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ALIZARIN DEPOSITION BY CORALS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY MAY 1973

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Abstract

The hydroquinone dye, alizarin red S, was employed to visualize sites of calcification in reef corals under controlled laboratory and aquarium conditions using the reef coral *Pocillopora damicornis* Lamarck and ten other species found in Kaneohe Bay, Oahu, Hawaii.

Alizarin is taken up by living corals most effectively if added to the intake of a running seawater aquarium giving a final concentration of 10ppm.

Reef corals deposit alizarin in a pattern corresponding to the calcium deposition during the test period, to produce a visible magenta stain. Areas of uptake were erratic and did not follow polyp boundaries. Deposition was often most marked in areas far removed from zooxanthellae concentrations.

The deposited alizarin was redissolved from the coral skeleton with 10% EDTA solution which had been adjusted to the pH of seawater and quantitative measurements were made spectroscopically. Simultaneous uptake studies employing alizarin and radio-calcium in the form of $^{45}$Ca indicate a high correlation between these methods and that alizarin uptake is an accurate measure of calcification in reef corals. Statistical regression analysis of the two methods gave $r = .987$, $p = .01$.

Incorporation of the dye reflects the biological activity of the coral and was deposited most actively by the youngest growth forms, followed by a plateau of activity
which indicated that radius of growth was about equal in all medium sized colonies studied.

In light-dark experiments, heads of coral were split and half was preconditioned in total darkness for 48 hr. Both halves were then subjected to alizarin during the following 48 hr. The dye uptake in the halves subjected to dark almost equaled and at times exceeded the alizarin uptake in the halves subjected to light.

Alizarin incorporation was assessed in *P. damicornis* subjected to incremental increases in inorganic phosphate. The dye uptake decreased linearly with the logarithmic increase in the ambient phosphate but was still apparent to a concentration of $10^4$ times the normal for seawater.

Various reef corals were compared as to their ability to deposit alizarin over a given period of time. They could then be ranked as to growth potential. Some specimens were marked and returned to the reef for long term growth records. A large colony of *P. damicornis* showed a 1 cm linear growth in five months.

Calcification was observed in newly settled polyps of *P. damicornis*. In laboratory experiments the available calcium in seawater was decreased. Active deposition of alizarin occurred when calcium level was lowered to one third of seawater normal.

Coral polyps can live for 48 hr. but do not deposit alizarin in seawater diluted to 50%. Normal alizarin deposition occurred in seawater diluted or concentrated 10%
from normal but was decreased with dilutions or concentrations of 20% normal.
INTRODUCTION

Modern reef corals have inhabited this earth since the Triassic age and through the immensity of their numbers have laid down the foundations that constitute the essence of the dynamic structure known as the coral reef. The anatomy of the living coral has been recorded and morphological classifications have been made on the basis of their skeletons. Their metabolism has been investigated and many investigators are scrutinizing the ecology of coral reefs. Considering that a coral reef community is one of the most successful biological assemblages that this earth has ever known, astonishingly little is known of the coral animal itself and how this creature can construct in the face of seemingly insurmountable obstacles, a frail skeleton which can endure for eons.

Coral growths have been known and studied from antiquity but there was a heightening of interest in the 18th and 19th centuries when seamen brought back specimens to be examined by naturalists such as Linnaeus and Lamarck. Corals were not even known to be animals until so reported by Peysonnel in 1735. Nineteenth century exploring expeditions carried naturalists of whom the best known is Darwin (1842) who developed a profound and lifelong interest in coral reefs and atolls. Many naturalists recorded their impressions, made collections, and reported their findings. Several of these men came to the eastern and central Pacific basin and
examined the coral growths with which we are concerned (Stutchbury, 1830; Dana, 1846; Verill, 1870; Agassiz, 1889).

Histological studies on coral polyps were first made in the middle of the 19th century and thereafter a wealth of information relating to the coral animal and its skeleton was published so that Vaughan and Wells (1943) could append a bibliography of 1000 references to their monumental revision of coral taxonomy. Comprehensive review articles are available on all aspects of coral and coral reefs and no attempt will be made here to survey coral reef literature. A brief review of the anatomical terminology relating to corals is presented in Appendix A.

Many aspects of the process of calcification of corals have been studied. See Muscatine (1971) and Vandermeulen (1972). The process itself has by no means been explained. Goreau (1959a) made the following observations and drew some conclusions: (1) Corals that contain the symbiotic algae called zooxanthellae calcify faster in the light than they do in the dark; (2) Corals with zooxanthellae calcify faster in both light and dark than controls without them; (3) Corals incubated in carbonic-anhydrase inhibitors calcify at greatly reduced rates. Goreau assumed that the calicoblastic layer of the polyps secreted a muco-polysaccharide matrix and when carbon dioxide was removed by the zooxanthellae through photosynthesis, the calcium carbonate precipitated in this matrix.

Simkiss (1964) did not propose a theory of calcification
per se but suggested that zooxanthellae remove phosphate ions which he termed "crystal poisons" and their removal allowed spontaneous precipitation of calcium carbonate to form the aragonite coral skeleton.

Further investigation by Muscatine and Cernichiari (1969), Lewis and Smith (1971), Trench (1971 a,b,c), Young et al. (1971), and Muscatine et al. (1972) demonstrated that zooxanthellae in hermatypic corals were able to fix carbon photosynthetically and from these symbiont algae it moved to the host coral where it was incorporated into the skeleton and the matrix.

Calcium carbonate deposition by reef corals is obviously a highly effective process and it is not known whether the various hypothetical processes proposed are all operative, or if some of them are mutually exclusive.

Chave (1971, personal communication) pointed out that reef corals live in a supersaturated calcium carbonate environment and apparently can regulate skeletal production. It has not been determined whether aragonite deposition by corals is an active secretory process or a passive phenomenon whereby coral tissues remove inhibitory agents from seawater as it passes through them with subsequent spontaneous precipitation. It is known that in tropical seas where coral reefs occur, seawater is characteristically greatly super-saturated with aragonite (Pytkowicz, 1963; Lyakhin, 1968). Is it possible that this super-saturation is essential for coral reef calcification?
When newly settled coral polyps were viewed with magnification it was not always possible to determine when skeletogenesis had begun. Techniques for visual enhancement of the skeleton in situ were sought. Kendall (1971, personal communication) suggested alizarin as one means to effect this.

I have studied alizarin as an agent to demonstrate calcium carbonate as it was being deposited by corals. The solubility of alizarin in seawater was measured. The toxic properties of the dye on corals were studied and the optimum level of alizarin in solution was determined so that the most satisfactory deliniation of the newly deposited skeleton would result. An absorption maxima of alizarin in seawater was determined. Techniques were developed for spectrophotometric quantification of alizarin that had been deposited. This was combined with radio-calcium studies to demonstrate that alizarin is deposited through biological activity in the same pattern as calcium.

Coral growth was investigated by adding alizarin to running seawater using adult forms of reef corals to study growth patterns and for comparative studies relating growth to colony size. Light-dark experiments were formulated to test the effect of light deprivation on alizarin deposition. The level of ambient seawater phosphate was increased and the effect on alizarin deposition was noted and measured. Techniques for marking corals were developed for use in long term growth studies.
Newly settled coral polyps were raised in quantity and were subjected to a series of laboratory studies in which various seawater parameters were changed. Seawater calcium and salinity levels were changed to study the effect on the alizarin deposition by newly settled coral polyps.
II. MATERIALS AND METHODS

Facilities

The Hawaiian Institute of Marine Biology (H.I.M.B.), a facility of the University of Hawaii, is located on Moku-o-loe Island (Coconut Island), Kaneohe Bay, Oahu, Hawaii. It served as a base for all experiments performed in this series. All laboratory experiments were done at a constant temperature of $25^\circ C \pm 0.5^\circ$ and a relative humidity of 70%. Immediately adjacent were sheltered racks with a capacity to hold 12 or more 24 liter aquaflair plastic aquaria. Running unfiltered seawater was pumped through polyvinyl pipes and was available at all times. Natural light was supplemented by banks of white fluorescent lamps suspended 50cm above the water. These were controlled on a 12 hr on 12 off cycle approximating that of sunlight. Water was aerated with airstones.

Kaneohe Bay is situated on windward Oahu and the prevailing winds are such that within a mile of the laboratory luxuriant coral growth could be obtained that was relatively free of the effects of urban pollution. All specimens were gathered in water of less than 3 m and only mask, snorkel and fins were required. When corals were attached to pieces of rubble they were picked up with their base of attachment, otherwise they were dislodged with a geological hammer and were kept under water as they were transferred in plastic pails to the seawater tables of the
laboratory. All investigations in this study were performed during the summer of 1972 and water in the open aquaria ranged from 24°C to 26°C with less than one degree variation in any 24 hr period.

Experimental Animals

_Pocillopora damicornis_ Linnaeus is widespread in the Indo-Pacific and is readily available in Kaneohe Bay. It planulates spontaneously at the time of the waning moon (Edmondson, 1929; Harrigan, 1972) especially during the summer months. Techniques for raising planula larvae and the newly settled polyps of _P. damicornis_ as developed by Reed (1971) were used and modified as necessary.

Large heads of _P. damicornis_ were placed in an aquarium and when spontaneous planulation occurred, the drifting planulae were carried with the overflow into a plastic catch-basin. This was a 15 x 15 cm cylinder with the lower end fitted with fine-mesh plastic gauze. It was lowered into a bowl so that a constant water level of 5 cm was maintained. Glass microscope slides and plastic slides of the same size were suspended vertically in the seawater from slotted blocks of floating polyurethane foam. Many of the planulae settled on these slides which could then be examined quickly by lifting out the slotted blocks. Those slides to which healthy polyps adhered were put in holding tanks or taken to the laboratory for immediate use. During experiments the slides were held in position in small
plastic slide holders which could be lowered into the experimental tank.

In certain areas of Kaneohe Bay continuous water currents over coral rubble permit myriads of *P. damicornis* to settle. They grow to about 5 cm across but are then washed away and destroyed. Many small heads weighing from 6 to 10 g wet weight could be picked up with their basal attachment within an area of 10 m$^2$ and approximately 1 m deep at low tide. Such heads were used in studies of uptake of alizarin of radio-calcium.

Edmondson (1929) demonstrated that *Cyphastrea ocellina* Dana would planulate when subjected to mild stress by elevating the water a few degrees. Some experiments were carried out using polyps of *C. ocellina* as an experimental animal. It was found that these planulae were not always available during winter and spring months and when the planulae are released following heat stress settling was often delayed and erratic and subsequent growth was very slow. Not enough settled polyps were ever obtained for the type of comparative studies planned.

*Fungia scutaria* Lamarck form solitary polyps the largest of which measure about 20 cm in length and weigh about 1 kg. It grows on reef flats and was used in experiments on alizarin deposition as related to size of the corallum and in light-dark experiments.

Seven other species of hermatypic reef corals and the ahermatypic coral *Tubastrea aurea* were used in an experiment
on growth patterns as shown by alizarin deposition.  
(Chap. IV)

Laboratory Equipment

Standard pyrex glassware was used except for some experiments where commercially available plastic refrigerator containers were found to be satisfactory. Seawater was obtained from the aquaria and was filtered through a Whatman #1 filter paper in laboratory experiments. For alizarin concentrations to be used in coral studies and in pH variations the seawater was also filtered through a Millipore filter (Millipore Filter Co.) with a pore size of 0.9 μ. In all experiments seawater from Kaneohe Bay was used as a standard incubating medium and when the effect of changing some of the constituents of seawater was being investigated, this standard medium was modified.

pH determinations were made with a Beckman pH meter (678 D) with an expanded scale and standardized with two buffers. Salinity was determined with an AO-T/ refractometer. Centrifugation of specimens was by table model centrifuge (International Clinical Centrifuge, Table model CL) with a maximum speed of 3000 rpm.

Chemicals employed were of reagent quality. Alizarin (9,10 anthraquinone 1,2 di-hydroxy, Elseviers, 1948) is very slightly soluble in water; however, the sodium sulfalizarinate is much more soluble (7.69%) so this is commonly used in biological studies. It is referred to as alizarin -s,
alizarin red, alizarin red-s, or alizarin. The product used and found to be satisfactory was alizarin red-s certified for staining bone, C.I. number 58005.

Photography in these studies was performed using a Honeywell Spotmatic camera with various supplemental lenses and flash equipment. Some of the photomacrography was done through a Wild dissecting microscope and a monocular camera attachment. Kodachrome-II and SuperEktachrome film were used.

Time-lapse microcinephotography was performed with a Bolex 16 mm camera attached to a Sage series 500 cinephotomicrography apparatus. The camera was mounted above a Wild dissecting microscope with a monocular tube and iris diaphragm. Kodak Ektochrome film with an ASA rating of 160 was used. A synchronized Norman A-150 electronic flash 15 cms and at an angle of $45^\circ$ above the subject gave sufficient light for a photographic image through a 12 power enlarging objective.

A plastic container holding 5 liters of seawater was beneath the microscope. The organism to be photographed was suspended just below the water level or if it had settled on glass, the slide was reversed and supported so that the lower face was in seawater and the upper surface was above the water level. Convection currents were produced by a fine stream of air to the water surface. Photographic sequences were begun at 8 frames/min after the organism had been acclimated for 15 min. This run was continued so that
screen time would be at least 10 seconds. Stock solution of alizarin in distilled water to give 10 ppm total concentration was added and the photographic sequence was resumed at once for a comparable projection time after exposure to alizarin. Photographic sequence was then shifted to 1 frame/min for 24 hr or longer to observe the skeletal formation. Before discontinuing an experiment another run was obtained at 8 frames/min.

**Techniques**

A stock solution of alizarin was prepared with 4 mg of the dye in each ml of solution in distilled water. When a range of values was necessary, various amounts of this stock solution were pipetted into the seawater. In experiments with coral in aquaria supplied with running water, alizarin was added continuously to the intake to give an ambient concentration of 10 ppm. To effect this the water intake was adjusted so there was a seawater residence time in the aquarium of 2 hr. Alizarin was mixed with distilled water in a large plastic Clorox container with a stop-cock cemented to the bottom. Tubing led to a buret partially filled with glass-wool as a filter. An intravenous drip chamber, plastic tubing and hypodermic needle permitted accurate regulation of the alizarin flow. See Appendix-B, Figure 36.

In experiments where ambient phosphate concentration was changed, sodium mono-phosphate was mixed with alizarin
in distilled water and entered the aquarium through the same tubing. Alizarin did not appear to be altered by this procedure.

Determinations of calcium were made using the methods of Tsunogai et al. (1968) which permits a titrimetric determination of calcium in the presence of large amounts of magnesium such as is found in seawater. Calcium was extracted into a small volume of organic solvent as its glyoxal-bis (2-hydroxanil) complex. Calcium was then titrated with EGTA (ethyleneglycol-bis (2-aminoethylether)-N,N,N',tetra-acetic acid). Ordinarily the end point is sharp and occurs when the red color of the organic layer vanishes; however, because of the alizarin the method as described became unsuitable. The red color of alizarin in solution at the pH used, was virtually identical with the complexed calcium salt being titrated. Even though calcium had been extracted in butanol and floated on the larger volume of substrate, an end point determination was at first not possible. The procedure was altered so that titration was carried out in 150 ml beakers. A plug of commercial paraffin wax about 13 cm thick was fashioned to fit snugly within the beaker; the sides of the plug were fluted and there was a central perforation so that when this plug floated on the substrate it could be immersed easily by tapping it with a stirring rod. This served to keep the solutions mixed. A V slot was cut in the upper surface of the wax and the organic solvent collected into this.
Paraffin is heavier than butanol so the two layers were effectively separated by a barrier and the red complexed calcium could be titrated against a white background.

Spectroscopic determinations of the amount of alizarin dissolved in seawater or EDTA (ethylenediaminetetraacetic acid) were made with a Bausch & Lomb Spectronic-20 instrument at a reading of 548 µm unless otherwise specified. Radio-calcium was in the form of $^{45}$CaCl$_2$ (New England Nuclear) 10 mCi/m mol diluted to yield a final activity of 3 µ Ci/ml of medium. Levels of radio-activity were determined with an end window Geiger-Müller tube (LND-773) and a scaler (Nuclear Supplies, Model-250, Ancino, Calif.).

