S\%$) = 0.030 + 1.805 Cl
2. $\Delta t_F = 0.0966 \text{ Cl} - 0.0000052 \text{ Cl}^3$
3. $\Delta t_F = K_F M G$

$M =$ molality (for each ionic species present)
$K_F =$ 1.858 = molal freezing point constant
$G =$ Cryoscopic coefficient (isotonic coefficient)
chloride ions being present in all sea water solutions used. This introduces a source of potential error into the calculations when using artificial sea water solutions, whose chloride ion percentages might be slightly different from those present in the sea water standard on which these formulae are based. Formula (3) is expressed in terms of molality, and the $M$ term in this equation must include all ionic species present in the sea water. Thus the concentrations of the various trace elements present would need to be known, since sodium and chloride ions together comprise only around 85% of the ions present in normal sea water (see appendix). The concentrations of the different ions would either have to be measured or obtained from the manufacturer of the artificial sea salts, and even then, it must be assumed that there are no differential solubility effects at different salinities for the trace elements. To avoid such cumbersome measurements, samples of the appropriate external medium were estimated along with the body fluid samples, and all values for $A_t^r$ were obtained from the equivalent sodium chloride molarities extrapolated from the standard curve. The average values for the standard curve for $A_t^r$ are depicted in Figure 1.

**Na⁺ DETERMINATIONS**

The determinations for Na⁺ concentrations were made in the following manner. Five microliter samples of body fluid and an equal amount of the appropriate sea water were taken from the pooled storage vials and diluted 1:1000
in 7% ethanol in 5 ml polyethylene containers. A series of standard solutions of sodium chloride in 7% ethanol were also prepared: 0.1mM, 0.25mM, 0.5mM, 0.75mM, 1.0mM, and a blank of 7% ethanol. The standards were determined using a 5-second response time on a Zeiss Spectrophotometer Model QII equipped with a flame photometer attachment. Absorbances were plotted versus concentration and a standard curve was obtained. The experimental samples containing hemolymph or appropriate sea water were then measured and their Na⁺ concentrations determined from the standard curve.

Weigh Determinations

For the determination of wet weights, the animals were blotted dry with absorbent paper and weighed on a torsion balance.
RESULTS

EXPERIMENTS INVOLVING TRANSFER OF ANIMALS INTO DIFFERENT SALINITIES

This group of experiments was designed in an effort to determine the animal's osmotic behavior when transferred from normal sea water to sea water of a "stress salinity," that is, outside the range normally encountered by the animal. The experimental conditions were quite similar to those used by Segal and Dehnel (1962) and by Harry and Bucher (unpublished). An effort was made to determine if the total osmotic behavior of the animal was reflected by the behavior of the chlorides (the major ionic species present in sea water) determined by Harry and Bucher (loc. cit.).

Animals were removed from the control tanks (24%) and placed into experimental plastic containers, twenty animals per container. These vessels contained different salinities, that ranged from 5% to 60% at 10% intervals. Thus each experiment involved eight containers holding salinities 5, 10, 20, 30, 40, 50, 60%, and one container for the control salinity 24%. After 24 hours exposure to the experimental salinity, five animals were removed, body fluids withdrawn and pooled, and the Δt values determined. Percent attachment to the bottom of the container after 24 hours was recorded, as was percent survival. Those animals which did not respond to tactile stimulation at the mantle fringe by localized retraction
of the mantle in the area of stimulation were considered dead. Surviving animals after the first 24 hours exposure were left in the experimental salinities for three more days, and percent survival after each additional 24 hours was recorded. The results are summarized in Table 2 and Figure 1.

Table 2—Percent survival and percent attachment
Animal Transfer Experiments

<table>
<thead>
<tr>
<th>%S</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
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<tr>
<td></td>
<td>%S*</td>
<td>%A**</td>
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<td>5</td>
<td>10</td>
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<td>24</td>
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* percent survival
** percent attachment

During transfer of the animals during the experiments above, it was noticed that each animal had to be placed into the plastic containers foot down; when held above the bottom of a container and dropped, the animal will always land foot up, resting on the shell. Furthermore, the animals have no ability to right themselves when in this position. These observations open to question the true validity of experiments involving the animals in which they are removed from the substrate, placed in the experimental solutions, and then observed. The point is, the animals
Figure 1—Results, Animal-transfer experiments

- Hemolymph
- Sea water
will rarely encounter conditions in nature paralleling those which it encounters in any experiment in the laboratory in which the animal is removed from the substrate, placed in the experimental solution, and the entire surface of the foot exposed to the environment. In fact, in the few cases in which the animal would be knocked or washed from its rock in nature, it would almost certainly be doomed since, upon reaching the bottom, it could not right itself and death would result from predation or silting. These thoughts, coupled with the results obtained in the preceding group of experiments, lead to questions concerning the ability of the animal to reattach after exposure of the foot to stress salinities. The possibility of lengthening survival time under stress conditions by gradual acclimation of the animal in the attached state was therefore investigated. Therefore, slow dilutions or concentrations of the environment were carried out with no mechanical disturbance to the attached animals.

After a few of these acclimation studies had been carried out, it was noticed that those animals which were attached seemed to be much more firmly bound to the bottom of the tanks in the stress salinities than in the control tanks. Attempts were made to measure force of attachment of acclimated animals in various salinities, but these met with little success. No suitable method for the attachment to the shell of some sort of halter with which
upward force could be exerted was found. However, the following experiments provide indirect evidence for increased force of attachment were found to be feasible.

This group of experiments consisted of short term experiments and physical observations of the animal under several different conditions.

CROSS-TRANSFER EXPERIMENTS

The salinity in one control tank was diluted gradually from the initial salinity of 24% to a final salinity of 12% over a period of 24 hours. This was done by slowly adding fresh water into the tank, using an electric stirrer to ensure proper mixing. Percent survival after this 24 hour "dilution period" was 48%. The animals were then left in this "50% sea water" for an additional 24 hours, after which time an additional 5% of the animals died. Ten animals were then carefully removed from the experimental tank and returned to a control tank. Ten animals were removed from the bottom in the experimental tank, lifted upwards to expose completely the ventral surface and soft parts to the surrounding water, and returned to the bottom of the tank. Animals in the control tank were treated in the same manner, with ten being transferred into the experimental tank, and ten being lifted up and replaced. All forty animals were then left undisturbed for six hours to allow time for reattachment. After this period, all forty animals were examined by careful nudging, and the percentage of each of the
four groups which had reattached was recorded.

In this hypoosmotic situation, it was found that, in all cases, those twenty animals which had been entirely exposed to the experimental sea water (12%) could not reattach. This included ten animals from the control salinity as well as the ten animals which had been acclimated to the stress salinity and then lifted up to completely expose the ventral surface. By contrast, all twenty animals which had been completely exposed to the control salinity, whether they had come from the experimental salinity or had been removed from the bottom of one of the control tanks and replaced, retained the complete ability to reattach.