Light intensity at the water surface in the experimental tanks was measured with a Weston Illumination meter, model 756; Daystrom Incorp. Newark, N.J. The incident light at the water surface was approximately 1000 footcandles in all experiments using supplemental lighting.
III. ALIZARIN AS A CORAL MARKER

History and Properties of Alizarin

Alizarin was used as a textile dye throughout the ancient world. The orgins of the art are lost although it was known in India, Mesopotamia, and Egypt where it was used many centuries before the Christian era. Remnants of burial garments dating from that period still retain the characteristic red-purple color imparted by treatment with extract of madder root. This plant, indigenous to the above-mentioned countries, is referred to in the Greek Herbal of Eionecious written in the first century A.D. as a multi-purpose crop (Gunther, 1934). He noted that the Romans called the plant *rubia pasiva*. The roots were used for dyeing and medicinally and some of the toxic properties were understood for it was used as an abortifacient.

The present name is derived from the Roman designation of the root, *lizera*. The common name of this plant became madder and in later years the species of the near east was classified as *Rubia tinctorum* L and that of the Indian subcontinent became *Rubia cordifolia* L. For centuries the peoples of the near east have regarded madder as having magi-cal properties and used it medicinally to ward off witchcraft. Steckoll et al. (1971) reported that very ancient bones of human origin excavated in the Qumran region of the Jordan valley had traces of red pigment which were identified as alizarin. He claims that the Arabs living in the vicinity
still use madder extract to ward off the evil eye.

Madder root continued to be used as a vegetable dye. A surgeon, Mr. Belchior, noted that some pork he had eaten had red bones (DuHamel, 1739). He investigated and described his findings by saying "Bran, after it had boiled in a copper with calicoes in order to clean them from the dirty color occasioned by an infusion of madder root, was fed to hogs and stained their teeth and bones." Subsequently, DuHamel began active experimentation and demonstrated that when madder root was fed to birds, only the growing bones incorporated the dye but if fed in excessive amounts the birds sickened and died. With the beginnings of histological studies, vegetable dyes including that prepared from madder became important. These were discussed by Cameron (1930) and later by Putchler (1968) who noted that the dye prepared from madder root actually consisted of six separate anthraquinone variants of which only is today known as the dye alizarin.

When Perkin discovered the aniline dye mauve, the dyestuff industry was revolutionized. Alizarin was synthesized scarcely a decade later by Graebe and Lieberman (1868), as were many of the anthraquinone derivatives which became the mainstay of the dye industry and the base for such common dyes as turkey red. Sodium alizarin-sulfonate or alizarin red has been used as a vital stain (Ball, 1926) and as one of the better stains for calcium (Cameron, 1930).

In the past century it has been used in anatomical
studies of bone growth (Hoyte, 1960, 1968). Dixon and Hoyte (1963) used alizarin in combination with radio-calcium in auto-radiographic preparations of growing bone to show that both had the same qualitative pattern of deposition. No quantitative estimates were attempted.

Meanwhile, alizarin continued to be used extensively for in vivo staining of calcified tissues but for the most part, these were in terrestrial vertebrates and the dye was usually given by injection. It was also used in solution for fresh water aquatic animals. Kendall (1961) fed alizarin to sharks to show calcium deposition in scales and cartilage. Hidu and Hawks (1968) and others, demonstrated that shellfish could incorporate alizarin. Barnes (1971) used alizarin in corals. He also found that it would color the tubes of sepulid worms when used in concentrations from 10 ppm to 150 ppm. He found alizarin solutions in seawater to be stable at 37°C for two days. He noted a flocculent suspension occurred when the concentration was more than 30 ppm and he believed it was due to a combination of the dye with calcium. His relatively short term studies did not demonstrate any markings in a bivalve mollusc or in a hydrozoan. In subsequent experiments he used alizarin at 20 ppm in seawater and found that it was incorporated in the newly formed skeleton of corals without distorting or disrupting the continuity of the skeleton. He referred to "semi-stable units of fanning" which comprise the microscopic architecture of coral septa. These had previously been described as
sclerodermites by Milne-Edwards and Haime (1867). Barnes showed that these were examples of competitive crystal growth and when corals were kept in alizarin solutions, crystals of the skeleton had bands of the characteristic color of alizarin as if the dye molecules had been incorporated within the aragonite crystal matrix with no visible change except in color.

Alizarin has the formula \( \text{C}_{14} \text{H}_3 \text{O}_4 \) and a molecular weight of 240.3. The structural formula of the more soluble sulfalizarinate is given in Figure 1. It is ordinarily precipitated as orange or red needle-like crystals which are very slightly soluble in water and slightly more soluble in alcohol. The form used in these studies is an indicator dye with a color range from yellow to violet between pH 5.5 and 6.8. It changes from violet to purple in the range pH 10.1 to 12.1. When dissolved in seawater it imparts a magenta when compared to standard Kodak color filters.

Alizarin is an acid dye which forms salts with metal cations. Such chemical combinations often form insoluble substances this being the characteristic reaction in textile dyeing. In such instances ferrous iron, aluminum salts, or others are applied to the textile as a mordant which serves to hold the dye in an insoluble and permanent deposit. Such an insoluble combination of dye and metal cation is called a lake. Certain dyes form soluble lakes, calcium sulfalizarinate being of this group. It can be used, however, in combination with aluminum salts and then becomes the basis
Figure 1. Structural formula of alizarin-red-s.

1,2 dichlorbenzene  Sodium 2,3 dihydroxy-4,5 dicarboxy benzenesulfonate

Figure 2. Breakdown products of Sodium alizarin sulfanate
for such old favorites such as turkey red dye.  

Alizarin is a hydroquinone ketone and when in solution with a strong oxidizing agent such as sodium hypochlorite (Clorox) or hydrogen peroxide, it breaks down to give colorless breakdown products (Morrison, 1966). Some representative ones are shown in Figure 2.

The colors of the organic molecule are evoked by varying degrees of chemical unsaturation which gives rise to molecular resonance but also makes them chemically reactive. When the resonance matches in frequency and absorbs certain fractions of the visible spectrum the residual or reflected light gives the compound its characteristic color (Fox, 1972). Elsvier's (1948) gives the absorption maxima for alizarin in alkaline solution as 557 μm. This is in the red or purple range and Fox points out that many living organisms employ quinones in this color range. The purpose is often obscure but he believes they may act as catalysts. Corals also have these colors; however, Fox (1953) states that these are not due to quinones. I have found no references to the specific agent responsible for the color of the corals being studied.

The nature of the combination of alizarin and bone has been discussed since the time of Belchior (DuHamel, 1739) who decided that it had to do with pore size of the tissues involved. After alizarin was synthesized, extensive studies were done by textile chemists and it was known that alizarin could combine with earthy metallic cations. Ercoli (1943)
decided that the dye was held in combination with the phosphate in the bone mineral apatite. Myer (1968) remarked how little was known of the chemical reactions of alizarin and bone. He realized that alizarin was bound to the calcium and stated that the dye was capable of forming a chelate ring between the carboxyl and the adjacent hydroxyl and that metallic ions were held in that position. He believed that the dye then assumed the position on the surface of the calcium carbonate crystal so the long axis of the dye molecule was parallel to the surface of the crystal.

Putchler (1969) said that chemical and infra-red spectroscopic data showed that calcium sulfalizarinate was a salt and not a chelate. He said that alizarin does not have a specific affinity for calcium and he offers a number of hypotheses as to how alizarin might be bound to calcium and thus precipitated with the calcium in its various compounds.

Despite the fact that the mode of action of alizarin is not understood, it remains a satisfactory material for coloring calcium compounds that are deposited biologically.

Coral Growth in Alizarin Solutions

Some marine organisms can extract alizarin and calcium from seawater and deposit them as a colored form of calcium carbonate. Alizarin is toxic in large quantities but there has been no unanimity of opinion as to the amount of dye to
add to solutions to give maximum coloration with minimum toxic manifestations. I did three types of studies each using a different stage of coral growth to determine an optimum. These are labeled I, II and III.

Method
I. Seven finger bowls each containing 200 ml of seawater were set before a window facing north which received natural but no direct sunlight. Alizarin solution was pipetted into the seawater to give a range of concentrations from 1 ppm to 40 ppm. Table 1. One slide with healthy, newly settled polyps of P. damicornis was lowered into each bowl and left for 24 hr. These slides were then soaked in fresh water until all organic material was washed away from the polyps. They were immersed briefly in dilute Clorox to destroy passively adherent alizarin, and were air dried.

II. Ten plastic deep freeze cartons were labeled and in the first seven, one liter each of seawater was poured. Seawater which had been passed through a 0.9 μ Millipore filter was used in the last three cartons. Alizarin was added to give a series of concentrations from 2 ppm to 40 ppm. Filtered air was bubbled through each container using airstones. Constant illumination was supplied by two fluorescent lamps suspended 50 cm above the water level. pH of seawater before and after the 24 hr experiment remained at 8.15 ± 0.02. Glass slides with newly settled polyps of P. damicornis were placed in each container.
A freshly collected head of *P. damicornis* was broken up so a seven cm central branch weighing from 10-12 g wet weight could be placed in each tank. After 24 hr the specimens were cleaned as in Series I.

Table 1. Varied concentration of alizarin in seawater in three series to evaluate the deposition by corals.

<table>
<thead>
<tr>
<th>Specimen Number</th>
<th>Series Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Newly settled</td>
</tr>
<tr>
<td></td>
<td>polyps</td>
</tr>
<tr>
<td>1.</td>
<td>1 ppm</td>
</tr>
<tr>
<td>2.</td>
<td>2 ppm</td>
</tr>
<tr>
<td>3.</td>
<td>5 ppm</td>
</tr>
<tr>
<td>4.</td>
<td>10 ppm</td>
</tr>
<tr>
<td>5.</td>
<td>20 ppm</td>
</tr>
<tr>
<td>6.</td>
<td>40 ppm</td>
</tr>
<tr>
<td>7.</td>
<td>0 ppm</td>
</tr>
<tr>
<td>8.</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td></td>
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<tr>
<td>10.</td>
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</tr>
</tbody>
</table>

(M. indicates ultrafiltration)

III. The same containers and lighting were used with alizarin concentration from 1 ppm to 40 ppm in seawater as in Table 1. One small head of *P. damicornis* weighing from 6 - 10 g wet weight was put in each tank and after 24 hr the specimens were cleaned as in series 1.
Results

The magenta color from dissolved alizarin was less intense at the termination of all experiments than it was at the beginning, presumably due to oxidation of the alizarin and by absorption by the corals. This was most apparent in vessels having the least original dye content but it was not predictable. The coral skeletons showed dye incorporation in those containers with 1 ppm of alizarin even though seawater was colorless before 24 hr had elapsed. The receptacles with more than 20 ppm of alizarin usually contained dark red flocculent precipitant often enmeshed in mucus stands. The coral animals remained alive throughout the experiments.

Alizarin was incorporated in the calcium carbonate skeleton of the newly settled polyps in all solutions and on both glass and plastic slides. A light pink color in the skeletons was apparent with the lowest concentrations used. The skeletal color deepened to red with increasing concentrations of dye. Those polyps grown in seawater with 10 ppm of alizarin had skeletons with uniform pink-red color whereas those subjected to 20 ppm exhibited a deep red skeleton with mottling. In concentrations greater than this the color was irregularly distributed and sparse.

In series II, all pieces of coral exposed to alizarin appeared to be living and had evidence of dye incorporation. The bases of the stalks that had no coenosarc along the
fracture line were also colored. When cleaned, that portion without coenosarc retained a purple-pink hue which did not disappear with washing in fresh water for 12 hr but it did so immediately when the specimen was dipped in dilute Clorox. When fully cleaned, a white coral branch remained that was red only at the tip.

Almost all dye had been deposited on the distal portion of the coral stalk. When viewed with magnification the dye appeared to be incorporated in the septa and theca at the rim of the calyces but the lower portion of the septa was often uncolored. The deposition of the alizarin did not conform to the polyp boundaries though the color generally seemed to be a fairly uniform magenta. The polyps on the edges of any involved areas tended to be half colored indicating that part of the polyp could participate in the alizarin deposition while another part apparently did not do so. The transition from colored to uncolored skeleton occurred over a distance of less than 0.25 mm and the borders of the stained areas were irregular.

On inspection of an area with alizarin incorporation it was usual to find that when the upper part of the calyx was colored there would also be a uniform deposit over the dissepimental surface. There was some disparity in that some calyces with good deposition showed no dissepimental coloration and vice versa. There seemed to be no relationship between adjacent calyces in this regard. Some dissepiments in the process of formation retain a central
perforation and alizarin, at times, was deposited as a bright magenta ring on the inner margin of this opening.

In concentrations of alizarin of 30 ppm or more, deposition was spotty so that several areas in the septotheca of the same calyx showed concentrations of dye with none in other areas. In such calyces the distribution of alizarin in the dissepiments was also spotty and usually the skeleton beneath the coenosarc showed no evidence of alizarin deposition. The higher the concentration of alizarin, the smaller the patches of color became and at 40 ppm concentration the upper portion of the calyx was usually uncolored with deposition only within the calyx and on the septo-theca.

There was such a lack of uniformity in alizarin deposition in the coral branches in series II that only approximate ranking could be made in terms of visual coloration. The first seven specimens of this series were about the same size and shape though some obviously had more total alizarin content than others. There was actually little difference in hue. Three examiners ranked the specimens according to the amount of alizarin that had been deposited in each from most to least. Table 2 A.

During this experiment the last three specimens were held in water from which particulate matter larger than 0.9 μ had been removed. Examination of these specimens with magnification showed no gross difference from those reported in the previous paragraph. The same examiners then ranked
Table 2. Coral branches of Series II ranked according to color.

A. Ranking of the first seven branches according to total color from most to least by ppm of alizarin in solution.

<table>
<thead>
<tr>
<th>Specimen Number</th>
<th>Examiner Number</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
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<tr>
<td>1.</td>
<td>2 ppm</td>
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<td>2.</td>
<td>10 ppm</td>
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<td>3.</td>
<td>5 ppm</td>
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<td>4.</td>
<td>20 ppm</td>
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<tr>
<td>5.</td>
<td>30 ppm</td>
</tr>
<tr>
<td>6.</td>
<td>40 ppm</td>
</tr>
<tr>
<td>7.</td>
<td>0 ppm</td>
</tr>
</tbody>
</table>

B. The same group ranked as above but with added specimens that had been subjected to alizarin in seawater which had been passed through Millipore filters. These are marked M. All ranking from most to least coloration expressed in ppm of alizarin in the solution.

<table>
<thead>
<tr>
<th>Specimen Number</th>
<th>Examiner Number</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>1.</td>
<td>2 ppm</td>
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<tr>
<td>2.</td>
<td>10 ppm M.</td>
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<tr>
<td>3.</td>
<td>10 ppm</td>
</tr>
<tr>
<td>4.</td>
<td>30 ppm M.</td>
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<tr>
<td>5.</td>
<td>5 ppm M.</td>
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<tr>
<td>6.</td>
<td>5 ppm</td>
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<tr>
<td>7.</td>
<td>20 ppm</td>
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<tr>
<td>8.</td>
<td>30 ppm</td>
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<tr>
<td>9.</td>
<td>40 ppm</td>
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<tr>
<td>10.</td>
<td>0 ppm</td>
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</table>
all ten specimens. Table 2 B.

Series III coral heads showed a color gradation from very light pink to red. There was uneven distribution in alizarin so that in some heads almost the entire skeleton was colored and in others only the tips of the branches were involved (Figure 3). The irregularity of the surface precluded any accurate measurement of area of alizarin uptake or color intensity. Both of these varied among the specimens. Three examiners evaluated each specimen for color intensity and for area of alizarin deposition. When the specimen was white a value of one was assigned. The colors ranged through pink to red which was given an arbitrary value of five. A similar ranking was done for area of alizarin deposition. A grade of one was assigned when up to 20% of the specimen was colored. There was a gradation so when 80 - 100% of the surface was colored by alizarin a value of five was given. For any one specimen two arbitrary values were thus given, for intensity and for area. The product of these two values gave a specimen rank which could range from one to 25. There was remarkably little variation in the scoring so all products were averaged for comparison (Figure 4).

This study indicated that the greatest area of involvement was attained with 10 ppm of alizarin in seawater or less. Maximum intensity of color was also attained with 10 ppm of alizarin in seawater.
Figure 3. Alizarin deposition in the reef coral *Pocillopora damicornis*. Concentration of the dye is expressed in parts per million in the seawater medium used.
Figure 4. Corals grown in various concentrations of alizarin in seawater were ranked in five grades as to color attained in 24 hr. They were also ranked in five grades as to percent of the total area stained. The recorded alizarin score is the product of these two estimates for each concentration.
Discussion

Alizarin was incorporated in the skeletons of newly settled polyps on both glass and plastic slides. Glass slides had the advantage in that the skeleton could be viewed from below and the septal growth patterns were often strikingly delineated even when the polyp was alive. The polyps settled more abundantly on frosted glass than on plain glass though the frosted surface interfered with viewing the skeleton from below. Plain glass or very lightly frosted slides that had been aged for days or weeks in seawater were most suitable for these experiments.