The same sort of experiment as that just described was repeated, again using forty animals, under hyperosmotic conditions. The salinity of one control tank was slowly concentrated from the initial 24% to a final salinity of 49%, over a period of 24 hours. This was accomplished by slowly adding concentrated artificial sea water to the tank. Observations similar to those above were made, after the 48 hours exposure followed by a 6-hour reattachment period after transfer.

The results obtained were essentially identical to those observed under hypoosmotic conditions, i.e., all animals which had been completely exposed to the experimental sea water showed no ability to reattach, while the animals which were exposed to the control salinity reattached in all cases. The average (of four replicates)
percent survival observed after the 24 hour concentration period was 80%, and an identical value was observed after the additional 24 hour period just prior to cross-transfer.

In the course of the above experiment, it was noticed that those animals which had been acclimated to stress salinities seemed more firmly attached than the control animals. Therefore, some method for measurement of activity under stress and control conditions was sought. Considering the fact that reduced motility seemed to be coupled with this increased force of attachment, the following experiment was conducted.

ACTIVITY MEASUREMENTS

Activity of the individual animals in the different salinities was recorded in the following manner: Animals in the control tanks were placed under gradual stress over a 24-hour period until values of 12% in the hypo-osmotic tank and 49% in the hyperosmotic tank were obtained. Animals not firmly attached to the bottom of the tanks with a pencil, outlining the shell margin of each animal. All animals were then observed periodically; the percentages which had moved perceptibly from their original positions in the experimental as well as the control tanks were recorded:

<table>
<thead>
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<td>12%</td>
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<td>49%</td>
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For further observation, animals were allowed to attach to glass microscope slides in the control tanks.
Then, salinities were dropped to 12% or raised to 48% over a period of 24 hours. Control animals were allowed to attach to slides in the control tanks, where the salinity was unaltered. After the exposure period, the slides were inverted into finger bowls containing water from the appropriate tank from which the animals had been taken. In this position, the undersurface of the animal could be observed in the attached position. A dissecting microscope with 50X magnification was positioned over the slide. In order to observe water circulation beneath the shell, finely divided carmine particles were introduced into the water near the pneumostome. The animals were then observed for a while and the distribution of the red color was noted for animals in the two experimental salinities and the controls.

In the control animals, the carmine particles were seen to circulate freely beneath the shell, and the external water was obviously bathing most of the lateral margins of the foot and the groove lying between the mantle fringe and the edge of the foot. However, all acclimated experimental animals, whether in hypoosmotic (12%) or hyperosmotic (48%) were tightly clamped to the petri dishes. A barrier was apparently formed by the mantle fringe and the shell, and the animal appeared to be firmly attached to the substrate. No carmine particles were observed to pass beneath the shell at any time during these observations, with the exception of
the area adjacent to the pneumostome.

REATACHMENT AFTER STRESS

Animals in the control tanks were subjected to sudden osmotic stress by a dilution of the sea water to \( \frac{1}{2} \) normal (12\%) or a concentration to twice normal salinity (48\%) and left until a few animals in each tank had become unattached. The salinity in each experimental tank was then readjusted to the normal value and those animals which recovered their ability to attach after the stress was removed were noted. This was done in an effort to investigate any correlation between the ability to attach and the ability to survive.

It was found that, in all cases, those animals which had become released from the substrate could not, under any circumstances, recover from the stress after a return to a normal salinity.

Considering the fact that these animals live in an environment where dessication at low tides may be encountered, experiments were designed to study the responses of Siphonaria pectinata to the effects of dessication, in hopes of determining the maximum period which the animal may withstand these effects under laboratory conditions and still recover when resubmerged in sea water.

DESSICATION EXPERIMENTS

Animals were removed from the control tanks and
wet weights were taken. The weighed animals were then placed in petri dishes, the bottoms of which had been frosted by grinding with fine carborundum particles. The dishes containing the animals were then submerged in the control tanks for 24 hours to allow for attachment to the frosted glass substrate. The petri dishes were then removed from the tank, the water poured off, and the dishes with animals placed on a shelf in the laboratory. Two of the animals were then removed from one of the dishes every six hours over the next 72 hour dessication period; one was placed back into the control tank. After 4 hours exposure to the control sea water, the animal was examined and its condition noted. The other animal removed at each 6-hour period was weighed, the weight change from the original value recorded, the hemolymph collected, and the $\Delta t_p$ of the hemolymph determined. The results are summarized in Figure 2.

In the recovery experiments after dessication, it was found that animals survived up to 5½ hours dessication and could still recover completely when returned to the control tanks. These animals, upon return to the sea water, regained their initial weights in approximately 45 minutes to one hour after rehydration. However, many animals exposed to dessication for 5½ hours or more in these experiments did not recover. It was observed that in those animals which did not recover, the body had become shrunken to such a great extent that the mantle had been stretched to the point that it had pulled free
Figure 2—Results, dessication experiments

•• Δf fluctuations

•• Weight fluctuations
from its point of insertion along the inner margin of the shell.

STUDIES ON ATTACHED ANIMALS

These experiments were designed to investigate the osmotic behavior of the hemolymph and salinity tolerance of those animals which had been allowed to acclimate and become attached to the substrate before exposure to sudden or gradual salinity alterations. The experiments were conducted using procedures quite similar to those employed in the experiments involving transfer of animals; in this case, however, the animals were not transferred into the stress salinities; they were left undisturbed and the salinity of the surrounding sea water was altered. The survival averages and $\Delta t_f$ and $Na^+$ concentrations of the hemolymph will be compared with the values obtained in the animal-transfer experiments.

Experiments were carried out using one 15-gallon tank as a control, and four 15-gallon tanks as experimental. Two of the experimental tanks, designated tanks I and II, were used for hypoosmotic conditions, and the other two, tanks III and IV, were used for hyperosmotic studies.

All five tanks initially contained the same number of animals and were maintained at the control salinity. Seventy-five animals were placed in each tank and allowed to acclimate for several days. After this period, the following experiments were performed.
The salinities in tanks I and II were slowly diluted at the rate of 5\% per 24 hour period from the initial salinity of 24\% to a final salinity of 5\% over a period of four days. Simultaneously, tanks III and IV were concentrated at the rate of 10\% per day until a final salinity of 75\% was reached after five days. The control tank was maintained in the usual manner. Hemolymph and appropriate sea water samples were collected at the end of each day just prior to the next adjustment in salinity in each of the four tanks. Thus four pairs of samples, two of which came from different tanks of identical salinity, as well as a control pair of samples, were collected each day. Measurements of the $\Delta t_f$ and $Na^+$ concentration for each sample were conducted. Survival data in the different salinities were also collected daily and expressed as percentages. The results of this experiment are given in Figure 3.