Alizarin will adhere to a coral skeleton as a passive absorption phenomenon but this is prevented by the presence of an intact coenosarc. There is no evidence that alizarin diffuses through intact coral tissues and in this matter it behaves as does calcium which likewise cannot diffuse passively through coral tissues (Goreau, 1960b). Alizarin that is bound into the skeletal substance through biological deposition, differed in hue from coral skeletons which had been used as controls or which had been allowed to stand in alizarin solutions. The latter were highly colored but had a purple tinge. This color disappeared immediately when specimens were dipped in Clorox whereas alizarin deposited through biological activity showed no color change.

Experimental solutions with small amounts of dye were usually decolorized before the end of 24 hr. This was
spontaneous and depended on the amount of suspended organic matter in the seawater, on the area of living coral depositing calcium carbonate, on the vigor with which air was bubbled through the solution and possibly on other unrecognized factors. With low concentrations of alizarin in seawater, active incorporation was generalized in single polyps and in most small colonies. Active incorporation of alizarin was reduced at the base of the stalks of _P. damicornis_ with a dye level of 10 ppm. At greater concentrations, larger areas of the stalks remained color free. Deposition seemed to be most consistent and most intense on the tips of the stalks regardless of the amount of dye used.

Alizarin is soluble in seawater to about 15 ppm. With greater concentration a flocculent precipitation occurred which remained suspended if the water were moving. Microcinephotography showed that both polyps and coenosarc of the corals are in continual motion (Unreported observations). The rhythmical contractions and twistings of the polyps of _P. damicornis_ as well as the undulating movements of the coenosarc of _C. ocellina_ probably aid in seawater circulation through the ramifications of the gastrovascular system. Depression of alizarin deposition in the higher concentrations used may be due, in part, to mechanical interference with this circulation within the polyps. I suggest that patterns of deposition with increasing concentrations of alizarin show a gradient so that areas at the base are the most sensitive and resistance increases peripherally, reaching
its maximum at the tips of the stalks.

No attempt was made to feed the corals either prior to or during the experiments. The data listed in Table 2-B includes tests in which seawater had been passed through a Millipore filter. Results indicate that *P. damicornis* can function satisfactorily for a time without additional particulate food supplements. The ranking of the specimens which had been tested in Millipore filtered seawater tend to be higher than their counterparts processed in standard seawater. As a result of these experiments, 10 ppm of alizarin in seawater were employed in all subsequent studies. This concentration seemed to give the deepest magenta color attained in any specimens as well as the maximum area of deposition. These studies were interpreted as showing some toxicity by alizarin but little suppression of biological function.

**Toxicity Studies of Alizarin**

References to specific toxicity studies of alizarin on corals have not been found. Ball (1927) tested the effect of alizarin on *Paramecium caudatum* and noted that this animal could live and appeared normal if the cytoplasm did not stain; however, when using alizarin at 30 ppm over half of the animals were dead within 12 hr although the control animals survived. Barnes (1971) showed lessened uptake of calcium by serpulid worms when exposed to 20 ppm of alizarin in a closed container. These experiments were made in
conjunction with others and were to test the toxic manifestations of alizarin on the marine organisms at hand.

Methods

Healthy heads of *P. damicornis* about 15 cm in diameter and polyps of *F. scutaria* about 15 cm long were placed in running seawater aquaria and after a day or two they had expanded their tentacles and seemed to behave much as did their counterparts on the reef. *P. damicornis* did not planulate during this interval. Alizarin was then allowed to enter the seawater intake using the technique previously described. The ambient concentration of alizarin in the aquarium was about 10 ppm at all times. Changes in behavior were noted during the following 24 hr.

Sepulid worms of genus *Spirorbis* frequently settled on slides prepared for coral planulae. Seven such slides with *Spirorbis* *sp* were held in an outdoor aquarium with running seawater and were exposed to continuous concentration of 10 ppm of alizarin for 24 hr under intermittent lighting. The alizarin was then discontinued and the worms remained in running seawater for an additional 24 hr after which they were cleaned with fresh water, immersed in dilute Clorox and air dried. These studies were not all done at the same time and the seven slides represent multiple hatches of *Spirorbis*.

Studies of coral planulae, newly settled polyps and adult coral polyps of *P. damicornis* and *C. ocellina* were
made to observe the effect of alizarin on calcium deposition using time-lapse micro-cinephotography. The organisms were photographed, alizarin was added to give a concentration of 10 ppm in the seawater and changes in behavior were noted.

**Results**

Adult polyps of *P. damicornis* raise themselves well above the level of the corallum and expand their tentacles during daylight hours on the reef and in aquaria. When viewed with magnification the mouth can be seen and it is open. *Fungia scutaria* also projects its tentacles as much as 1 cm during the daylight hours and the mouth gapes widely. When alizarin is added to seawater slowly there was no observable changes in the polyps of *P. damicornis* although if there were mature planulae they tended to be released at that time and settled on prepared slides much the same as did planulae released spontaneously. The tentacles of *F. scutaria* were partially withdrawn and remained so during contact with alizarin. The mouth opening was not as wide as when the animal had been in clear seawater. After exposure to alizarin these polyps recovered completely in 24 hr if returned to a seawater table.

Serpulid worms exposed to alizarin in these experiments incorporated the dye in their calcareous tubes through their entire circumference. There was an abrupt transition from white calcium carbonate to the deep red area where alizarin had been incorporated. The change back to white when the
worm was returned to the seawater table was much less abrupt. In this study seven microscope slides of alizarin marked calcareous tubes were examined. There were a number of species of *Spirorbis* represented but generally, all tubes from any one slide were of the same species and were similar in the manner of tube coiling and in the amount of alizarin deposited. The number of tubes per slide varied from five to 15. The arc length of the colored portion of each tube was compared with the arc length of the succeeding section of uncolored tube. Invariably the colored section was shorter than the colored section. The ratios between the colored and uncolored segments were specific for each species and differed from slide to slide. The highest ratio was 1:6 and the lowest was 2:3 in this experiment. No attempt was made to identify the organisms my species.

Micro-cinephotography did not show any obvious behavioral changes in any of the coral forms studied.

**Discussion**

Edmondson (1929) found that corals would release planulae when subjected to mild stress such as raising the temperature of the water slightly or by increasing the salinity. My experiments showed that if this is carried to near the lethal point of the corals, immature planulae were eventually released if they were present. These did not settle immediately as did mature planulae. Alizarin appears to act as a mild stress on corals and in this way may be
considered to be toxic. It also appears to decrease the amount of calcium carbonate deposited by sepulid worms under conditions of this experiment.

In all studies which involved exposure of an animal to alizarin followed by a return to running seawater, additional uncolored skeleton was deposited. The surfaces of these skeletons were so irregular especially in the case of corals, that areas could not be measured or even estimated quantitatively. It was my observation that more uncolored skeleton than colored skeleton was formed in comparable lengths of time. In the various studies done it was noted that coral calcification as demonstrated by alizarin uptake was more effective when it took place in running seawater. This must be considered if the deposition of alizarin is compared in specimens treated by different methods.

The deposition of aragonite by corals is decreased by the presence of alizarin in seawater; however, the use of low concentrations and relatively short exposure to the dye presumably does not necessitate a change in the interpretation of the data presented in these studies.
Alizarin in Seawater

Solubility and Spectrophotometric Studies

Barnes (1971) noted that alizarin produced a flocculence in seawater at 30 ppm. There have been no studies on the solubility of the dye in seawater nor have there been studies of quantification on alizarin deposition in either bone or invertebrate skeletons. Dixon and Hoyte (1963) used auto-radiography in a study on bone but did not believe quantification of alizarin was possible using that method.

Beer's law (Evans, 1948) states that if a colorant is added to a solution the increased absorption of the light through a standard thickness of solution will be directly proportionate to the amount of colorant added. It was essential to study the solubility of alizarin in seawater before attempting to develop a spectroscopic method to quantify alizarin deposited in corals. Beer's law was the foundation for these experiments.

Methods

A series of alizarin in seawater was prepared in 5 ppm increments from 0 ppm to 45 ppm. A spectrophotometric scan was performed with the 10 ppm tube using a Beckman model DU-2 ultra-violet spectrometer. This was a single beam, null balancing model measuring from 190 µm to 1000 µm at a slit width of 0.08. Millipore filtered seawater was used as a blank.
After an absorbance maxima had been established all samples were measured with a Spectronic-20 instrument. Because of flocculation in some tubes, all were centrifuged for 5 minutes at 3000 rpm. The supernatant was pipetted into a clean tube and a second measurement made.

A colony of *P. damicornis* 15 cm across was immersed in 10 ppm of alizarin in running seawater for 48 hr. The skeleton was cleaned with fresh water, dipped in dilute Clorox and oven dried overnight at 60°C. A control of the same size but without the dye was cleaned and dried in the same way. Fragile tips of the coral columns were cut off with a bone cutting rongeur and the fragments were crushed. Six combinations of colored and uncolored fragments were weighed out and each put in a 10 ml beaker. Table 3.

Table 3. Weights of alizarin colored and uncolored coral fragments used in spectrophotometric studies.

<table>
<thead>
<tr>
<th>Specimen Number</th>
<th>Alizarin Weight</th>
<th>Uncolored Weight</th>
<th>Total Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0 g</td>
<td>1.0 g</td>
<td>1.0 g</td>
</tr>
<tr>
<td>2</td>
<td>0.2 g</td>
<td>0.8 g</td>
<td>1.0 g</td>
</tr>
<tr>
<td>3</td>
<td>0.4 g</td>
<td>0.6 g</td>
<td>1.0 g</td>
</tr>
<tr>
<td>4</td>
<td>0.6 g</td>
<td>0.4 g</td>
<td>1.0 g</td>
</tr>
<tr>
<td>5</td>
<td>0.8 g</td>
<td>0.2 g</td>
<td>1.0 g</td>
</tr>
<tr>
<td>6</td>
<td>1.0 g</td>
<td>0.0 g</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

A 10% solution of EDTA in distilled water (w/v) was brought to pH 8.2 by adding NaOH pellets then it was filtered. To each of the above combinations 5 ml of EDTA solution was added and allowed to stand for 30 min with
occasional swirling. Four ml of solution of each solution was decanted into a clean tube. The colors ranged from colorless through pink to red. The fourth and sixth tubes were selected for an absorbance maxima deter determination.

An incremental color series with EDTA and alizarin was set up in the same range as that for seawater. To give a calcium carbonate content comparable to the above experiment, the EDTA solution was poured over crushed coral fragments in a proportion of 8 ml of solution to 1 g of coral. This stood for 1 hr with occasional swirling. The solution was filtered and mixed with alizarin. Each sample was measured spectrophotometrically at 548 μm with EDTA-calcium carbonate solution as a blank. Each sample was then centrifuged for 5 min at 3000 rpm and the supernatant was measured at 548 μm.

Results

When alizarin is added to the EDTA solution to form a color series the same range of colors are obtained as with alizarin in seawater. The absorbance patterns are identical. Spectrophotometric studies of alizarin in seawater showed an absorbance peak in the visible range centering about 548 μm wave length. The results of a spectrophotometric scan carried out at 50 μm intervals is shown in Figure 5. The peak for the two samples was identical. An absorbance scan of alizarin EDTA measured at intervals of 10 μm was also performed (Fig. 6).
Figure 5. Spectrophotometric absorbance of two samples of alizarin in 10% EDTA.
Figure 6. Spectrophotometric absorbance of alizarin in 10% EDTA measured at 10 µm intervals.
The optical density at 548 μm of alizarin-seawater and alizarin-EDTA are compared in Figure 7. There is a linear relationship between the absorbance and the concentration of the dye from 0 ppm to 15 ppm. Above this concentration Beer's law does not hold. After centrifugation there was a visible precipitation in all tubes with concentration more than 20 ppm of alizarin. There was also a change in the absorbance of alizarin-seawater. In all tubes with concentration of alizarin of 15 ppm or more, the supernatant specimens were visually identical and spectrophotometrically they were approximately the same. An absorbance reading of about .170 was recorded from all tubes (Figure 8).

Alizarin in EDTA was also centrifuged and there was no precipitation in any tube (Figure 9).

Discussion

Alizarin in seawater and in EDTA solutions has an absorbance maximum 10 μm less than that recorded for alizarin in distilled water. The lessened solubility of alizarin in seawater as compared to fresh water is due to a salting out effect by competing ions in seawater (Edsall, 1958). The absolute solubility would change with the particular seawater used in the experiment. The use of 10 ppm of alizarin is well within the linear relationship to justify the use of such amounts in other experiments.

There was no precipitant with centrifugation of alizarin in EDTA solution. A linear relationship exists between the
Figure 7. Comparison of the absorbance measured at 548 μm between solutions of alizarin in seawater and in 10% EDTA.
Figure 8. The effect of centrifugation on the absorbance at 548 μm of alizarin dissolved in seawater.
Figure 9. The effect of centrifugation on the absorbance measured at 548 μm of alizarin dissolved in 10% EDTA.
dye concentration and the absorbance from 0 ppm to 15 ppm. Above 15 ppm Beer's law is no longer applicable. Probably the alizarin was being held in colloidal suspension at these higher concentrations and could not be brought down by the centrifugation.

If the quantity of solution used in relation to the dye is kept in such proportion that the absorbance will fall below .100 Beer's law can be applied and the absorbance figure will reflect the amount of alizarin in the sample. Using this method samples from various coral colonies can be compared if conditions of the tests are standardized. The EDTA as used in these experiments presumable removes only the outermost calcium carbonate deposit. In the living animal calcium carbonate is deposited in a spotty manner which is not predictable and may sequester alizarin bound calcium carbonate if it is present. For this reason in such experiments no additional calcium carbonate should be allowed to form after the alizarin has been deposited if an accurate determination of the amount of alizarin is desired.

**Measurement of Deposition of Calcium Carbonate in Reef Corals determined by uptake of $^{45}$Calcium and Alizarin**

Edmondson (1929) recorded the various ways in which growth rates in reef corals were measured prior to his time and added his information on shallow water Hawaiian corals. He measured annual vertical growth and gains in weight of
coral colonies for definite time periods. Maragos (1972) using similar methods with transplanted corals measured the effects of many environmental variations on coral growth, Bosch (1963) measured all specimens of *F. scutaria* in selected areas and seven months later he remeasured them and recorded the gains in various size classes. Knutson *et al.* (1972) also depended on accumulated skeletal mass for his studies which used radio-active fallout materials from the nuclear tests of the 1950's to provide a marker which could be disclosed by auto-radiography.

Goreau (1959a) first used radio-isotopes to evaluate coral growth. He used $^{45}$Calcium and this has distinct drawbacks. Radio-isotopes such as this can be used only by investigators trained in handling and disposing of such substances. The measurements of radio-activity require elaborate and expensive electronic equipment for reproducible results. The half life of $^{45}$Ca is 165 days and when deposited in a coral skeleton it cannot be seen and the specimen must be destroyed in the course of measurements.

The exact patterns of alizarin incorporated in the calcium carbonate lattice of the coral skeleton can be seen and photographed. The dye is chemically stable and it does not fade. Quantitative measurements of the alizarin can be performed spectrophotometrically and this can be done with minimal equipment. The dye itself is inexpensive and it can be used in virtually any laboratory with only very basic equipment. The use of alizarin does not require any special
special training but it has never been ascertained whether alizarin was deposited in the same patterns as the calcium carbonate of the skeleton. The purpose of this experiment was to determine whether the radio-calcium deposited by living corals could be correlated with the visible deposition of alizarin laid down at the same time.

Methods

A number of small colonies of *P. damicornis* weighing from 8 - 10 g wet weight were held overnight in running seawater. A dozen heads were supplied with newly hatched *Artemia salina* nauplii and in an hour, four symmetrical heads were selected that had actively ingested *Artemia*. They were washed in filtered seawater to remove excess nauplii and were allowed to stand an hour to make sure that all nauplii had been engulfed. The colonies were transferred to 150 ml beakers which contained 10 ml of Millipore filtered seawater. They were labeled I, II & III. The fourth coral head was broken into fairly symmetrical halves of about 7 g each. These were put in similar beakers and labeled IV & V. Table 4 lists the additions of alizarin and $^{45}$Ca to each.
Table 4. Seawater media with varying amounts of alizarin and \(^{45}\)Ca added for quantification studies on coral growth.