In another experiment, the salinities of tanks I and II were quickly diluted from the initial value of 24\% to a final value of 4\% by the addition of fresh water. Similarly, the salinities in tanks III and IV were quickly concentrated to a value of 74\% by the addition of a concentrated sea salt solution. The salinity changes were effected as quickly as possible in order to obtain an approximately instantaneous change to a stress condition. After the alterations in salinity, hemolymph samples and a water sample were taken from
Figure 3—Results, responses of attached animals to gradual salinity change

- •——• Hemolymph

- •——• Sea water

- ▲——▲ % Survival
each experimental salinity at the following time intervals: 0.25, 0.5, 0.75, 1.0, 3, 6, 12, 24, 48, and 72 hours. The $\Delta t_f$ and $Na^+$ concentrations of the hemolymph and sea water samples were determined and their changes with time in the different salinities recorded. Percent survival in each tank at each time period was also recorded. The results are summarized in Figure 4.

COMPARATIVE STUDY BETWEEN ATTACHED AND UNATTACHED ANIMALS

This group of experiments was conducted with the purpose of showing similarities and differences in the osmotic behavior of the animal in the attached and unattached state. By means of a set of experiments conducted in petri dishes, measurements were made of weight changes in the different salinities in attached and unattached animals. Sodium concentrations of the external milieu were conducted and these findings were correlated with the $\Delta t_f$ measurements which were carried out on both sea water and hemolymph.

The bottoms of petri dishes were frosted to facilitate attachment by the animals. Individual animals were weighed and marked and placed in these dishes, five animals per dish. The dishes were then placed in one of the five recovery tanks and allowed to acclimate for 72 hours. During this period, animals were observed closely and those which crawled out of the dishes were replaced. After the acclimation period, dishes were removed from the tanks and any accumulated feces were
Figure 4—Results, responses of attached animals to sudden osmotic stress

- Hemolymph, tanks I and II
- Hemolymph, tanks III and IV

Survival curve
- Tanks I and II
- Tanks III and IV
washed out with freshly prepared sea water of the control salinity. The wash solution was poured off, and the dishes and attached animals were blotted dry with absorbent paper. Fifty milliliters of sea water of the following salinities were added to the dishes, two dishes per experimental salinity: 5, 10, 20, 30, 40, 50, 60, 70%, and the control salinity 24%. One blank control dish containing 50 ml of water and no animals was prepared for each experimental salinity and the control salinity to allow for any evaporation effects. At time intervals 1 hr, 3 hr, 6 hr, 12 hr, and 24 hr, one animal from each dish from each salinity was removed and its hemolymph collected, along with a sample of the surrounding experimental water. Insufficient hemolymph was obtained to determine both the $\Delta t_f$ and $Na^+$ concentration in the low salinities; therefore, only the $\Delta t_f$ was determined for the hemolymph. The $\Delta t_f$ of the surrounding medium was determined, as well as the initial and final samples taken from each of the blank control dishes. The remaining set of dishes were left covered for the full twenty-four hours, and the five animals in each dish were observed periodically in an effort to detect any swelling, shrinking, or other visible changes in the physiological state during the stress period. After 24 hours, a sample of the surrounding water was taken from each dish and the $Na^+$ concentration of each; these concentrations were compared to those obtained for the water from the blank
control dishes containing no animals. The five animals in each of these dishes were then weighed and their final weights compared to the original (pre-stress) weights.

A similar experiment was then conducted using petri dishes; however, in this experiment, animals were removed from the dishes after acclimation and a small piece of plastic screen was placed in each dish. The animals were then placed on this screen and the test solution (50 ml) added. All dishes were then covered. In this fashion, the animals were submerged in the water but were unable to make contact with the bottom of the dish, making attachment impossible. Thus the complete surface area of the foot and soft parts was exposed to the experimental sea water. Two dishes were again used at each salinity with five animals per dish. One dish was again used for determinations of changes in $aT_f$ of the hemolymph with time, while the other dish was maintained until the end of the 24-hour period. At this time, the $Na^+$ fluctuations of the external milieu were measured. Weight fluctuations were recorded just prior to removal of hemolymph samples, at the same time intervals used in the preceding experiment. The results are summarized in Table 2 and Figures 5-9.
Table 2--Results, Comparative Study

ATTACHED ANIMALS

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SEA WATER SAMPLES

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No changes found in absolute control dishes for Δσ or Na⁺

Above values represent averages of three experiments.
## Table 2 (cont.)

**UNATTACHED ANIMALS** (on plastic screen)

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</table>

Above values represent averages of three experiments.
Figure 5—Results. Comparative Study

Responses under hypoosmotic stress

△ — △ Attached animals, 5%
△ — △ Unattached animals, 5%
⊙ — ⊙ Attached animals, 10%
⊙ — ⊙ Unattached animals, 10%

% W = percent change in weight
Figure 6—Results, Comparative Study

Responses in the normal range

- Attached animals, 20%
- Unattached animals, 20%
- Attached animals, 30%
- Unattached animals, 30%
- Attached animals, 40%
- Unattached animals, 40%

\( \% W = \text{percent change in weight} \)
Figure 7—Results, Comparative Study

Responses under hyperosmotic stress

- ■ - Attached animals, 50%
- □ - □ Unattached animals, 50%
- ◆ - ◆ Attached animals, 60%
- ○ - ○ Unattached animals, 60%
- ▲ - ▲ Attached animals, 70%
- △ - △ Unattached animals, 70%

% W = percent change in weight
Figure 8—Results, Comparative Study
Comparison of weight fluctuations, attached and unattached animals

•——• Attached animals

○——○ Unattached animals
DISCUSSION
The data compiled in Figure 1 indicate that Siphonaria pectinata shows no ability to osmoregulate over the range of salinities tested under the defined experimental conditions. The freezing points depicted for hemolymph and sea water are not considered to be different, since the values represent averages of fourteen determinations with a standard deviation of 0.07 and 0.09 for sea water and hemolymph, respectively. It was therefore concluded that the hemolymph remains essentially isosmotic with the external milieu at all salinities between 5% and 60%, under the defined conditions.

From the survival data compiled in this experiment, it is proposed that the animal can tolerate salinities of between 20% and 40% with apparently no ill effects. This range of salinities will be tentatively be referred to as the "normal range." Above and below the limits of this normal range of salinity, it was found that the animals could not reattach to the substrate when placed in the experimental sea water. Furthermore, no animal which was unable to effect reattachment survived for longer than 24 hours after exposure to the sea water above or below the normal range.

A comparison of these results with those found by Harry and Bucher (unpublished) show a close correlation between the fluctuations in total osmotic pressure and those of the chlorides of the hemolymph in different
salinities. It is thus concluded that a measure of the chlorides is a good approximation of the total osmotic behavior of the hemolymph of *S. pectinata* under the defined conditions.

The animal-transfer experiments and those reported in the literature which were conducted under similar conditions (Segal and Dehnel, 1962) are considered to be unphysiological and artificial. There are several reasons for questioning the validity of such experiments, especially in the case of *S. pectinata*. The animal rarely encounters in the environment such sudden salinity changes as it was subjected to in the laboratory in these experiments. Salinity extremes are occasionally encountered in nature (see Table 2, Appendix), but they normally occur gradually over hours or even weeks' duration. The second objection to these experiments lies in the methods used for exposure of the animals to the experimental salinities, that is, by placing them directly into the test solutions. The shell of the animal is its only means of protection from predation; if the animal is dislodged from the substrate and allowed to drop into the water, it lands on the bottom with the foot up. In this position, it is unable to right itself and would therefore be particularly susceptible to predation, as well as the ever-present danger of being silted under. Therefore, experiments in which the animal is fully exposed to the surrounding medium, even for a few seconds, are considered
to be a poor representation of the natural habitat. The questionable value of total exposure of the foot of the animal to the environment is further substantiated by the observation that the foot of the animal shows a slight tendency to swell in salinities of 15-20‰ after only a momentary exposure of the soft parts to the surrounding water. Accompanying this swelling is an inability to reattach when placed on the substrate. The ability to reattach is apparently regulated by the amount of swelling observed after exposure, with more swelling being observed with decreasing salinity. By contrast, animals were often collected in the field in salinities of 15-20‰, and no swollen or poorly attached animals were ever seen on the jetty. It might be argued that the apparently conflicting observations could be evidence that the artificial sea water used in these experiments was not ideally suited for the support of this species, and thus could be the reason for increased swelling and inability to remain attached. However, it was observed that animals which had been collected in the field in 16‰ and transported back to Houston in this water showed little tendency to attach to the sides of the transporting container, whereas those collected in higher salinities attached both to the sides of the container and to the shells of neighboring animals. Furthermore, initial mortality rates during the 24 hour recovery period were considerably higher than normal in these groups of animals.
In the course of the cross-transfer experiments, several observations were made. In both the hypoosmotic and hyperosmotic conditions, percent survival after 24 hours exposure was greatly improved over the results reported in the animal-transfer experiments, which involved a removal of the animal from the substrate. No better than 25% survived after 24 hours exposure to a salinity below the normal range in the animal-transfer experiments. In the cross-transfer experiments, in which the animals were placed under stress without the accompanying removal from the substrate, 45% of the animals had survived 12-18 hours of the dilution period during which the salinity had passed out of the normal range, as well as 24 additional hours at a constant salinity of 12%. In the hyperosmotic situation, 80% of the animals had survived a similar length of exposure to water considered to be above the normal range of salinity. Furthermore, in both cases (hypo- and hyperosmotic), all the animals which died during the experiment died within 6-12 hours after the salinity had passed out of the normal range. This is interpreted to mean that those animals which did not survive the initial dilution in the attached state lacked some sort of mechanism for adaptation to the stress salinity which the survivors possessed. Presumably the ability to withstand the initial stress was lost through previous injury.

After the initial dilution (concentration) period,
the cross-transfer experiment was conducted. The results suggest that all animals which are exposed totally to a hypo- or hyperosmotic stress salinity show no ability to reattach. This held true for animals which had been transferred into the stress salinity from the control tank, as well as those which had been attached in the stress tanks and simply lifted up and placed back on the substrate. However, all animals in the control tanks reattached after similar manipulations, as did those animals which were taken from the stress salinities and placed in the control tanks.

These results are interpreted to mean that the animals show improved survival if undisturbed from the attached state on the substrate. Furthermore, mere exposure of the soft parts of the body seems to be the key to the reason the animals in the stress salinities could not reattach. The possibility that the animals were injured during removal from the substrate may be safely discarded as a reason for inability to reattach, since all animals in the control tanks, which were treated in the same manner as those in the stress salinities, reattached.

The results of this experiment imply that only a few seconds' exposure of the soft parts (head-foot complex and mantle extension) to an extreme salinity outside the normal range renders the animal unable to reattach to the substrate. It would appear that such a short exposure to the stress salinity would yield a similar effect in those animals which were removed from these salinities.
and transferred to the control tanks, since they experi-
enced a similar duration of exposure to the stress salinity
during transfer. Presumably the immediate and sustained
exposure to the control salinity after this brief period
of total exposure to the stress solution is sufficient
to reverse the effects of the stress salinity. This
hypothesis is supported by the fact that all these animals
did, in fact, reattach.

The preceding results prompt the formulation of an
hypothesis to explain the discrepancy in survival ability
in salinity extremes outside the normal range between
attached animals and those which had been completely
exposed to the stress salinity. It was noticed in the course
of the above experiments, that the animals seemed much
more firmly attached in the stress salinities than in the
normal range. Animals in the hypo- and hyperosmotic sal-
inities were much more difficult to remove from the
substrate—so difficult, in fact, that they seemed almost
to form a "water-tight bond" between the outer margin of
the shell and the substrate.

It is thus postulated that attached animals, when
faced with an environmental salinity outside the normal
range, respond to the stress by active contraction of the
shell, foot, and visceral muscles, effecting a tight union
between shell edge and substrate. It is further proposed
that such a "clamping response" of the animal followed by
the maintenance of tonus of the muscles involved, could
greatly reduce water flow between the foot and the environment during periods of stress when the osmotic gradient is high due to salinities outside the normal range. Furthermore, previous attachment to the substrate seems to be obligatory, since animals which have been removed from the substrate during periods of osmotic stress show no ability to elicit the proposed response.

Indirect evidence for the existence of the clamping response comes from observations gathered during the animal-transfer and cross-transfer experiments. Animals which had been previously attached showed much less tendency to swell or shrink in hypo- and hyperosmotic stress salinities, respectively, than did those animals which had been removed from the substrate and completely exposed to the stress salinity.

The results of the activity measurement experiments also support the notion that the animal elicits the clamping response during periods of osmotic stress. The reduced activity observed shows a direct correlation with the proposed response, which is plausible since any active foraging on the part of the animal would tend to sacrifice the tight bond with the substrate which had been established.