<table>
<thead>
<tr>
<th>Specimen Number</th>
<th>Seawater</th>
<th>Alizarin Added</th>
<th>(^{45})Calcium Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>100 ml</td>
<td>10 ppm</td>
<td>10 (\mu\text{Ci})</td>
</tr>
<tr>
<td>II</td>
<td>100 ml</td>
<td>10 ppm</td>
<td>10 (\mu\text{Ci})</td>
</tr>
<tr>
<td>III</td>
<td>100 ml</td>
<td>10 ppm</td>
<td>10 (\mu\text{Ci})</td>
</tr>
<tr>
<td>IV</td>
<td>100 ml</td>
<td>none</td>
<td>10 (\mu\text{Ci})</td>
</tr>
<tr>
<td>V</td>
<td>100 ml</td>
<td>10 ppm</td>
<td>none</td>
</tr>
</tbody>
</table>

Air was bubbled to the base of each beaker through a fine glass tube and all were placed in a water bath to which pieces of ice could be added to keep temperature constant. Lighting was by a bank of white fluorescent lamps suspended 60 cm above the water level and was constant.

At the end of 24 hr all corals had extended tentacles and the water temperature had risen from 25\(°\)C to 26\(°\)C. The corals were placed in dilute NaOH and bubbling was continued until all organic material had separated. The skeletons were washed in distilled water, immersed in dilute Clorox and oven dried overnight at 60\(°\)C.

One half gram portions of these skeletons were then procured by cutting off the tips from all columns with a rongeur until sufficient material had been taken for that sample. Alizarin deposition occurred in all corals but IV. The first sample included only material from the colored tips. The second sample was of skeletal material from beneath the first. A third was taken from surface material farther down the column. The samples varied in color from white to pink.
and red. Similar samples were procured from heads II and III. EDTA solution 10% (w/v) was adjusted to pH 8.14. The 10 samples were placed in 10 ml beakers and each was covered with 4 ml of EDTA solution. They stood for one hr with occasional swirling. The fluid was decanted, centrifuged for 5 min at 3000 rpm and an absorbance was measured at 548 \mu m. Each reading was preceded and followed by a standardization with a blank of EDTA-calcium.

From the same ten samples 100 ul aliquots each were placed in a standard aluminum planchette utilizing a circular piece of lense tissue and a drop of wetting agent to insure uniform dispersion. They were dried on a hot plate and were immediately counted for radio-activity. The counts were for 2 min each and results of the two series were averaged. Background counts were subtracted. I had performed previous studies and these showed that no correction for self absorption was necessary if the counts using this technique did not exceed 3000/min.

A similar procedure was then followed with corals IV and V. In head IV the tips of the columns were colorless and in head V they were brilliantly colored. Two samples from each head were processed for spectrophotometric studies and for radio activity.

**Results**

Coral heads labeled I, II, & III were brightly colored with alizarin and showed maximal deposition of the dye at
tips of the columns. When the samples had been processed with EDTA the supernatant solutions ranged from red to colorless. Table 5 gives the visual color, the absorbance measurement, and the GM counts of the radio activity.

Table 5. Spectrophotometric absorbance and radio activity data on samples of EDTA-calcium carbonate dissolved from an array of corals.

<table>
<thead>
<tr>
<th>Coral Number</th>
<th>Specimen Number</th>
<th>Visual Color</th>
<th>Absorbance at 548 μm</th>
<th>GM counts per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.</td>
<td>red</td>
<td>.070</td>
<td>2221</td>
</tr>
<tr>
<td></td>
<td>2.</td>
<td>pink</td>
<td>.020</td>
<td>735</td>
</tr>
<tr>
<td></td>
<td>3.</td>
<td>pink</td>
<td>.017</td>
<td>680</td>
</tr>
<tr>
<td>II</td>
<td>4.</td>
<td>red</td>
<td>.048</td>
<td>2130</td>
</tr>
<tr>
<td></td>
<td>5.</td>
<td>pink</td>
<td>.016</td>
<td>618</td>
</tr>
<tr>
<td></td>
<td>6.</td>
<td>pink</td>
<td>.016</td>
<td>546</td>
</tr>
<tr>
<td></td>
<td>7.</td>
<td>white</td>
<td>.009</td>
<td>220</td>
</tr>
<tr>
<td>III</td>
<td>8.</td>
<td>red</td>
<td>.042</td>
<td>1857</td>
</tr>
<tr>
<td></td>
<td>9.</td>
<td>pink</td>
<td>.028</td>
<td>995</td>
</tr>
<tr>
<td></td>
<td>10.</td>
<td>white</td>
<td>.007</td>
<td>209</td>
</tr>
</tbody>
</table>

When these data were graphed a line drawn by method of least squares showed a linear relationship with a correlation coefficient $r = .987$, $p < .01$ (Figure 10).

Table 6 gives the color, absorbance measurements and GM counts from heads IV and V. It was assumed that the red tips of the columns of half head V were as active as the tips of the columns of the other half labeled IV. These were combined and were expressed graphically along with the regression line of Figure 10 (Figure 11).
Figure 10. Regression analysis by method of least squares of radioactivity found in coral heads I, II & III expressed in counts / min against spectrophotometric absorbance measured at 548 µm from the same samples expressing alizarin content.
Table 6. Spectrophotometric absorbance and radio-activity data from comparable samples from each half of the same coral head.

<table>
<thead>
<tr>
<th>Coral Number</th>
<th>Specimen Number</th>
<th>Visual Color</th>
<th>Absorbance at 548 μm</th>
<th>GM counts per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>11. white</td>
<td>.000</td>
<td>2700</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12. white</td>
<td>.000</td>
<td>1348</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>13. red</td>
<td>.060</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14. pink</td>
<td>.019</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Goreau (1959a) assumed that $^{45}$Calcium when incorporated through biological activity represented the precise pattern of the deposition of that element. In this experiment it also presumed that $^{45}$Calcium was deposited in an identical manner to all calcium deposited during the 24 hr time span. The close correlation found in this combined experiment permits us to conclude that alizarin and calcium carbonate are deposited in the same pattern and in proportionate amounts.

Some measure of the toxicity of alizarin was gained from the study of depression of the calcium deposition when alizarin was present in the seawater. The assumption was made that metabolism of halves of the same coral head were comparable, especially so in a colony such as was chosen. It had been growing in an upright position in open water away from large heads of the same species. In half head V, alizarin deposition was present in all column tips. It
Figure 11. Data from radioactivity and absorbance measurements at 548 μm from separate halves of the same coral head (IV & V) processed separately and here combined with line from Figure 10 of similar data from coral heads I, II & III.
was assumed that $^{45}$Calcium had been deposited similarly. For my purpose I combined the absorbance measurement from one half head with the GM count from the comparable area of the other head, i.e. #11 with 13 and #12 with 14. There were thus only two points available to plot and they are of no statistical significance. However, it is of interest to note that the slopes of the two lines are about the same.

Alizarin is mildly toxic to coral growth even in moderate dosages such as 10 ppm in seawater. This experiment again tends to confirm this.
IV. CALCIUM DEPOSITION IN ADULT CORAL COLONIES

Patterns of Growth in some Reef Corals

The profusion of forms found among reef corals enables the most casual observer to distinguish differences in growth patterns. Taxonomists have used these obvious distinctions but have relied on the patterns of asexual reproduction and the character of the septal margins for specific identification (Vaughan, 1919b). The adult skeleton represents the end product of a lengthy process and does not reveal the sequence of calcification or the day to day patterns of growth.

Most corals assume a general form that is genetically determined and consistent for the species (Finckh, 1904; Wood-Jones, 1910; Vaughan, 1915; Mayor, 1924; Edmondson, 1929, 1946; Stephenson and Stephenson, 1933; Goreau, 1956; Bosch, 1967; Maragos, 1972). However, there can be tremendous variations among the colonies of the same species depending on the local environment. Transplanting colonies from one area to another can result in unusual growth forms caused by changes in the amount of light, salinity, sedimentation, wave action, temperature and other local phenomena. Many erratic responses do not seem to be related to physical or environmental factors (Maragos, 1972).

Wood-Jones (1910) remarked that corals grew by fits and starts with no apparent external cause for this variation. Goreau (1959b) also suggested that calcification was
discontinuous in corals and that this gave rise to growth fluctuations. This view is supported by Barnes (1971) who stated that when maximal asexual division occurred in corals, and was accompanied by maximal calcification, a hemispheric colony would result; however, there were many factors that prevented this, especially in shallow water, so that random differences in growth rates eventually lead to the surface becoming bumpy. Before this, Moto-da (1938) recorded the extensions of the branches of 81 small heads of P. damicornis. He showed very clearly the tremendous variation in growth rates among similar coral colonies raised in the same environment. These opinions have not gone unchallenged for Shinn (1966) and Lewis et al. (1968) are of the opinion that corals grow constantly with few interruptions. Their studies were performed by measuring annual growth rates and weight gains over the same time span.

Goreau (1959a) using radio-isotopes measured total calcification in a coral colony and was able to relate the gain in colony weight to the area of the growing surface. By his sampling techniques he was able to show that the highest growth rates occurred at the tips of the branches. Buchsbaum-Pease (1971) likewise showed that there was a gradient in the calcification rates from the tips of the stalks of Acropora sp downward.

Reed (personal communication) showed that corals will live in an aquarium for months and will grow vigorously
provided they have a constant source of seawater. Under such circumstances a planula of P. damicornis will settle on a glass slide and will eventually develop a typically branched pattern with hundreds of living polyps. There are individual differences among the colonies of various corals; Cyphastrea ocellina will spread out laterally from a single polyp to a flat symmetrical pavement type of colony. These colonies are remarkably similar when they grow along the surface of a glass slide. Similarly, Fungia scutaria can be raised in an aquarium. The planulae of this species have never been identified though it is not unusual to find long dead skeletons of this coral with a profusion of young polyps growing from the surface. These must have either grown from settled planulae or they may have arisen by asexual reproduction in a dying adult coral. Such fungid corals if kept in an aquarium continue to grow and show their characteristic mushroom pattern and eventually separate spontaneously to begin an existence as an independent polyp. Still, when they are all growing together each has its own form and identity determined by its position in the group and probably other factors. Corals such as this have been protected from wave action, from sedimentation, from excessive sunlight and dessication and from temperature extremes but they still produce skeletons that are characteristic for the species but with individual variations.
The present studies were undertaken to determine the pattern of alizarin deposition in growing corals over a short period of time and to see if such patterns can be predicted and also to determine whether the growth rates of the various colonies can be compared using such a method.

**Method**

Small heads of the following corals were lifted from a protected area in Kaneohe Bay within a radius of 10 m and at a depth of less than 1 m low tide:

- *Pocillopora damicornis cespitosa* Dana
- *Pocillopora meandrina* var. *nobilis* Verrill
- *Montipora verrucosa* Lamarck
- *Psammocora stellata* Verrill
- *Pavona varians* Verrill
- *Cyphastrea ocellina* Dana
- *Porites compressa* Dana
- *Fungiia scutaria* Lamarck

All of these were growing independently. They were taken on an early morning low tide and transferred to the laboratory in plastic pails. In addition, some other shallow water specimens from near-by reefs were included:

- *Porites lobata* Dana
- *Leptastrea bottae* Milne-Edwards & Haime
- *Tubastrea aurea* Quoy & Giamard (ahermatypic)

The colonies were placed in 24 liter tanks and were propped on old *P. damicornis* skeletons so there would be no crowding
or mutual interference. Light was supplemented by four cool fluorescent lamps suspended 60 cm above the water lighted for the duration of the experiment. Water flow was adjusted to give a complete turn-over every hour.

Alizarin in distilled water was prepared so that a regulated drip would give 10 ppm of ambient concentration to the tank. At the end of 24 hr all corals were living and appeared healthy. At the end of 48 hr alizarin was discontinued and the corals were kept in running seawater for an additional 48 hr. All corals appeared healthy when they were placed in fresh water until organic material could be removed with a strong spray. They were washed in dilute Clorox and air dried.

Results

Reef corals in this experiment absorbed alizarin in different amounts so it was possible to determine qualitatively how much this varied from species to species and where the most growth took place during the course of the study. The coral specimens were ranked visually according to the amount of alizarin uptake as follows:

(1) _F. damicornis_; (2) _Montipora verrucosa_; (3) _F. meandrina_; (4) _F. scutaria_; (5) _Porites compressa_; (6) _Psammocora stellata_; (7) _Pavona varians_; (8) _Cyphastrea ocellina_; (9) _Porites lobata_; (10) _Tubastrea aurea_; (11) _Leptastrea bottae_. The following section describes the alizarin uptake as it appeared in these specimens.
1. *P. damicornis* skeletons showed the greatest magenta coloration in this series and appeared to have taken up the largest amount of alizarin (Figure 12). These bushy colonies consist of numerous columns arising from a solid base generally forming a hemispheric shape. The greatest alizarin deposition was at the tips of the ascending columns with coloration being present in most central papillae; however, the lateral papillae on the same stalk may be devoid of color. This varied considerably among colonies so that an estimate of the percent of uncolored papillae near the tip may be less than 5% in a small colony to 50% or more in the large colony. This tremendous variation among specimens can be seen to some extent by perusing the photographs (Figures 4, 20, 27, 28) of *P. damicornis* colonies.

The margins of the calyces as well as the coenosteum were brightly colored at the tips of the stalks. In general the coral surface of the central part of the stalk showed the least amount of alizarin deposition so that neither calyces nor coenosteum were involved though occasionally a streak of color descended from the top of the colony to its base along one stalk. In other areas there was occasionally a blush of color without sharp limits.

The coenosteum at the base between major stalks was usually highly colored with the dye incorporation but again, this was variable. In some instances the dissepiments at the base showed no color though the coenosteum surrounding these calyces did so. Examination with magnification led me to
Figure 12. *Pocillopora damicornis* skeleton showing alizarin incorporation (2/3 natural size).

Figure 12a. Sketch of *Pocillopora damicronis* skeleton. Stippling represents alizarin deposition.
Figure 12.

Figure 12a.
believe that this was not an artifact due to later deposition of calcium carbonate. The alizarin incorporation was variable in the basal area and much more so at the margins than in the central region.

A gall forming crab, *Haplocarcinus marsupialis* Stimpson (Hiatt, 1954) infests many of the colonies of *P. damicornis* and when the galls are on the periphery, the growing edges are usually uniformly and deeply stained with alizarin. When the same form of gall was on another column which was below the hemispherical surface, it was usually void of color. It is not known whether the individual crabs were living at the time of the experiment.

2. *Montipora verrucosa* demonstrates a highly erratic pattern of alizarin uptake which may be in keeping with the multitude of growth forms encountered in this species of coral. In some specimens with a hemispheric contour, (Figure 13) there was heavy alizarin incorporation in some areas while adjacent areas had no color. The transition from heavy deposition to none could occur in the space of 1 mm. The areas of involvement varied from colony to colony and in some growth forms where ridges were apparent, all deposition was at times along the summit of these ridges. Some colonies showed a heavy uptake while a similar colony of the same size and shape and treated in the same way showed relatively slight uptake. As nearly as could be ascertained, the areas of alizarin staining did not correlate with any recognizable anatomical feature nor could it be ascribed to
Figure 13. *Montipora verrucosa* skeleton showing alizarin deposition (2/3 natural size).

Figure 13 a. Sketch of Montipora verrucosa skeleton. Stippling on summits represents alizarin.
Figure 13.

Figure 13a.
any specific environmental influence. When viewed under low power magnification the papules of this species often showed an intense coloration at their tips but others showed only the base of the papule to be involved. Some showed one side colored only and this varied throughout the colony so that color distribution was very patchy with no regard to polyp or papule configuration. Position on the convexity of a hemispherical colony seemed to have no bearing on the amount of coloration though generally the underside of the specimen had little alizarin uptake. The dye was deposited superficially only.

3. Verill describes P. meandrina by saying that the corallum is firm and dense ... branches nearly equal... separated by regular intervals. They are regularly forking and not enlarged at the obtusely rounding ends. The summit of the branches are generally strongly verrucose (Vaughan, 1907). Alizarin deposition tended to be most intense along the ends of the large stalks (Figure 14), with the one side being much more stained than the other. The ends of the columns were often free of color as were the sides while the coenosteum at the base between the columns was usually alizarin stained. In general, the alizarin distribution in this coral species is very similar to that found in P. damicornis; likewise, under low power magnification the two species appear very much alike though alizarin deposition in these experiments was less in P. meandrina which may be an indication of slower overall growth in this species.
Figure 14. *Pocillopora meandrina* skeleton showing alizarin incorporation (1/2 natural size).

Figure 14a. Sketch of *Pocillopora meandrina* skeleton. Stippling represents alizarin.
Figure 14.