Further evidence that the clamping response is operative was obtained from direct microscopic examination of the animals acclimated in hypo-, hyperosmotic and control salinities. Carmine particles were added near submerged animals which had attached to microscope slides. The
so-called barrier erected between the mantle fringe-shell complex and the slide appeared near-perfect in the stress salinities; the only bridge across this barrier lay in the region of the pneumostome, where water was observed to be circulating in and out at regular intervals. Except in this region, the red color was not observed to cross the shell margin. By contrast, in the control animals, the red color was seen to pass freely beneath the shell and much of the foot margin acquired a red hue, due to the carmine particles being trapped in the mucus covering the lateral margins of the foot. These observations lend further evidence for reduced ciliary activity and muscular contraction in response to the stress salinity.

Those animals which do not show the clamping response and become dislodged from the substrate due to salinity stress show no ability to recover and survive when placed into a salinity of normal concentration. These animals have presumably lost, through injury, the ability to clamp themselves down in response to the stress. If these animals are exposed to the stress for more than a few minutes, they are irreparably injured by the stress medium. This experiment points out the obvious adaptive advantage held by those animals which possess the ability to show the clamping response. Furthermore, it lends evidence to the premise that ability to attach in stress salinities is directly correlated with the ability to survive.
The most striking result of the dessication experiments is the finding that the animals, when attached, could withstand dessication for up to 72 hours. Furthermore, the limiting factor here seemed not to be one of water loss so much as rupture of the mantle itself, that is, mechanical injury. This finding points up the adaptation with *S. pectinata* has acquired for an existence in a zonation along the jetties where the animals might be subjected to periods of dessication during low tides. It is unlikely that the animal would ever be exposed for longer than 12-18 hours in the Galveston region; however, on a warm day, evaporation could be increased to the point that exposure under field conditions would be more detrimental than those in the laboratory at 25°C. The results of the dessication experiments will be discussed in more detail in a later section.

Arnold (1957, 1959) made note of the fact that the prosobranch limpet, *Patella vulgata*, during dessication, tends to raise the front margin of the shell. This behavior was also noticed by Segal and Dehnel (1962) to occur in *Acmaea limulata*; they termed the phenomenon the "stilting response." Presumably the function of this response is to permit air to circulate over the tissues, thereby inducing evaporation and lowering the temperature. There is no evidence for the existence of such a response in *S. pectinata*. This is ascribed to the fact that the extrinsiceral water in *Patella* and *Acmaea* lies, for the most part,
within the mantle cavity or pallial groove and is therefore enclosed; thus the animal would be in little danger of losing much water by raising the front of the shell, with the exception of that lost due to evaporation. However, most of the extravisceral water of *Siphonaria* is held between the shell and the lateral margins of the foot. Any release of the shell from the substrate would release this water, thereby complicating the problems of dessication.

The findings of these experiments support an hypothesis that the clamping response may have evolved in this animal to the point that it might be a mechanical method of temporary osmoregulation, by means of a short-term ability to prevent water from leaving or entering the tissues during osmotic stress.

It has been demonstrated that the animal remains isosmotic with the environment at all salinities under the conditions defined in the animal-transfer experiments. It is proposed that *S. pectinata* functions superficially as an osmometer when faced with salinity variations. This implies that the animal behaves as an approximately semipermeable membrane, completely permeable to water and relatively impermeable to salts. Thus, there would be little salt exchange with the environment, and all volume changes, be they swelling in hypoosmotic conditions, or shrinking in hyperosmotic solutions, could be explained in terms of water flow. Further discussion of these considerations appears in a later section.
The next group of experiments, conducted entirely with attached animals, was conducted in an effort to produce evidence to substantiate or refute the hypotheses presented. An effort was made to show the osmotic fluctuations of the hemolymph when attached animals were presented with hypo- and hyperosmotic stress and allowed to show the clamping response. In addition, it was felt that some ionic species should also be determined and its fluctuations measured in the different salinities. It has been previously demonstrated that the chloride ion concentrations of the hemolymph fluctuate in response to different salinities in a fashion directly proportional to the total osmotic concentration. Therefore, one of the monovalent cations was considered to be the obvious ion of choice for these experiments, since $Na^+$ is second only to chloride ion concentration in terms of the major species present in sea water (Appendix).

The first of these experiments using attached animals involved a gradual dilution (concentration) to a hypoosmotic (hyperosmotic) salinity extreme over a period of four (five) days. Examination of the survival data show that the animals survived much better in the attached state under gradually increasing osmotic stress than was found after instantaneous exposure in the animal-transfer experiments. Assuming the concentration did not reach a stress level in the hypoosmotic tank until the second day when the level reached 20%, 19% of the animals managed to survive three
additional days of increasing stress to a final level of 5%. In the hyperosmotic tank, the salinity was concentrated over a period of five days to a final value of 74%, after which time 43% of the animals were still alive. This a 43% increase in survival over the animals in the animal-transfer experiment, which after exposure to 60% survived no longer than 24 hours. Furthermore, percent survival showed no large decrease in the hyperosmotic tanks until the fifth day, a point 24 hours after the concentration had reached 74%. This is considered to be very significant, since the upper limit of the normal range had been attained 48 hours previously. Therefore, it is concluded that the clamping response is more beneficial to the animals under hyperosmotic conditions than hypoosmotic ones. This does not imply that the clamping response is ineffective in hypoosmotic salinities; it merely hints that it is more effective in allowing the animal to survive salinities above the normal range than below it.

Examination of the data obtained for $\Delta t_f$ for hemolymph and sea water at the different salinities (Figure 3) lend further evidence to this hypothesis. According to the proposed hypothesis, any deviations from the external $\Delta t_f$ (isosmotic line) by the hemolymph would indicate some sort of control mechanism is operative. Furthermore, these deviations should be primarily due to restriction of water movements due to the clamping response. The values
for $\Delta f_1$ and $Na^+$ concentration were found to be essentially identical as regards increments of deviation from the external milieu. This indicates that measurement of the $Na^+$ concentration of the hemolymph is directly proportional to the total osmotic concentration at all salinities tested. This conclusion agrees with that proposed earlier concerning hemolymph chloride concentration.

A closer examination of the data composing Figure 3 shows that within the range of $20-45\%$, the hemolymph $\Delta f_1$ values correspond very closely with those of the external milieu. However, the values are markedly higher than the sea water below $20\%$, and are lower than the sea water above $45\%$. It is further noticed that when the salinity was diluted from $10\%$ to $5\%$ over an additional $24$ hours, the hemolymph values drop back to a point equal to that of the external milieu. No such drop was observed at the opposite end of the salinity spectrum, after the final salinity adjustment in the hyperosmotic tanks.

These results are interpreted to mean that when the dilution of the hypoosmotic tank reached a level of $20\%$, the animals clamped down, hindering equilibration of the hemolymph with the sea water as the environmental dilution was continued. The same phenomenon apparently has taken place in the hyperosmotic situation. As the level of concentration reached $45\%$, the animals elicited the clamping response; thus equilibration was hindered due to restriction of water movements. It must be pointed
out that the restriction of water flow is not a perfect process; if it were, the hemolymp curves would level off when the clamping response occurred, indicating total blockage of water exchange. However, the curves do not demonstrate this in either the hypoosmotic or hyperosmotic salinities. The rate of change of hemolymp concentration is obviously greatly reduced, but it is by no means stopped completely.

The above values imply that the limits for the tentative "normal range" determined by percent survival in the animal-transfer experiments, i.e. 20-40%, should be amended. The animal clamps down at salinities below 20% or above 45%; it is therefore proposed that the range of salinity between 20% and 45% be designated the normal range for optimum survival. Percent survival begins to fall off when the salinity passes out of the normal range, at both ends of the curve. Furthermore, there is a good correlation between these curves and the proposed notion that the clamping response works more efficiently in hyperosmotic than in hypoosmotic salinities. This is evidenced by the fact that in the hyperosmotic stress salinity, the hemolymp concentration remained markedly below the external concentration throughout the experiment, indicating that the clamping response was still operative. However, after 48 hours exposure to hypoosmotic stress, the Δr of the hemolymp suddenly dropped back to equality with the sea water. Furthermore, the animals which were
left when the salinity had finally reached 5%. were all unattached and swollen; only 19% were still alive. This result indicates that the clamping response becomes inoperative when the salinity, after 48 hours dilution outside the normal range, finally reaches a level of 5%. This inability to maintain the clamping response may be accounted for in a variety of ways. One explanation would be that salinities below 10% are unequivocally lethal to the animal. A more plausible explanation would be that, after 48 hours exposure to a stress salinity (below 20%), sufficient water had entered to allow the foot and soft parts of the animal to swell to the point that maintenance of muscular contraction was rendered impossible. In this partially swollen condition, the animal could thus no longer maintain the clamped state, and would become released. Thus the tissues would be completely exposed to the environment; the end result would be a return to isotonicity.

In either case, the curves do substantiate the clamping hypothesis, as well as the notion that the animals are more tolerant to hyperosmotic than to hypoosmotic conditions. The actual mechanism responsible for the loss of the clamping ability in hypoosmotic salinities remains somewhat obscure.

The second of the experiments conducted entirely with attached animals was done in an effort to show the responses of the attached animals to sudden osmotic stress.
Again, both $\Delta t_F$ and $\text{Na}^+$ concentrations were measured for hemolymph and sea water.

The survival curves (Figure 4) show that the animals survived the hyperosmotic stress reasonably well; after 72 hours exposure, 27% had survived. Furthermore, the plot of percent survival versus time would show a linear drop. Those animals in the hypoosmotic tanks show near-identical survival values through the first twenty-four hours exposure; however, at this point, there is a sharp break in the curve; over 50% of the animals died after between 24 and 48 hours of exposure at 4%.

These results are interpreted as lending support to the hypothesis that the animals elicit the clamping response when subjected to osmotic stress. In this experiment, any unattached animals were considered dead, due to the findings earlier that animals which had become unattached due to salinity extremes could not recover. Therefore, the initial 5-6% of the animals which had become released due to stress after 30 minutes exposure were presumed to be those which could not effect the clamping response.

The approximate linearity of survival with time in the hyperosmotic salinity is to be expected; it simply suggests that with increasing exposure, more and more animals pass the salinity tolerance limit, become unattached, and die. Those animals which die first presumably have more imperfections along the margin of the shell. Thus, they are presumably less efficient at restricting
water entry than are those animals with more uniform shells which form a more efficient bond with the substrate. This argument is strengthened by the observation that the longer an animal had been maintained in the laboratory before it was used in an experiment, the more efficient was the clamping response. Observations showed that after 2-3 weeks of maintenance in the control, irregularities in the shell edge had been largely repaired through shell regeneration.

The sharp break in the survival curve in the hypo-osmotic salinities (Figure 4) shows a strong resemblance to that observed in the first experiment reported conducted entirely with attached animals. It seems probable that this break in slope represents the point at which the animals begin to swell to the point that the clamping response is interfered with. This is quite similar to observations made in the first experiment, involving gradual osmotic stress (Figure 3).

The survival curves shown (Figure 4) also support the notion that animals are more tolerant to hyperosmotic than hypoosmotic conditions. Only 4% of the animals survived the entire 72 hours in a concentration of 5%, a value approximately 20% that of the normal salinity. In the hyperosmotic tanks (74%), 27% of the animals survived, after the full 72 hours exposure.

This point is well taken by close examination of the $\Delta t_x$ values measured at the various time intervals. The
hypoosmotic curve shows a drop for the first 12 hours, to a \( \Delta t_f \) value of 0.60 (\( Na^+ = 55 \text{mM} \)). After this, the curve levels off and then drops again at between 24 and 48 hours until a final value of hemolymph identical to that of the external milieu is reached (\( \Delta t_f = 0.30, \ Na^+ = 24 \text{mM} \)). It is therefore proposed that when the blood concentration has reached a stress value, the clamping response occurs. The drop after 24-48 hours to a final value equal to the external milieu supports the idea that the clamping response is overruled by gradual swelling; this would occur after 24-48 hours of exposure to the stress salinity. Therefore, the animal appears to be able to survive at least 24 hours of extreme hypoosmotic stress and possibly as much as 48 hours. This lengthy tolerance may be attributed to the clamping response.

The curves for the hyperosmotic tanks show similarities to those just discussed; again, the clamping response occurs, at a hemolymph \( \Delta t_f \) value of approximately 3.77 (\( Na^+ = 340 \text{mM} \)) after approximately 24 hours. In this case, the curves drop very slightly once the clamping response has been initiated; the curves are still significantly below the values for the external milieu after 72 hours exposure.

In summary, the experiments conducted with attached animals under either gradual or sudden osmotic stress have added evidence to the response hypothesis. It has been established that this response is apparently highly useful, especially under conditions of hyperosmotic stress, in preventing sudden weight losses or gains and the accom-
panying cellular damage due to strong osmotic gradients between body fluids and sea water. The response allows *S. pectinata* to survive 24-48 hours of hypoosmotic stress, after which time sufficient water has entered to allow the animal to swell to the extent that it cannot maintain the clamped condition. Under hyperosmotic stress, the animal can survive for periods of 72 hours or longer by aid of the clamping response.

RELATIONSHIP BETWEEN DESSICATION AND OSMOTIC DEHYDRATION

It has been shown that the animals which exhibit the clamping response show tolerance to hyperosmotic conditions for periods of 72 hours or longer. Similarly, the dessication experiments (Figure 2) have indicated a maximum survival time of approximately 54-72 hours at room temperature. In view of these similarities, it is proposed that dessication during exposure and water loss due to hyperosmotic dehydration are similar phenomena when faced by *S. pectinata*. This notion is substantiated by the fact that in both cases, the animal exhibits the clamping response. That this response was operative during the dessication experiments is not obvious from the curve in Figure 2; however, during these experiments the animals were observed to cease all movement and to adhere very strongly to the substrate, two factors characteristic of the proposed clamping response.