Figure 14a.
4. *Fungia scutaria* looked to be more highly colored on the aboral surface than on the oral surface especially in the smaller specimens (Figure 15 a & b). In part this is because most of the dye on the oral surface is deposited deeply between the septae and is not readily visible though it tends to be distributed symmetrically. The corallum has a flat aboral surface which is somewhat irregular. It is costate with irregular dentate margins. Figure 15 b shows that the pattern on the aboral surface may be uneven with large areas showing much alizarin uptake while similar areas unrelated to the physical features of the corallum were relatively free of color. This was an individual phenomenon peculiar to each polyp but it could be observed in most medium sized specimens so treated.

Occasionally one of the *F. scutaria* showed very marked uptake in only one or two areas on the edge of the corallum. Under low power magnification areas of the aboral surface showed a generalized, rather granular type of deposition with many spots of intense dark red coloration which were sharply circumscribed, irregularly lobulated and from 0.1 to 0.25 mm in diameter. The total combined area of all of these spots was perhaps less than 5% of the entire aboral surface. It is not known what these represent. In some instances the spines on the inferior surface showed no alizarin. Their size and distribution made it unlikely that the post alizarin exposure to normal seawater had any influence on this or that the lack of visual color was due to a coverup by newly deposited
Figure 15 a. *Fungia scutaria* skeleton showing alizarin incorporation, oral side, 1/2 natural size.

Figure 15 b. Same skeleton from aboral aspect.

Figure 15 c. Aboral surface of skeleton of *Fungia scutaria* sketched with stippling representing alizarin.
calcium carbonate.

5. Porites compressa, as might be expected, showed most of the alizarin uptake at the tops of the various columns. Many of the colonies tested failed to take up much of the dye and when they did most of the color was at the tops of the columns but never so that the entire top was uniformly involved. Very frequently only about 2/3 of the area of the top was dye colored and this was a general marking involving all structures and fading gradually at the edges over a distance of 1 mm. In some specimens there was no uptake in the polyps on the base or on the edges of the colony whereas other colonies of the same size showed active incorporation in the polyp skeletons at the base and at the edges of the base (Figure 16).

6. Psammocora stellata consists of numerous columns with flared tips. This coral species showed fairly regular alizarin deposition over the extent of the hemispheric surface of the colony with uniform involvement of all surface structures. There was much less uptake along the column walls. Unfortunately, the only satisfactory specimen of this species that showed good alizarin uptake was collected (by a visitor) as a souvenir before it could be photographed.

7. Pavona varians is an incrusting form, in this specimen, with angular or convoluted crests arising from a gently rolling surface. These serpentine collines showed marked alizarin uptake which was evenly disposed along the ridges. With magnification small areas of deeper coloration were
Figure 16. *Porites compressa* skeleton showing alizarin incorporation. (1/2 natural size)

Figure 16 a. Sketch of skeleton of *Porites compressa*. Stippling represents alizarin distribution.
Figure 16.

Figure 16 a.
seen here and there among the septae with no apparent relationship to anatomical structures (Figure 17).

8. *Cyphastrea ocellina* showed tremendous variation in amount and distribution of alizarin uptake in the various aspects of these studies. Figure 18 shows strikingly how one half of a colony may be alizarin colored and the other half may be uncolored. The transition is often gradual but it can occur in the space of 0.2 mm. Under low power magnification the coenosteum in these specimens was more involved than were the septa which in many instances in these experiments were almost uncolored. Alizarin uptake may be colony dependent and not polyp dependent though the higher the magnification the more spotty the alizarin deposition appeared. Figure 20 demonstrates uptake of alizarin in various sizes of *P. damicornis* colonies. The largest colony on this photograph is fixed to a piece of coral rubble and on close inspection of the base of this dead coral two colonies of *C. ocellina* are visible. One showed intense generalized uptake by alizarin and the other showed none at all. Similarly, in the present experiments one specimen of *C. ocellina* had much uptake and another of the same size and shape showed no coloration at all. There was no obvious explanation for this.

9. *Porites lobata* is often such a massive structure that suitable pieces for inclusion in an aquarium study are difficult to obtain. There are a bewildering number of growth forms in this species (Vaughan, 1908) but the
Figure 17. Skeleton of *Pavona varians* showing alizarin incorporation. (Enlarged by 1/2)

Figure 17 a. Sketch showing skeleton of *Pavona varians*. The area between the stippled lines along the ridges of the collines represents the region where most of the alizarin deposition occurred.
Figure 18. Skeleton of *Cyphastrea ocellina* showing alizarin incorporation. (About twice natural size)

Figure 18 a. Sketch of *Cyphastrea ocellina* skeleton enlarged several times. Stippling represents alizarin deposition.
Figure 18.

Figure 18 a.
specimens included in this series were obtained from tips of columns or columnar protrusions of a colony of 1 m in diameter. There was very little uptake of alizarin and that only on the summit, all structures being weakly and evenly stained with alizarin. This was so sparse that it was barely visible with low power magnification and photographs showed no evidence of staining.

10. *Tubastrea aurea* is a slow growing coral. At one time the writer succeeded in getting the planulae of *T. aurea* to settle and they were observed for several months. The young polyps readily took up alizarin but their skeletal growth was relatively slow so that the experiment was terminated. Other colonies were observed for months in laboratory aquaria and none showed evidence of growth. In this experiment there was no visible deposition of alizarin by the polypary structures themselves, at least no color was visible in the calyces but there was slight evidence of deposition in the coenosteum between the calyces (Figure 19).

11. *Leptastrea bottae* is one of the less frequently found corals in Kaneohe Bay and never formed large colonies. The specimens of this coral that were treated with alizarin showed no uptake at all.

**Discussion**

It is very apparent from reviewing the data from these experiments that growth of these reef corals if measured over a short interval is erratic and relatively unpredictable.
Figure 19. Skeleton of *Tubastrea aurea*. Alizarin incorporation was on the base and cannot be seen. (2/3 natural size)

Figure 19 a. Sketch of *Tubastrea aurea*. Stippling represents area of skeleton which showed alizarin incorporation.
Figure 19.

Figure 19 a.
Some colonies deposited much more alizarin than comparable colonies of the same species subjected to alizarin in seawater under the same conditions. It may be that some corals may cause a depression of alizarin uptake in neighboring corals. In the various experiments carried out with alizarin I often noted that individual heads of *P. damicornis* would survive in aquaria but if several were put together, often all of them died; whereas if it were a mixed assemblage estimated to be about the same surface area of living tissue, all would remain healthy. I also noted that newly settled polyps of *P. damicornis* often died if left in the overflow water from an aquarium containing large heads of the same species. Lang (1970) discussed interspecific competition at close range; however, the effect I noted may be from metabolites or other substances in the water which has a deleterious effect on other corals of the same species.

*Porites compressa* is a dominant coral species in the northern end of Kaneohe Bay but it showed relatively little alizarin uptake in the present experiments. Franzisket (1969) found that that species had a high respiration rate and did not lend itself to aquarium living, in spite of vigorous aeration and rapid water turn over. This may be one reason why the colonies of this species tested deposited so little alizarin compared to *P. damicornis*.

*Porites lobata* in the form selected for these experiments tends to form massive barrel shaped structures.
This species probably has many of the same metabolic features of *P. compressa*. The lack of alizarin deposition in quantity may be an expression of this.

*Cyphastrea ocellina* and *Leptastrea bottae* belong to the same subfamily, Monastreinae (Vaughan & Wells) and often grow in proximity on the reef flats. I have examined their planulae and they look very much alike except those of *L. bottae* lack the glistening white blobs that identify the oral end of *C. ocellina*. It may be assumed that the two species behave much alike in their ability to deposit alizarin. The colonies of *L. bottae* deposited no alizarin but this was also noted in some specimens of *C. ocellina*. The reason for this is not known.

Franzisket (1969b) in measuring the growth rates of five species of Hawaiian corals recorded the greatest increase in "*Pocillopora elegans*" followed in order by *Fungia scutaria*, *Porites compressa*, *Montipora verrucosa* and *Dendrophyllia* sp. He identified the fastest growing coral in his series as *P. elegans*. Vaughan (1907) in considering the *Pocillopora* series indicates that *P. elegans* is very similar to *P. meandrina* and this is generally considered to be the species growing in Kaneohe Bay (Vaughan, 1907; Edmondson, 1929; Powers, 1970; Maragos, 1972) where his series and mine were collected.

Maragos (1972) in his study on growth rates of Hawaiian corals lists the growth rates in his series from fastest to slowest as follows: (1) *Montipora verrucosa*;
(2) *Porites compressa*; (3) *P. damicornis*; (4) *Porites lobata*; (5) *P. meandrina*; (6) *Fungia scutaria*; (7) *Pavona varians*; (8) *Cyphastrea ocellina*; (9) *Tubastrea aurea*; (10) no growth in *Leptastrea bottae*.

There is a difference in arrangement in these listings, however, these studies are not comparable except that all specimens were taken from the same geographic area. Franzisket's figures are from a laboratory experiment of four months duration while Maragos' study measured average growth rates on the reef itself during the course of a year.

My experiments give some indication as to what is happening within a few hours to a number of small select colonies with regard to alizarin uptake. All of these colonies were transplanted shortly before the experiments and they were all subjected to a mildly toxic substance, alizarin. It was assumed that all corals behave alike to stresses applied.

Growth patterns of the various corals are highly irregular if measured over a short period of time but this would balance out over a longer period of time. Several entirely distinct regions in any one colony may be depositing calcium carbonate at any one time whereas other regions in the same colony may be depositing no calcium carbonate. This is unpredictable for any one short period but in general, it follows a genetically determined pattern which accounts for recognizable growth forms. Many other factors obviously must
be taken into consideration to explain why *P. compressa* is the fifth fastest growing coral in this series and still is the dominant species in northern Kaneohe Bay.

The irregularity and inconstancy of alizarin deposition probably is a true reflection of calcium deposition during the course of these experiments. This irregularity and inconstancy seems to increase with the size of the colony—especially *P. damicornis*. Only in rapid growing species can it even be assumed that calcium carbonate is being deposited if the experiment is a short term study. Even in the faster growing corals such as *P. damicornis*, random sampling of a few colonies might give extreme variations in calcium carbonate deposition. Some recent studies on coral growth have employed radio-calcium in short term experiments, some of them of an hour or less. It is unknown at present what difference such a short time would make but it might compound the difficulties mentioned above.

Short term studies as those done here and those employing $^{45}$Calcium as a tracer, must of necessity be done with caution. Specimens of coral should be chosen with care as small colonies would show less variation in uptake than would larger colonies. To be meaningful for an estimation of calcification, numerous samples of the actual surface material would have to be taken, unless of course, the entire coral colony is used as a sample.

The above conclusions are made on the basis of the findings of these alizarin experiments using a limited
number of reef corals. It is unknown if the same phenomena occurs in all corals though it probably does so. This experiment was to determine if the short term growth of corals could be outlined by a permanent marker such as alizarin. It is my conclusion that it serves this purpose.

**Coral Growth in Relation to Colony Size**

There is a difference in opinion among investigators whether or not coral growth is slowed with age. Earlier opinions were all that initial growth was rapid and that it leveled off with time (Vaughan, 1915; Mayor, 1918; Edmondson, 1929; Stephenson & Stephenson, 1933; Motoda, 1940). Bosch (1967) chose nine size classes of the coral *Fungia scutaria* and showed a linear decrease in rate of arc length increase for the larger sized specimens over a period of seven months. Maragos (1972) found no difference in growth rates of five species of reef corals that he measured though he was measuring adult colonies of unknown age. He also measured *F. scutaria* and selected nine size classes, the smallest class consisting of small specimens of less than 50 g and the largest class weighing over 800 g. His pooled data showed no difference in growth rates among the nine classes. He further noted that the growth of *Fungia* was very erratic at all stations to where he had transplanted specimens except for his station #9 in Kaneohe Bay where *Fungia* seemed to grow faster at larger sizes. He postulated that this may have been the result of surf conditions at that station.
Maragos recalculated the data of Bosch in terms of weight to conform to his own equations. This showed that an opposite trend was encountered. Maragos believed that the most interesting finding was that interpretation of growth patterns could be influenced by how growth rates were expressed. His results support the hypothesis that radius growth is nearly constant with time in such species as *P. damicornis* but that radius growth is decreased with increasing size in the solitary polyps. There are size limits to which some corals may grow and many branched or solitary corals have a maximum size (Bosch, 1967; Edmondson, 1929; Grigg and Maragos, 1973).

Goreau and Goreau (1960a) using $^{45}$Calcium found that on the basis of nitrogen content, branched corals grew faster than massive ones and they found considerable variation in growth rates among similar colonies. They noted that there was a slowing with increasing size in the reef coral *Manicina areolata* and suggested that this was due to clone aging which is comparable to the expression used by Motoda (1940), physiological senescence. Connell (1973) believed that coral growth may be determinate or indeterminate depending on the species. He found, for the most part, growth of corals was at first rapid but in larger colonies it had slowed to a constant rate. His studies were for over seven years but it is probably that few or none of the colonies being observed had reached the phase of physiological senescence. Knutson *et al.* (1972) in their radiographic studies of massive corals,
did not refer to this specifically but their photographs do not suggest slowing of growth over the 20 or more years that these massive colonies had been growing after marking.

The present study was undertaken to determine whether colony size and growth could be correlated by measuring the alizarin deposition.

**Method**

During one collection trip, an assortment of colonies of *P. damicornis* were collected from Kaneohe Bay. The largest was 15 cm in diameter; twelve others taken formed a growth series down to less than 1 cm. Typically, they were hemispheric, they had no dead branches and they were all growing in the same environment on the same reef. They were all placed in a plastic aquarium and exposed to concentration of alizarin, 10 ppm, in running seawater. Four heads were removed at the end of 24 hr and the rest at 48 hr. All were cleaned in fresh water, immersed in dilute Clorox and dried.

On another occasion, specimens of *F. scutaria* were taken from the reef flats for a similar study. The smallest and largest specimens of this series were procured from the vicinity of Maragos’ station #9 described above. All were exposed to continuous concentration of alizarin, 10 ppm, for 48 hr. They were then cleaned, immersed in Clorox solution, dried and photographed.
Results

When the processed specimens of *P. damicornis* were compared, all of them had a vivid magenta color and appeared to show on superficial examination that growth in all colonies was continuous and rapid. On closer examination of each head it was noted that in colonies of about 10 g and smaller, alizarin deposition was very marked and uniform over the entire colony surface. In the intermediate sizes, the uptake of alizarin was regular at the tips of the columns but larger and larger areas on the column surfaces showed no alizarin deposition or, at most, only a slight tinge. Intermediate sized heads often showed papillae that remained uncolored. With increasing head size these became more and more frequent. The tips, however, of all columns in this series generally showed good alizarin deposition (Figure 20).

On inspection of the various *Fungia* specimens the alizarin deposition seemed to vary depending on whether the examination was from the oral or aboral view, the color appearing much more marked on the latter. In part this is due to the deposition of alizarin in lower parts of the septa so that it is not visible except with good lighting. It has been pointed out that the deposition of alizarin in *Fungia* can be irregular and patchy but this is not apparent in the smallest specimens in this series. The smallest specimen shows a uniform magenta color to the edge of the anthocaulus from which it has not separated. This structure showed no alizarin deposition. The irregularity of deposition is more
Figure 20. Alizarin deposition in varied sizes of *Pocillopora damicornis* colonies.
apparent in the medium sized specimens labeled 2 - 6 in Figure 21. These polyps weighed from 50 to 100 g. The largest specimen in the illustration weighed 1001 g and the alizarin deposition was so sparse as to be questionable even on a minute examination though it was obviously present in the two young corals sprouting from the aboral surface.

Discussion

When the various specimens of *P. damicornis* were examined it appeared that growth was mainly from the tips of the columns outward and was continuous; however, with increasing colony size the number of uncolored papillae increased. This suggests that a stage may be reached when most or all of the terminal papillae would be uncolored with resulting discontinuous or absent growth. No maximal sized colonies were tested and it is unknown why such colonies attain a maximum size. This may be determinate growth but no conclusions can be reached from this study.

When *P. damicornis* grows in shallow waters such as the area where these specimens were collected, maximum size is not reached. The currents are swift and those colonies which settle on coral rubble are soon washed away. The larger colonies probably cannot stand the wave action. In deeper waters, colonies may reach a diameter of 30 cm but at that size the central branches are often dead. It has not been ascertained to what size a colony of *P. damicornis* might attain if it were in an area with adequate but no excessive
Figure 21 a. Photographs showing the alizarin deposition in a series of varied sized polyps of *Fungia scutaria*. (a) oral view of skeletons, (b) aboral view of same skeletons.
water flow, good nourishment, lack of predation and the optimum conditions for growth.