The best line of evidence for these two phenomena being essentially identical would be a conclusive docu-
mentation that the animal does, in fact, behave as an osmometer. If such were the case, then any weight loss in hyperosmotic salinities could be explained completely in terms of water loss. This would of course be identical to weight loss during dessication. In keeping with these considerations, weight fluctuations in the different salinities were measured in the course of the experiments involving a direct comparative study between attached and unattached animals' responses to osmotic stress.

The results of these experiments are summarized in Figures 5-8 and Table 2. These experiments were designed to show behavioral differences between attached and unattached animals after sudden exposure to a variety of salinities. Experiments were conducted for only twenty-four hours in order to obtain a direct comparison between the two groups of animals over a time period considered to be the maximum period which the animal would encounter severe stress conditions in nature. The experiments were conducted in petri dishes, in hopes of showing any differences in external $\Delta t_f$ or Na$^+$ concentrations or other factors which might possibly be ascribed to ion movement or lack thereof. However, the final values did not differ significantly from those found initially; it was concluded that the volume in which the animals were placed (50 milliliters) was too large to detect any ion or water movements in terms of fluctuations in the external medium. No conclusions concerning ionic fluctuations of the medium could
therefore be drawn.

The curves illustrating $\Delta t_F$ values for attached and unattached animals in Figures 5-7 are essentially self-explanatory. These results once more reiterate the efficiency of the clamping response in restricting hemolymph concentration changes outside the normal range. Furthermore, since the foot and soft parts of half the animals used were completely exposed to the sea water throughout the experiments, examination of the $\Delta t_F$ values lends information concerning the length of time necessary for equilibration of the hemolymph with the sea water. In all cases, this equilibrium was established within one hour. This serves to illustrate how permeable to water the foot actually is when completely exposed to concentration differences. In the salinities below 20% (Fig. 5) and above 40% (Fig. 7), the clamping response is seen to markedly restrict this equilibration from occurring.

Examination of the survival curves shows that among the attached animals, at least 85% of the animals survived after 24 hours. This is in direct contrast to those animals, which because of the presence of the plastic screen, were prevented from effecting the clamping response. Consequently, survival values for those animals were poor in the salinities above and below the normal range. The fact that all animals in 5% and 10% were dead after 24 hours, while complete mortality in the hyperosmotic range was observed only in the 70% salinity supports further the
notion that the animals are more tolerant to hyperosmotic conditions than to hypoosmotic ones.

Examination of the weight fluctuations in the attached and unattached animals (Fig. 5-7 and Fig. 8) again supports the clamping response hypothesis. In all cases of salinities outside the normal range, the weight fluctuations for those animals which were allowed to remain attached were markedly less than were the fluctuations found in the unattached group. Since both the attached and unattached groups were maintained under identical conditions for similar time periods, the weight changes of the unattached animals (Fig. 8) may be considered as ideal behavior for the animal, in the absence of any control factors. By contrast, the weight fluctuations of the attached group represent the animal as it would behave under approximately normal physiological conditions. In the latter case, it is evident that the clamping response is operative. This is most obvious in those salinities hyperosmotic to the normal range; here, the weight variations have a marked tendency to level off. The discrepancy between weight variations is also evident in the hypoosmotic stress salinities, but the differences between attached and unattached animals are not as pronounced in this case. This is due to the fact that the experiment was conducted for 24 hours. It has been demonstrated that in some cases 24 hours is sufficient time for the slow inward movement of water to cause sufficient swelling of the foot to nullify the
clamping response, depending on the degree of irregularity in the shell margin.

An examination of the weight fluctuations of the unattached animals (Fig. 8) shows the curve produced to be asymptotic about the acclimation salinity of 24%. Furthermore, little change in weight is observed between the salinities of 20 and 40%. This is approximately the same range defined earlier as normal, or the "non-stress" salinity range for the animal.

Gross (1954) and Segal and Dehnel (1962) make use of the formula for the volume of mobile (osmotically active) water:

\[
V = \frac{C_2 (\Delta W)}{C_1 - C_2}
\]

\(C_1\) = the original acclimation salinity (100% sea water)

\(C_2\) = the concentration of sea water into which the animals are transferred

\(\Delta W\) = the change in weight of the animal after equilibration of the body fluids has been attained after transfer

If the animal in question were behaving as a perfect osmometer, i.e. permeable to water but impermeable to salt, the volume of osmotically active water would be a constant at all salinities. This is based on the assumption that given any increment of change in external concentration, there will be a corresponding proportional increment in weight change observed.

A simpler method for demonstration that an animal is behaving as an osmometer would be to show that the product of the osmotic pressure of the body fluids and the
water content of the body is a constant at all salinities. Assuming a constant weight of the animal is non-water in all salinities, it follows that, if weight fluctuations were due solely to water movements, the product of the weight of the animal and the osmotic pressure of the body fluids should be constant at all salinities. Since the osmotic pressure is directly proportional to the freezing point depression ($\Delta T_f$), the product of the $\Delta T_f$ of the hemolymph and the weight of the animal at all salinities should be a constant, if only water movements are occurring.

Although application of either of these two methods for determination of mobile water yields non-constant values for the different salinities in the case of S. pacinata, the possibility that the animals are behaving as osmoconformers outside the normal range cannot be ruled out, although it would be unique among the molluscs if such were found to be the case. Within the normal range of 20-45%, the animals show little predictability regarding weight fluctuations. This holds true even though the animals have been acclimated to the 24% control salinity for at least one week. Such a wide range of salinity tolerance in the unattached state may possibly imply that some sort of ionic regulatory mechanism is present and operative within the normal range. In the stress salinities, the animal shows considerable and fairly consistent weight gain or loss. This implies that water movements are occurring, although no conclusions may be drawn from this study.
concerning the reasons why the volumes of mobile water differ in the different salinities. Evidence has been presented that the total osmotic concentration, the \( \text{Na}^+ \) concentration, and the \( \text{Cl}^- \) concentration of the hemolymph closely approximates that of the external medium over the range of salinities tested. In light of these findings, the assumption is made that any sort of ionic regulation which may be occurring does not involve \( \text{Na}^+ \) or \( \text{Cl}^- \) ions. There are two possible alternatives to explain the reason why these animals show different weight loss or gain in hyperosmotic or hypoosmotic salinities than that predicted for a perfect osmometer.

One explanation would be that some osmotically active constituent in the hemolymph appears in disproportionate concentrations in different salinities, that is, some sort of ionic regulation is occurring which cannot be explained in terms of \( \text{Na}^+ \) or \( \text{Cl}^- \) ion fluctuations.