This experiment was performed in the summer months when the water temperature was such that growth would be most rapid. Clausen (1971) working at the H.I.M.B. with P. damicornis using $^{45}$Ca showed that maximal growth rates occurred at 25°C to 27°C, the highest temperatures reached at Kaneohe Bay. He showed a marked decrease in growth at 20°C. The water temperatures during my experiments were near the upper level but the water temperature in the Bay decreases to 22°C or lower in the winter months. A seasonal variation in calcification rates is to be expected. Growth rates as measured by alizarin deposition, as in these experiments, are not comparable with growth rates measured over a year's time but they do give indications of growth trends.

On viewing the various Fungia specimens, the alizarin deposition suggests that initial growth is rapid and generalized. When the specimen reaches a size somewhat less than 50 g the dye is dispersed over a larger area and is patchy so that in the larger specimens a smaller percent of the surface appears to be colored. The actual deposition may be larger but by contrast it looks smaller. In the largest specimen in this series, there seemed to have been no alizarin deposition at all. This coral may have reached the stage of physiological senescence so that growth had ceased. It is not uncommon to find areas in Kaneohe Bay with large
numbers of *F. scutaria* skeletons all of about this size. It is possible that these were polyps that had reached a maximum determinate age with subsequent polyp death. The sprouting of young corals may be an "agonal" expression in certain species of *Fungia* so that they appear at the time impending death of the parent.

These experiments suggest that both *P. damicornis* and *F. scutaria* have a determinate type of growth. Alizarin provides a method which gives some information as to the amounts and patterns of growth taking place and is a satisfactory adjunctive alternative to other experiments. These were very short term experiments but to the extent of my findings, they concur with those of Bosch and Maragos.

**Light-Dark Studies of Alizarin Deposition in Reef Corals**

Many studies have shown that calcium carbonate deposition in reef corals is light dependent. The most recent of these are those of Goreau (1959b, 1961, 1963), Yamazato (1966), Buchsbaum-Pearse (1970), Buchsbaum-Pearse and Muscatine (1971), and Vandermeulen (1972). Maragos (1972) found *P. damicornis* to be more light dependent than other species of corals in his series. It has been known for years that corals generally do not live in perpetually shaded areas and later it was found that the maximum depth at which hermatypic corals are found is about 100 m. The lack of sunlight at this depth is probably one of the limiting factors. Vaughan (1915) placed corals in total darkness for many months and they survived though the zooxanthellae were eliminated.
Yonge and Nicholl (1932) found similar results as did Goreau et al. (1971). Franzisket (1970) demonstrated that corals can survive for months in total darkness. The hermatypic corals showed no increase in weight presumably because all calcification had ceased. Several other recent studies have employed a procedure in which reef corals were maintained in the light and then were placed in darkness with an isotope tracer for a specific length. In some of these there was no preconditioning to the dark and in others it was a matter of minutes or a few hours. Buchbaum-Pearse & Muscatine (1971) performed experiments on Acropora sp and indicated that light enhancement at the tips of the corals where most of the growth occurred, resulted from activity further down the stalk so that there was a translocation of algal products of photosynthesis which effected the enhancement of the calcification rate. They did not speculate on how this transaction occurred, the speed of movement or the route traversed.

In the following experiments various corals were maintained in darkness before they were subjected to experiments with alizarin to see how profound the diminution of calcium production would be when compared to light treated controls.

Methods

Specimens of P. damicornis of various sizes, the largest being 15 cm in diameter, were placed in a plastic aquarium
fitted with an overflow pipe which led to a lower level so the overflow could enter an opaque 12 qt (11.35 l) pail with an airstone for aeration (Figure 36, Appendix B). A stainless steel milk carton crate was covered with opaque plastic so that when it was placed over the pail, the system was lighttight. An opaque plastic tube 30 cm long and 1 cm in diameter entered the top of the crate and was taped tightly to exclude all light. The overflow water from the first tank entered this tube and was directed deeply into the pail to insure good circulation.

The larger head of *P. damicornis* was broken into two nearly equal parts and one was put in each container. Likewise, a *Montipora verrucosa* colony was broken in two and a portion placed in each container. Small polyps of *Fungia scutaria* weighing 50 g or less were put in both containers and the system was closed. Water entered the aquarium at the rate of 12 l/hr giving a theoretical turnover time of 2 hr, and the entire overflow entered the dark container. The system was allowed to run for 48 hr without disturbing either tank. Alizarin was then added to the intake to give seawater in the aquarium an ambient concentration of about 10 ppm for the following 48 hr without at any time exposing the corals in the lower container to any light. At the end of that time the corals were all placed in fresh water, they were cleaned, labeled and air dried. The same experiment was repeated on several occasions using these coral species with approximately the same environmental conditions prevailing for
all experiments.

Results

The dried halves of *P. damicornis* colony weighed 137 g and 101 g. When they were fitted together, Figure 22a, the entire reconstructed colony was of a uniform light magenta tinge similar in color to many other specimens that had been alizarin treated but lighter than most. Alizarin was deposited generally, most of it appearing in the rims of the calyces between the papillae at the tips of the columns but also along the column walls and at the base. There were few areas of intense deposition and these were more frequent in the light confined than in the dark confined half. In the later portion, these areas were most noticeable on the growing cusps on *Haplocarcinus* galls. A casual observer could rarely see any difference between the two specimens which are shown separated in Figure 22b.

The visual color depth indicating alizarin uptake by *Montipora verrucosa* was more striking in the dark confined portion of the colony than in its light counterpart as shown in Figure 23. The same pattern of alizarin deposition was found in the dark confined specimen as was described in the light confined specimens of previous experiments in this report. The alizarin deposition patterns were not as generalized in the light confined specimens nor as intense as is usually found, but were characteristic for the species.
Photographs of *Pocillopora damicornis* colony. One half of colony was subjected to alizarin in the light and the other half in the dark. Figure 22 a shows the colony reassembled. Figure 22 b shows the halves separated. Dark treated half is on the right.
The *Fungi scutaria* showed generalized alizarin deposition which was intense on all surfaces of the light treated specimens but there was distinctly less alizarin deposition in all dark confined specimens when compared to their light treated counterpart.

**Discussion**

There is tremendous variation in calcification among corals as demonstrated by alizarin deposition. This is found in colonies within the same species though it is unlikely that this much variation would ordinarily occur between halves of the same colony as was observed in *Montipora*. This experiment was short when compared to the usual growth studies performed with corals though it was much longer than the recorded studies involving tracer substances other than alizarin. It indicates that hermatypic corals can deposit calcium carbonate in the dark even when they have been preconditioned for 48 hr.

Zooxanthellae in *P. damicornis* are most numerous in the gastrodermis of structures, especially along the column wall. Goreau & Goreau (1960a) noted that older corals in their series had more zooxanthellae than did the younger ones and Buchsbaum-Pearse (1971) remarked that the number of zooxanthellae increased from the tips to the base of the columns of the *Acropora aerolata* while active calcification decreased in that order. This is also apparent in *P. damicornis* with fewer algae in the younger corals and in the distribution of zooxanthellae in the adult colonies. Figure 24 presents a
photograph of a column of adult *P. damicornis* showing the brown color which is the result of the presence of the algae while Figure 25 indicates that many of the column tips of an adult colony lack this color indicating absence of zooxanthellae or a diminished number.

Visual inspection also shows this to some extent in *Montipora verrucosa* and *Porites compressa* even though many of the zooxanthellae in the latter species may be deep in the calyces. *Fungia scutaria* usually lies on a solid substrate so that the undersurface of the corallum is never exposed to the light. The coenosarc is thin and often the zooxanthellae are sparse, but calcium deposition may be marked. The area of minimal algal density as determined by the paleness of the area is often as much as 5 cm from an area of heavy density but still has maximal alizarin deposition. It was also apparent that colonies of *P. damicornis* absorbed alizarin equally well whether the apparent number of zooxanthellae as judged by color were few or many.

These experiments were formulated to determine the feasibility of a method. It was not within the scope of the studies to establish a final answer to many of the questions raised. The apparatus for these experiments as constituted would need considerable revision.

Frazisket (1969) in studying reef corals showed that very little ambient light is necessary for photosynthesis to reach its maximum and this level is reached soon after sunrise. Barnes and Taylor (1973) found that high light intensities,
Figure 23. *Montipora verrucosa* colonies subjected to alizarin in light-dark experiments. The upper colonies were dark treated, the lower two were light treated. The upper left colony and the lower right were originally one.
Figure 24. Adult polyps of *P. damicornis* enlarged x18 to show distribution of brown zooxanthellae in coenosarc and tentacles.

Figure 25. Photograph of adult colony of *P. damicornis* as it was transferred to an aquarium. The lighter areas at the column tips indicate fewer zooxanthellae.
above the saturation rate of symbiont photosynthesis actually inhibited the rate of calcification in the coral *Montastrea annularis*. This is in accord with the observation of many students of coral growth who have seen the flourishing reefs of the central Pacific. That growth is most luxuriant at a depth of about 5 m where ambient light is significantly reduced. Barnes and Taylor (1973) found a saturation level for photosynthetic CO$_2$ fixation of from 500-600 footcandles in their experiments. This approximates the 1000 footcandle surface illumination recorded from the aquaria used in these experiments. Excellent deposition of alizarin was attained in most cases using the lighting described. Alizarin at 10 ppm gives an intense color to seawater; however, transmission of light wave lengths essential for photosynthesis are virtually unimpeded (Hoar, 1966).

It is possible that the overflow water from the upper tank used in this experiment may have carried products of photosynthesis or dissolved residua to the lower tank where they had an enhancing effect on the coral calcification. To obviate such a factor, a double system of light and dark tanks would be necessary with identical seawater and alizarin flow. This would necessitate more accurate flow regulation than the present experimental design afforded.

The rate of translocation of photosynthetic products may be slower than previously estimated. Vandermeulen (1972) examined diurnal rhythm of calcification in *P. damicornis*
in which he maintained a colony of coral in a seawater table and at regular intervals over a 24 hr period he treated a portion of the colony with $^{45}$Calcium labeled seawater, each for 30 min in the same ambient light that was striking the parent colony. He found that the peak of calcification occurred during the early morning hours and it declined during the day. It was more marked with sunlight than a duplicate carried out under artificial light. His data indicate that calcification processes reach a peak shortly after sunrise but they decline throughout the entire day and reach a low point shortly after sunset. From that time on throughout the night there was an increase in deposition of skeletal calcium. It is presumed that this nocturnal enhancement is the result of slow translocation of the products of photosynthesis from the algal donor to the recipient calcifying host tissues. This slow translocation may account for satisfactory deposition of alizarin even after 48 hr preconditioning in total darkness.

Variation is such a common feature in all calcification experiments including this series that multiple replicates should be run before firm conclusions can be reached. The problems mentioned were encountered and would serve as further fields of investigation but were not within the scope of the present study and were not possible with the present apparatus.

Alizarin techniques are feasible for studying the light-dark processes of calcium deposition in corals. The
results of these experiments lead to far more questions on light enhancement than on problems solved.

The Influence of Phosphate Levels on Alizarin Deposition in Reef Corals

Phosphates have been used for many years commercially to prevent calcium carbonate scale from forming in the pipes of heating systems circulating fresh water. Bachra et al. (1963) in chemical precipitation studies noted that the calcium carbonate precipitation was prevented by the presence of the phosphate ions in concentrations too low for the precipitation of the calcium phosphate. They suggested that if the bicarbonate level were raised and the phosphate level in the medium reduced, an orderly precipitation of the various calcium carbonate polymorphs might take place.

Simkiss (1964) called phosphates crystal poisons for calcium carbonate crystals. He believed that the phosphates in seawater prevented the calcium carbonate deposition in corals. He postulated that one of the main functions of zooxanthellae in reef building corals was the removal of the offending phosphates so that calcification could then take place. Yamazato (1966) showed that very high levels of inorganic phosphate in seawater retarded calcification processes in Fungia scutaria and Porites compressa. A moderate concentration actually increased the calcification rate in Porites compressa.
Pomeroy (1969) reported that there was a net release of phosphates by reef corals that possessed zooxanthellae and that this was two or three orders of magnitude more than in ahermatypic corals without these algae. D'Elia (1972, personal communication) stated that corals raised in heavy phosphate concentrations had a tissue phosphate level higher than did the controls. Phosphate levels in and about reef corals may have a profound influence on the calcification processes. Caperon et al. (1970) report a wide variation in the phosphate levels in various sections of Kaneohe Bay with the H.I.M.B. being in a transition zone between high ambient levels and low concentrations of seawater phosphates. Caperon (1973), personal communication) gives the phosphate concentration for this transition zone as 0.05 \( \mu \text{g}/\text{l} \) whereas the level of phosphate in the open Pacific ocean was recorded as 0.1 \( \mu \text{g}/\text{l} \). Phosphates, he believes, are essential for coral growth but are probably not a growth limiting substance in Kaneohe Bay.

The present experiment was conceived as an attempt to formulate a method by which toxic agents could be applied to growing corals in various concentrations. The inhibition or concentration of precipitation of calcium carbonate could thus be studied. Phosphate is a normal constituent of seawater and it seemed a logical agent to alter in such a feasibility study.
Methods

Medium sized heads of *P. damicornis* 5 - 15 cm in diameter usually with their base of attachment were placed in an aquarium with a regulated intake of filtered seawater. After the corals had become acclimated, a mixture of alizarin and sodium hypo-phosphate (NaH$_2$PO$_4$) in distilled water was added to the intake. Because of the variable content in phosphate in and about Kaneohe Bay, an arbitrary level of 2 x 10$^{-7}$ M was chosen as a normal. A series of increasing concentrations of sodium hypo-phosphate was calculated so that when the solution was mixed with seawater there would be a logarithmic increase in the phosphate content in the aquarium and a continuous concentration of 10 ppm of alizarin.

The first control series was carried out for 24 hr with only alizarin. In part II the seawater content in the aquarium was 2 x 10$^{-5}$ M phosphate. In part III the phosphate concentration was 2 x 10$^{-4}$ M and in the final part (IV) the phosphate concentration was estimated to be 2 x 10$^{-3}$ M. When each run was finished the corals were removed to fresh water, cleaned, labeled and air dried. One head of *P. damicornis* used in the experiment with a phosphate content of 2 x 10$^{-3}$ M was returned to a seawater table for observation. pH was monitored before, during and at the termination of all parts of the experiment.

At the conclusion of the experiments, spectrophotometric absorbance studies were performed on representative heads of
coral. Two samples of 1 g each were procured from the column tips of each head. The superficial alizarin deposition was dissolved away with 5 ml EDTA 10% (w/v) in distilled water at pH 8.14. After one hour the solution was filtered, centrifuged at 3000 rpm for 5 min and an absorbance measured on the supernatant at 548 μm. EDTA-calcium solution was used as a blank.

Results

At the termination of the experiments all coral specimens appeared healthy. The head of *P. damicornis* that had been returned to the water table appeared as healthy as a newly collected specimen. The control (I) was a bright magenta color. There was an obvious visual difference in alizarin deposition in the coral skeletons in this series (Table 7).

Table 7. Spectrophotometric absorbance of alizarin dissolved in EDTA from corals raised in seawater with varying concentration of ambient phosphate.

<table>
<thead>
<tr>
<th>Number</th>
<th>Specimen</th>
<th>Phosphate Content (NaH₂PO₄)</th>
<th>Color</th>
<th>Absorbance (548 μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1, 2</td>
<td>2 x 10⁻⁷ M</td>
<td>red</td>
<td>.061</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.065</td>
</tr>
<tr>
<td>II</td>
<td>3, 4</td>
<td>2 x 10⁻⁵ M</td>
<td>pink</td>
<td>.038</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.035</td>
</tr>
<tr>
<td>III</td>
<td>5, 6</td>
<td>2 x 10⁻⁴ M</td>
<td>pink</td>
<td>.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.013</td>
</tr>
<tr>
<td>IV</td>
<td>7, 8</td>
<td>2 x 10⁻³ M</td>
<td>white</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.000</td>
</tr>
</tbody>
</table>
A logarithmic relationship is apparent between the ambient phosphate dissolved in the seawater and the alizarin deposited by *P. damicornis* with a correlation coefficient of $r = .99$, $p = .01$, Figure 26.

The pH of seawater in the open tanks during these experiments ranged from 8.13 to 8.20. At phosphate level of $2 \times 10^{-5}$ M the pH ranged from 8.02 to 8.06. At phosphate levels of $2 \times 10^{-4}$ M the pH remained almost stable at 7.5. In part IV, with phosphate levels of $2 \times 10^{-3}$ M, pH level in the aquarium quickly fell to 6.5. Other studies being run concurrently had shown that newly settled polyps of *P. damicornis* could deposit alizarin if kept at pH of 6.9 for 48 hr but could not do so at pH 6.5. During the next few hours the pH reached a low of 6.3 where it remained throughout the test period. The coral skeletons from part IV showed virtually no color though one skeleton did show a trace of alizarin color visually (Figure 27).

Unidentified coralline algae also deposited alizarin as they deposited calcium carbonate. In this series the alizarin deposition by the algae incrusting the basal attachments of the control specimen is not prominent. With increasing phosphate concentration there is also increasing alizarin deposition by the coralline algae. The most marked alizarin deposition by the calcifying algae was at a phosphate level of $2 \times 10^{-3}$ M. This gave an acid solution which was apparently too low for alizarin deposition in *P. damicornis*. This can be seen in Figure 27.
Figure 26. The effect of altered ambient seawater phosphate on alizarin deposition by corals as shown by spectrophotometric absorbance of the alizarin redissolved from the corals.
Colonies of *Pocillopora damicornis* were subjected to alizarin 10 ppm combined with varying strengths of ambient phosphate in seawater for 24 hr each.

1. Normal phosphate $2 \times 10^{-7}$ M
2. Phosphate level $2 \times 10^{-5}$ M
3. Phosphate level $2 \times 10^{-4}$ M
4. Phosphate level $2 \times 10^{-3}$ M

Figure 27
Discussion

Pocillopora damicornis can live in relatively high concentrations of sodium hypo-phosphate but they do not deposit alizarin, nor presumably, calcium carbonate at these levels. This decrease in alizarin deposition is in accord with the views of Simkiss (1966) that precipitation of aragonite by corals is prevented by phosphates and is accelerated by the removal of phosphates by the zooxanthellae. High levels of tissue phosphate would cause a chemical competition and would discourage calcification. This experiment lends credence to this view. I have concluded that the methods used in this experiment were satisfactory for determining the effects of chemical additives on the alizarin deposition by living corals.

Alizarin as a Marker for Growth Studies

Growth in reef corals has been measured by changes in weight and linear extensions over a span of time. Some workers have used ring markers or similar devices and have recorded growth from that point upward (Edmondson, 1929). Using available equipment, an experiment was set up to mark corals with alizarin for replacement on the reef.

Method

Adult heads of P. damicornis were collected and were marked with alizarin in running seawater at a continuous concentration of 10 ppm for 48 hr. They were then taken to an area in Kaneohe Bay where there was some protection from
high surf but in the vicinity of healthy heads of the species. They were identified with plastic labels attached by stainless steel wires and deposited on a firm substrate at a depth of 1 m at low tide.

Six weeks later one small head was dead so it was washed in fresh water and dilute Clorox. It was air dried. A large head about 20 cm across remained alive for five winter months. It had not shifted position and appeared healthy when harvested. It was cleaned as usual.

Slides on which planulae of <i>P. damicornis</i> had settled were taken into the laboratory and exposed to alizarin, 10 ppm in seawater in a closed container for 24 hr. They were then returned to a seawater table for 48 hr after which they were cleaned and dried.

**Results**

The small head of <i>P. damicornis</i> which had been on the reef for six weeks showed typical alizarin deposition on the terminal 15 mm of each column and somewhat less farther down the columns. Superimposed on the tip was a 4 mm extension of pure white skeleton the length of which could be made up of two calyx diameters. The calyces at the junctions of colored and uncolored skeleton were generally magenta colored on their lower margin and white on the upper rim and with colored dissepiment. The coenosteum in <i>P. damicornis</i> is characterized by many minute spines and in this specimen they were whiter than expected, suggesting
some superimposed calcium carbonate deposition. The dissepiments showed varying patterns, some showing a white outer rim with a pink center. Others were uniformly white although the calyx was colored so the dissepiment appeared to have developed in the interim. It is not known how long this specimen lived after it had been returned to the reef. All organic matter was gone when it was collected but algal growth had not yet appeared on the surface (Figure 28).

The larger colony of *P. damicornis* showed evidence of growth over the entire convexity ranging from 2-3 mm on one side to 10-12 mm on the opposite side with a gradation between. The new growth consisted of extensions of the column tips which were up to seven calyx diameters in length. There were many newly formed papillae. It had previously been noted that some heads of *P. damicornis* deposit alizarin rather evenly over the entire growing surface and not only at the column tips. The large head showed such a distribution when it was cleaned. None of this head was sacrificed at the time it was placed on the reef and it is assumed that a generalized distribution of alizarin was present from the onset.

With magnification new skeletal material could be seen on all surfaces but not enough to hide the magenta stain of the alizarin. This new skeleton was most apparent on the rims of the individual calyces although many dissepiments displayed a white central disk indicating new calcium carbonate deposition. This was distinctly different from
Figure 28. Photograph of two columns of *P. damicornis* skeleton. The colony had been marked with alizarin and returned to the reef for six weeks. Slightly enlarged.
a dissepimental extension in that the texture was more
granular and the color of the alizarin at the margins
blended instead of being sharply cut off. At fracture sites
alizarin deposition was grossly visible as a hair-line
streak between two white surfaces (Figures 29 and 30).

Newly settled polyps of *P. damicornis*, *Cyphastrea*
*ocellina* and *Tubastrea aurea* all deposit alizarin in their
skeletons. If they are returned to normal seawater the
colored portion is covered by white skeleton. At times
some areas lag in their development with a subsequent speed
up so that symmetry is preserved. Such areas are often
strikingly delineated when such specimens are viewed with
magnification (Figure 31).

**Discussion**

Mayor (1924) reported that the average linear extension
for *P. damicornis* was 23 mm in one year. I estimated that
the specimen examined in this experiment showed a linear
extension of about 1 cm average. This included growth in
cooler months and the yearly averages probably are comparable.
This technique is superior in that the exact site of onset of
new growth can be determined.

Kinzie (1973, personal communication) showed me a
specimen of *Goniastrea* sp that he had marked in situ on a
reef in Eniwetok. There was an inch of growth beyond an
excellent alizarin colored band, seen in vertical section,
that had occurred in one year.
Figure 29. Skeleton of *P. damicornis* marked with alizarin and returned to the reef for five months. Photograph of skeleton 1/3 life size.

Figure 30. Central area of skeleton shown in Figure 31. About natural size.
The method used in this experiment is convenient. It can be performed in the laboratory under controlled conditions and the corals can then be returned to near their normal habitat. It is versatile and can be used for most of the corals found in Kaneohe Bay.

Figure 31 Skeletons of *P. damicornis* showing alizarin stained skeletons with superimposed unstained calcium carbonate.
V. ALIZARIN DEPOSITION BY NEWLY SETTLED CORAL POLYPS

Many aspects of coral growth can be studied in an aquarium with running seawater although other studies can be better carried out in a laboratory where more conditions can be kept constant. These include studies of coral calcification in which seawater media is altered. A series of experiments were thus outlined in which various parameters were altered to study the effect on calcium deposition as indicated by alizarin uptake.

These experiments constitute a study in methodology of alizarin use. They were carried out concurrently with experiments performed and reported in previous sections. The optimum alizarin concentrations as well as methods for raising planulae for such experiments have been reported in previous sections.

The Effect of Altered Seawater Calcium Levels on Alizarin Deposition by Reef Corals

The biological influence of calcium has been the subject of many reviews (Simkiss, 1967; Budy, 1967; Cuthbert, 1970) and the biological transport systems have been much studied (Wasserman, 1963; Levin, 1967; Jarnfelt, 1968). The conclusion reached is that the mechanism by which calcium ions cross membrane barriers is imperfectly understood. Calcium is essential for the health and growth of all living organisms and to enter a cell it must pass through the biological membranes which bound such units.
Calcium ions function in the transmission of nerve impulses. They are essential for muscle relaxation. Calcium is necessary for ciliary function and it has an action in binding cells one to another. The amount of non-organelle fixed calcium must be stringently limited to a relatively low intracellular level and any excess is sequestered (Hurwitz, 1973). Hasselbach (1967) estimated that the concentration of the free calcium in the muscle cells of Balanus and Maia was $5 \times 10^{-7}$ M. If this level is surpassed it will "wreck" the enzyme systems (Howard, 1967).

All living cells possess active transport systems for calcium either to store it in the phospho-lipid fraction of the cytoplasm or to eliminate it. It has been assumed that the calcification processes in corals are active ones so that calcium must traverse at least two layers of cells and the mesoglea with ultimate deposition by the calicoblasts in the form of polygonal aragonite crystals to build up an ever enlarging corallum. Wise (1970) in his studies of P. damicornis with scanning electron microscopy, described these developing crystals as appearing in the form of lathes, blades or needles in arrays or clusters so they formed a uniform covering except where living polyps were attached by tiny strands of tissue.

Barnes (1971) suggested that the crystallization process takes place in a space created by the corals themselves and demonstrated how such spaces existed at the growing edge of the epitheca. This is consistent with the view that
calcification is to some extent a passive process, aragonite crystals forming in stacks as they precipitate from a supersaturated solution. This view is supported by Vandermeulen (1972) who found the calicoblast epidermis of the fully settled polyp of P. damicornis to be nonsyncytial, extremely flattened and of a single cell type. He found no surface specializations, no intracellular accumulations of calcareous particles and noted that the calicoblast epidermis was separated from the skeletal surface by a 1 - 5 μ gap devoid of electron opaque structures. There was no evidence of a partially calcified region between the tissues and the skeletal surface. The calcium, probably in ionic form, is deposited in this space where the calcium carbonate molecule is assembled and precipitation occurs on the corallum, the outline of the skeleton being determined by the coral polyp.

Yamazato (1966) studied ambient calcium concentrations in seawater and its uptake by corals. He exposed polyps of Fungia scutaria to varying concentrations of calcium and measured the uptake using 45Ca. He found the rate of uptake from artificial seawater to increase linearly as the calcium was increased from 0 to 400 mg l. He noted an acceleration over the linear increase with greater concentrations than this. His studies measured calcium uptake in the entire polyp and not calcium deposition in the skeleton alone.
The present experiments were designed to explore coral deposition of alizarin in seawater with an altered available calcium content.

**Method**

Seawater calcium was bound with a chelating agent, EGTA, to give a series of incubating media with low available calcium. NaOH was added to keep the initial pH at 8.2. Supplemental calcium and sufficient EGTA to bind it was added to three containers to rule out deleterious effects of the EGTA. In these containers the available calcium remained at normal levels.

The experiments were performed using one liter plastic containers with seawater media to which 10 ppm of alizarin had been added. One or more glass slides with healthy, newly settled polyps of *P. damicornis* were lowered into each; also, a 4 cm terminal branch of *P. damicornis*. All were taken from the same coral head. The preparations were maintained in full artificial light. pH was monitored. Replicate calcium determinations were made on the second day and were consistent to less than 1 mg/l variation. At the conclusion, all specimens were examined under low power magnification following which they were cleaned with fresh water and dilute Clorox.

A second series (II) using larger increments was established so that the test range extended from 0 mg/l to 500 mg/l. The same methods were used as in series I.
Results

Large colonies of \textit{P. damicornis} take alizarin stain very poorly at times, more often in stagnant than in running seawater. The adult specimens used in series I were uniform in that they failed for no obvious reason to deposit enough dye to be evaluated though all but one of the samples lived. These are reported in Table 8 with data on the estimated calcium levels, the actual calcium levels determined and pH changes. The polyps described in the table are those which had settled on glass slides and all of these revealed good alizarin deposition at all calcium levels studied. The pH values in this series remained within a range that would not significantly alter the calcification. The seawater dilution by the small amounts of distilled water used to dissolve EDTA and alizarin were too small to warrant concern. Water evaporation over the 48 hr period was less than 5%.

The behavior of the polyps used in series I, did not seem in any way different from the control specimens. There was no apparent evidence of a harmful effect on the polyps individually by the EGTA bound calcium. Calcium deposition was satisfactory at all levels of ambient calcium studied.

The data from series II of this experiment is divided for convenience and is presented in Tables 9 and 10. The first of these records the calcium levels estimated and the calcium levels determined titrimetrically, the physical condition of the adult polyp at the termination of the
Table 8. Data on Series I. The effect of altered seawater calcium on corals.
Polyp health concerns the polyps on adult columns of *P. damicornis* whereas
the alizarin deposition is for newly settled polyps of *P. damicornis*.

<table>
<thead>
<tr>
<th>Number</th>
<th>Estimated calcium mg/l</th>
<th>Measured calcium mg/l</th>
<th>pH changes</th>
<th>Polyp health 48 hr</th>
<th>Alizarin deposition in skeleton</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>460</td>
<td>402</td>
<td>Initial 8.00</td>
<td>24 hr 8.12</td>
<td>48 hr 8.03</td>
</tr>
<tr>
<td>2.</td>
<td>440</td>
<td>405</td>
<td>Initial 8.00</td>
<td>24 hr 8.10</td>
<td>48 hr 8.00</td>
</tr>
<tr>
<td>3.</td>
<td>420</td>
<td>403</td>
<td>Initial 7.97</td>
<td>24 hr 8.10</td>
<td>48 hr 8.04</td>
</tr>
<tr>
<td>4.</td>
<td>Normal</td>
<td>409</td>
<td>Initial 8.00</td>
<td>24 hr 8.20</td>
<td>48 hr 8.02</td>
</tr>
<tr>
<td>5.</td>
<td>Control</td>
<td>408</td>
<td>Initial 8.00</td>
<td>24 hr 8.18</td>
<td>48 hr 8.02</td>
</tr>
<tr>
<td>6.</td>
<td>380</td>
<td>382</td>
<td>Initial 8.28</td>
<td>24 hr 8.16</td>
<td>48 hr 8.12</td>
</tr>
<tr>
<td>7.</td>
<td>360</td>
<td>364</td>
<td>Initial 8.00</td>
<td>24 hr 8.10</td>
<td>48 hr 7.70</td>
</tr>
<tr>
<td>8.</td>
<td>340</td>
<td>334</td>
<td>Initial 8.03</td>
<td>24 hr 8.10</td>
<td>48 hr 8.10</td>
</tr>
<tr>
<td>9.</td>
<td>320</td>
<td>321</td>
<td>Initial 8.01</td>
<td>24 hr 8.03</td>
<td>48 hr 8.00</td>
</tr>
<tr>
<td>10.</td>
<td>300</td>
<td>316</td>
<td>Initial 8.01</td>
<td>24 hr 8.18</td>
<td>48 hr 8.16</td>
</tr>
</tbody>
</table>
Table 9. Data on Series II. The effect of altered seawater calcium on adult polyps of *P. damicornis*. See text.

<table>
<thead>
<tr>
<th>Number</th>
<th>Estimated calcium mg/l</th>
<th>Measured calcium mg/l</th>
<th>Polyp health</th>
<th>Alizarin deposition</th>
<th>Gall crab signs</th>
<th>Alizarin in gall</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>500</td>
<td>507</td>
<td>Normal</td>
<td>Good</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>450</td>
<td>464</td>
<td>Normal</td>
<td>Good</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>400</td>
<td>430</td>
<td>Normal</td>
<td>Slight</td>
<td>Empty</td>
<td>No</td>
</tr>
<tr>
<td>4.</td>
<td>Control</td>
<td>428</td>
<td>Tentacles extended</td>
<td>None</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>350</td>
<td>361</td>
<td>Normal</td>
<td>Excellent</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Empty</td>
<td>Yes</td>
</tr>
<tr>
<td>6.</td>
<td>300</td>
<td>301</td>
<td>Contracted</td>
<td>Slight</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>7.</td>
<td>250</td>
<td>250</td>
<td>Alive</td>
<td>Slight</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>8.</td>
<td>200</td>
<td>201</td>
<td>Contracted</td>
<td>No</td>
<td>Empty</td>
<td>No</td>
</tr>
<tr>
<td>9.</td>
<td>150</td>
<td>134</td>
<td>Giliary currents</td>
<td>No</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>10.</td>
<td>100</td>
<td>72</td>
<td>Alive?</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>11.</td>
<td>50</td>
<td>21</td>
<td>Dissolution</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>12.</td>
<td>0</td>
<td>0</td>
<td>Empty</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
experiment and the evidence of alizarin uptake in the adult skeletons. (Table 9 includes newly settled polyps.)