A second possible explanation of the phenomenon concerns the actual localization of water movements in \( S. \ pectinata \). The weight loss (gain) in hyperosmotic (hypoosmotic) salinities is not reflected in the hemolymph volumes. Rather, all swelling or shrinking takes place intracellularly. This has been shown for other species of molluscs as well (Potts, 1954; Krogh, 1939). All observations made during hemolymph collection in the experiments herein described with \( S. \ pectinata \) have revealed that in hypoosmotic salinities, the foot of the animal swells and thickens.
Concurrent with this swelling is a drop in blood volumes to the point that, in the extreme dilutions (5%), it becomes almost impossible to collect a measurable amount of hemolymph. Conversely, in the hyperosmotic salinities, hemolymph volumes increase with increasing salinity, while the foot itself shrinks to a point that, in the extreme cases (70%) it appears almost paper-thin. This phenomenon was also observed during the dessication experiments. In this case, the tissues had become dehydrated to the point of rigidity in the latter stages of dessication. However, with increasing water loss, hemolymph volumes remained fairly constant.

This observation indicates that weight fluctuations which occur in this animal are realized in the tissues rather than in the hemolymph. Therefore, responses to salinity gradients apparently are reflected in the tissues and the hemolymph itself appears to play a passive role in response to these concentration differences. This observation explains in part the reason for reduction in blood volumes with increasing total body water during hypoosmotic stress. The cells of the foot swell, and this swelling in turn leads directly to occlusion of the foot sinuses and hemocoel. Conversely, in hyperosmotic solutions, shrinking of the cells of the foot occurs, leading to an increased volume of available space in the foot sinuses and hemocoel and a corresponding increase in blood volumes.

With these observations in mind, the complexities
involved in any attempt at predicting weight fluctuations of the animal in different salinities become clear. Ity was mentioned earlier in connection with the mobile water considerations, that one method of demonstration that a given animal behaves as an osmometer consists of showing the product of the osmotic pressure of the body fluids and the water content to be a constant at all salinities. In the case of *S. pectinata*, this formulation would necessarily be required to include some consideration of the osmotic pressure of the tissues, i.e., of the cytoplasm of those cells which play an active role in the response to the osmotic gradient. Furthermore, only a discreet area of the animal, the foot, was observed to display obvious swelling or shrinking.

It is concluded from these considerations that no speculations can be made as to whether the animal is behaving as an osmometer. It has been shown, however, that water movements do occur, and that the clamping response acts as a mechanico-regulatory aid to the animal in restricting this flow. Any deviation from a theoretical value for mobile water calculations bears no direct relation to the clamping response, although the possibility of an internal ion regulatory mechanism which would complement this mechanical regulatory mechanism would certainly be advantageous and cannot be ruled out from the results provided.

In conclusion, the clamping response lends to the
false limpet, *Siphonaria testinata*, the potential to evade environmental stresses due to dessication or severe osmotic concentration fluctuations for reasonable periods of time. The animal is a pulmonate mollusc, a group composed mainly of terrestrial forms. Indeed, it may be argued that members of the genus *Siphonaria* are, in fact, terrestrial mollusces, since most members reside in the splash zone and are oftentimes alternately submerged and exposed with the tides. Considering the fact that the animal has been observed to respire air when exposed and water when submerged, it is suggested that this species is in a transitory phase of its evolution. The mantle cavity has been specialized into a highly vascularized "lung," similar to other pulmonates which show this unique anatomical feature. However, in *Siphonaria*, this lung has been further specialized and contains a pseudobranch, or secondarily derived gill. Thus the animal may be termed an "amphibious" mollusc, surviving under both aquatic and terrestrial conditions. It is therefore proposed that the clamping response has evolved in this species as a mechanism to prevent the effects of dessication during periods of exposure during low tides. The response, as the animal has become submerged for increasingly long periods of time, has been further specialized to the point that it has become a mechanism to guard against osmotic swelling or dehydration during periods when the salinity reaches stress levels outside the normal tolerance limits.
REFERENCES
REFERENCES


Review articles
Table 1

Average values for the ionic constituents of sea water

<table>
<thead>
<tr>
<th>Ion</th>
<th>Composition % Sea salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (Na)</td>
<td>30.61%</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>1.10</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>3.69</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>1.16</td>
</tr>
<tr>
<td>Strontium (Sr)</td>
<td>0.04</td>
</tr>
<tr>
<td>Boron (as H$_3$BO$_3$)</td>
<td>0.07</td>
</tr>
<tr>
<td>Chloride (Cl)</td>
<td>55.04</td>
</tr>
<tr>
<td>Sulfate (SO$_4$)</td>
<td>7.68</td>
</tr>
<tr>
<td>Bicarbonate (HCO$_3$)</td>
<td>0.41</td>
</tr>
<tr>
<td>Bromide (Br)</td>
<td>0.19</td>
</tr>
<tr>
<td>Fluoride (F)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

99.994%

Table II

Salinities measured at South Jetty, Galveston, Tex. on Collection days

<table>
<thead>
<tr>
<th>Date</th>
<th>S%</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/10/65</td>
<td>20.5%</td>
</tr>
<tr>
<td>6/15/65</td>
<td>28.8</td>
</tr>
<tr>
<td>6/24/65</td>
<td>25.2</td>
</tr>
<tr>
<td>6/28/65</td>
<td>27.7</td>
</tr>
<tr>
<td>7/7/65</td>
<td>25.2</td>
</tr>
<tr>
<td>7/14/65</td>
<td>26.9</td>
</tr>
<tr>
<td>7/20/65</td>
<td>31.0</td>
</tr>
<tr>
<td>7/25/65</td>
<td>21.1</td>
</tr>
<tr>
<td>8/2/65</td>
<td>19.5</td>
</tr>
<tr>
<td>8/8/65</td>
<td>16.4</td>
</tr>
<tr>
<td>8/14/65</td>
<td>17.2</td>
</tr>
<tr>
<td>8/20/65</td>
<td>16.9</td>
</tr>
<tr>
<td>8/29/65</td>
<td>27.2</td>
</tr>
<tr>
<td>9/20/65</td>
<td>24.4</td>
</tr>
<tr>
<td>11/13/65</td>
<td>19.4</td>
</tr>
<tr>
<td>12/6/65</td>
<td>20.7</td>
</tr>
<tr>
<td>1/8/66</td>
<td>20.3</td>
</tr>
<tr>
<td>1/30/66</td>
<td>21.6</td>
</tr>
<tr>
<td>2/15/66</td>
<td>24.4</td>
</tr>
<tr>
<td>3/11/66</td>
<td>25.6</td>
</tr>
</tbody>
</table>

Average   23.0%