It was previously noted that many adult colonies of *P. damicornis* harbor the gall crab *Haplocarcinus marsupialis*. Of the 12 stalks used in this experiment, eight of the galls were present and presumably the crabs were living in as much as several of the galls contained the dried remnants of an adult crab. In some instances there was increased dye uptake within the gall and on the edges of the cusps which was thought to be a reflection of the general excellent alizarin deposition in the entire specimen. In others there was increased dye uptake within the gall with evidence of crab occupancy but without alizarin uptake in the rest of the specimen. The gall on number 5 had crab remnants with no alizarin deposition whereas specimen 10 showed the reverse in that the column showed no alizarin but the gall with crab remnants had alizarin deposition within the cavity and at the edges of the excurrent water pores.

These galls are symbiotic phenomena and the microhabitat induced by the filter feeding crab may be considerably different from the environment of the experimental container. In specimen #10, alizarin was deposited in the gall at a very low level of ambient calcium in which coral tissues barely remained alive and did not show evidence of alizarin deposition in any other area.

The polyps of adult corals normally extend 1 mm or more above the calyx rim in these corals and the ring of tentacles when expanded doubles the visualized diameter.
Alizarin at 10 ppm is mildly toxic so the polyps do not fully extend their tentacles when in this solution though in other ways they appeared to behave normally. The physiological function of the polyps was gradually compromised with declining levels of ambient calcium so they contracted and, when severe, the entire polyp was below the calyx rim.

When ambient calcium levels were decreased to 300 mg/l the contraction of the adult polyp was noticeable. It became maximal when seawater calcium was 200 mg/l.

Newly settled polyps of *P. damicornis* were very vigorous in their uptake of alizarin as compared with adult polyps. The data for these are given in Table 9. When they were living the calcification could be seen either from above but better from below through the glass. Figure 32 is a photograph of a colony from below, at a calcium concentration of 507 mg/l. The group of three polyps shows a brilliant radial calcification with distortion in one of the skeletons. Such configurations are commonly seen where several planulae have settled together and in themselves do not constitute an abnormality. This photograph can be compared to Figure 33 which shows alizarin uptake by a newly settled coral polyp in unaltered seawater.

No attempt was made to quantify the amount of alizarin taken up by these minute skeletons and for that reason they were ranked in five grades so the best deposition with the most alizarin was considered to be excellent; less than this either in distribution or intensity was graded as good; a
Figure 32. Polyps of *P. damicornis* photographed through the glass slide to show radial calcification with alizarin incorporation in the septa. Calcium concentration was 507 mg/liter. (Enlarged x 36)
Figure 33. Skeleton of *P. damicornis* polyp. Alizarin deposition was at 10 ppm in normal seawater. (x36)

Figure 34. Deteriorating polyps of *P. damicornis* subjected to calcium levels of 1/3 normal for 48 hr. Very faint alizarin deposition. (x18)
third rank was labeled fair when the skeletal structures were pink or if the color was not extensive. The fourth rank was sparse when alizarin deposition was spotty or faint and the lowest rank was none. These rankings are listed in Table 10.

Traces of alizarin were visible in the skeletal septa of polyps raised in seawater in which the ambient calcium level had been reduced to one third normal (134 mg/l). Figure 34 shows a group of such polyps. At one stage in their lives they had deposited calcium carbonate which contained traces of alizarin but there is little doubt that these are deteriorating polyps and probably would not have survived. When ambient calcium levels had fallen to one fourth of seawater normal, individual polyps remained alive for 48 hr but these did not deposit calcium which had any alizarin stain.

Discussion

Calcium is necessary for cellular function but when seawater calcium is reduced to about one third normal the coral polyps were markedly contracted and did not respond to touch but ciliary currents were still present. It is apparent that coral polyps can live for a considerable time at calcium levels about half seawater normal and can form traces of a skeleton at these levels. They do not deposit calcium carbonate at calcium levels where they barely survive.
Table 10. Data on Series II. The effect of altered seawater calcium on polyps of *P. damicornis* which had settled on glass slides.

<table>
<thead>
<tr>
<th>Number</th>
<th>Estimated calcium mg/l</th>
<th>Measured calcium mg/l</th>
<th>Health pf polyps on slides</th>
<th>Alizarin deposition in skeletons</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>500</td>
<td>507</td>
<td>Clumps, good</td>
<td>Excellent</td>
</tr>
<tr>
<td>2.</td>
<td>450</td>
<td>464</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>3.</td>
<td>400</td>
<td>430</td>
<td>Good</td>
<td>Excellent</td>
</tr>
<tr>
<td>4.</td>
<td>Control</td>
<td>428</td>
<td>Good</td>
<td>Uncolored</td>
</tr>
<tr>
<td>5.</td>
<td>350</td>
<td>361</td>
<td>Good</td>
<td>Excellent</td>
</tr>
<tr>
<td>6.</td>
<td>300</td>
<td>301</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>7.</td>
<td>250</td>
<td>250</td>
<td>Good</td>
<td>Fair</td>
</tr>
<tr>
<td>8.</td>
<td>200</td>
<td>201</td>
<td>Alive</td>
<td>Fair</td>
</tr>
<tr>
<td>9.</td>
<td>150</td>
<td>134</td>
<td>Contracted clump</td>
<td>Sparse traces</td>
</tr>
<tr>
<td>10.</td>
<td>100</td>
<td>72</td>
<td>Alive</td>
<td>None</td>
</tr>
<tr>
<td>11.</td>
<td>50</td>
<td>21</td>
<td>Dead</td>
<td>None</td>
</tr>
<tr>
<td>12.</td>
<td>0</td>
<td>0</td>
<td>Dead</td>
<td>None</td>
</tr>
</tbody>
</table>
When artificial seawater is mixed, a calcium concentration greater than 240 mg/l results in calcium carbonate precipitation (Paasche, 1963). Krauskopf (1967) noted that seawater is a concentrated and exceedingly complex solution and ordinary laws of dilute solutions cannot be applied or at best, need great modification before being applied to specific problems. He observed that organisms effect the formation of insoluble compounds primarily by creating conditions favorable for their precipitation rather than by changing the solubility products. Weyl (1961) concurs that the equilibration of carbonate minerals with seawater is more complicated than "would have anticipated".

Although calcium carbonate deposition by corals can occur in artificial seawater (personal observation) and with ambient calcium levels of 200 mg/l, the rate is very slow and it is doubtful if any coral could produce a vigorous colony comparable to those found on a coral reef. The EGTA itself did not seem to be harmful to coral polyps. It is a chelator of metallic ions such as calcium, magnesium and strontium. The magnesium content in seawater is five times that of calcium. It is essential for cellular function but the requirements are probably much less than for calcium (Nicol, 1967) and I believe it can be ignored in this experiment. The same holds for strontium.

There is apparently an unexplained celluarly primed and controlled ion pumping mechanism so that calcium is
extracted from seawater even though the available calcium is at very low levels. It is probable that an efficient concentrating mechanism is present so that the calcium carbonate levels are raised in the space between the calicoblastic layer and the skeleton to a state of supersaturation. Aragonite precipitation can then occur. The calicoblastic layer apparently is of prime importance and may govern the entire process.

Alizarin deposition at levels of available calcium at levels of one third those ordinarily found seems to indicate that the organism actively extracts calcium from seawater, concentrates it and allows the calcium carbonate to precipitate. Alizarin participates in this process in that it is transported and precipitates with the calcium carbonate. It is not known whether or not it is bound to the calcium before it is absorbed; however, it does not seem to interfere with the process as such.

Corals can efficiently extract calcium from seawater with low levels of unbound calcium; but the efficiency of the coral animal in reef building is most likely coupled with the supersaturation of tropical seawaters. It is doubtful if a coral reef could be formed if this were not so. This experiment did not delineate the intracellular mechanisms by which alizarin molecules are transported; however, alizarin has been shown to be a valuable tool by which the transport abilities of these tissues can be studied.
Dilution and Concentration of Seawater
and its Effect on Alizarin Deposition

Scleractinian corals do not live in brackish water (Remane & Schlieper, 1971) and do not tolerate prolonged dilutions of seawater. It has been the observation of those who have lived on islands surrounded by fringing reefs that there is frequently a navigable break where fresh water streams debouch. Goreau (1964) and Banner (1968) have reported great destruction of reefs by inundations of fresh water; however, Edmondson (1929) found that Fungia scutaria would live for months when kept in 75% seawater, but it lost weight. He concluded that some corals could live indefinitely in this concentration provided they had food. Yamazato (1966) studying the uptake of $^{45}$Calcium by $F$. scutaria varied the salinity so it ranged from 125% to 25% of normal seawater. He found that altered salinities had an adverse effect on the calcium uptake and this was independent of the altered calcium concentration.

The present experiment was designed to study the alizarin deposition in $P$. damicornis in seawater with altered salinities and calcium concentrations.

Method

Plastic containers holding one liter each were used in this laboratory experiment which was done under the standard conditions described. To achieve a concentration of 120%
seawater, 6 g of NaCl and 1.6 g mgSO$_4$ were added to the seawater media. Distilled water was used for dilution to form an incremental series which was checked with a salinometer. Data are outlined in Table 11. To have ambient calcium levels as near normal as possible, CaCl$_2$ was added to some vessels. Alizarin 10 ppm was added. The ambient calcium was determined on the first day and pH was recorded daily. Glass or plastic slides on which newly settled polyps of *P. damicornis* had settled were lowered in each tank as well as a column tip from the central portion of the same colony of *P. damicornis*.

After 24 hr several of the tanks had lost their alizarin color so the alizarin content was renewed. At 48 hr all specimens were cleaned with fresh water, washed with dilute Clorox and air dried.

**Results**

The data (Table 11) show that adult polyps of *P. damicornis* have neither the tolerance to dilution nor to concentration of seawater as do newly settled polyps as determined by the visual health of the specimens after 48 hr and by their ability to deposit alizarin. Adult polyps in this series survived a dilution of between 60% to 70% for 48 hr while a newly settled polyp would tolerate a dilution of 50% for that period of time. At the levels given of extreme tolerance neither the adult nor the newly settled polyp deposited any calcium as determined by the lack
Table 11. Concentration-Dilution studies on alizarin deposition by P. damicornis. Data on salinity, calcium levels, pH, color changes and colony health.

<table>
<thead>
<tr>
<th>Number</th>
<th>Dilution or conc. % normal</th>
<th>Salinity 0/00</th>
<th>Estimated calcium mg/l</th>
<th>Actual calcium mg/l</th>
<th>Initial pH</th>
<th>24 hr pH</th>
<th>48 hr pH</th>
<th>Solution color in 24 hr</th>
<th>Health of adult polyps</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>120</td>
<td>40.5</td>
<td>411</td>
<td>412</td>
<td>8.00</td>
<td>7.70</td>
<td>7.92</td>
<td>Gone</td>
<td>Alive</td>
</tr>
<tr>
<td>2.</td>
<td>110</td>
<td>37.5</td>
<td>411</td>
<td>408</td>
<td>8.05</td>
<td>7.90</td>
<td>8.00</td>
<td>Gone</td>
<td>Alive</td>
</tr>
<tr>
<td>3.</td>
<td>100</td>
<td>35.0</td>
<td>411</td>
<td>408</td>
<td>8.07</td>
<td>7.97</td>
<td>8.03</td>
<td>Gone</td>
<td>Alive</td>
</tr>
<tr>
<td>4.</td>
<td>90</td>
<td>31.5</td>
<td>370</td>
<td>359</td>
<td>8.08</td>
<td>8.10</td>
<td>8.10</td>
<td>Gone</td>
<td>Alive</td>
</tr>
<tr>
<td>5.</td>
<td>80</td>
<td>27.5</td>
<td>329</td>
<td>330</td>
<td>8.09</td>
<td>8.00</td>
<td>7.92</td>
<td>Normal</td>
<td>Alive</td>
</tr>
<tr>
<td>6.</td>
<td>70</td>
<td>24.5</td>
<td>288</td>
<td>285</td>
<td>8.00</td>
<td>7.75</td>
<td>7.78</td>
<td>Normal</td>
<td>Alive ?</td>
</tr>
<tr>
<td>7.</td>
<td>60</td>
<td>20.0</td>
<td>247</td>
<td>240</td>
<td>7.92</td>
<td>7.72</td>
<td>7.50</td>
<td>Murky</td>
<td>Dead ?</td>
</tr>
<tr>
<td>8.</td>
<td>50</td>
<td>17.5</td>
<td>206</td>
<td>207</td>
<td>7.94</td>
<td>7.88</td>
<td>7.50</td>
<td>Murky</td>
<td>Dead</td>
</tr>
<tr>
<td>9.</td>
<td>120</td>
<td>40.0</td>
<td>492</td>
<td>472</td>
<td>7.92</td>
<td>7.60</td>
<td>7.30</td>
<td>Murky</td>
<td>Dead</td>
</tr>
<tr>
<td>10.</td>
<td>90</td>
<td>31.0</td>
<td>411</td>
<td>404</td>
<td>8.00</td>
<td>7.95</td>
<td>7.30</td>
<td>Murky</td>
<td>Dead</td>
</tr>
<tr>
<td>11.</td>
<td>70</td>
<td>24.5</td>
<td>411</td>
<td>403</td>
<td>7.97</td>
<td>7.88</td>
<td>7.70</td>
<td>Gone</td>
<td>Alive</td>
</tr>
<tr>
<td>12.</td>
<td>50</td>
<td>17.5</td>
<td>411</td>
<td>403</td>
<td>7.82</td>
<td>7.85</td>
<td>7.38</td>
<td>Murky</td>
<td>Dead</td>
</tr>
<tr>
<td>13.</td>
<td>Control</td>
<td>35.0</td>
<td>411</td>
<td>411</td>
<td>8.18</td>
<td>8.13</td>
<td>8.11</td>
<td>Clear</td>
<td>Alive</td>
</tr>
</tbody>
</table>
of alizarin deposition.

The seawater pH in these experiments did not vary greatly in 48 hr in those tanks that had a dilution or concentration 10% from normal. There was a fall in the pH during the 48 hr more marked with the greater dilutions. This seemed to be directly related to the declining well-being of the polyps. In this series decoloration of the solutions was apparent in all healthy colonies. The alizarin color did not disappear in those tanks in which the colonies eventually died. Neither did these colonies show alizarin deposition in the skeletons. Addition of calcium to bring the ambient level to normal or over normal did not alter the ability of the adult or the newly settled polyp to withstand the altered salinity and it may have had adverse effect.

In this experiment the alizarin deposition occurred in the adult polyps; it was not outstanding though there was a distinct difference among the specimens so that they could be ranked in approximate order as to the amount of alizarin deposition that had occurred. This ranking considered both the depth of color and the area involved. The results recorded in Table 12 show that the greatest alizarin uptake in this series was in newly settled polyps in water which had been diluted to 80% of normal seawater. Alizarin deposition in the adult polyps appeared to be most efficient when the seawater was diluted slightly and was 90% of normal. Newly settled polyps were able to deposit alizarin when the seawater was diluted to 70% of normal. After 48 hr in 70%
normal seawater the polyps gave very little reaction to
direct stimulation. The mouth gaped and the mesentarial
filaments were extruded. It is doubtful if these polyps
would have survived for an additional 24 hr. The newly
settled polyps of *P. damicornis* continued to deposit alizarin
in seawater diluted to 70% normal. Figure 36 is a photo­
graph of a polyp skeleton with alizarin deposited under such
conditions.

**Discussion**

The color loss in the test containers was apparent
only in those in which the polyps were not dead at the end
of 48 hr, due, probably in part, to active absorption by
the coral polyps. It is unlikely that the death of the
other polyps was due to a toxic reaction to the dye although
this, with the osmotic stress occasioned by the dilution,
may have contributed to their demise.

Maragos (1972) found that *Montipora verrucosa* grew
more rapidly in seawater where salinity was lower than normal.
These studies indicate that *P. damicornis* functions well in
seawater that is slightly dilute. The reasons are obscure
but, the ability to perform thus would enable a planula of
*P. damicornis* to be better able to colonize areas where
there was some dilution by fresh water.

This was a study in methodology to determine if alizarin
was satisfactory for delineating the calcification processes
under such altered conditions. Alizarin is a satisfactory
Table 12. Concentration-Dilution studies on alizarin deposition by *P. damicornis*. Data on deposition in adult and newly settled polyps with rank as to observed amount from most to least.

<table>
<thead>
<tr>
<th>Number</th>
<th>Dilution conc. % normal</th>
<th>Alizarin deposition by adult polyps</th>
<th>Rank among adult skeletons</th>
<th>Alizarin in newly settled polyps</th>
<th>Rank among newly settled skeletons of polyps</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>120</td>
<td>Sparse</td>
<td>7</td>
<td>Fair</td>
<td>6</td>
</tr>
<tr>
<td>2.</td>
<td>110</td>
<td>Fair</td>
<td>6</td>
<td>Fair</td>
<td>4</td>
</tr>
<tr>
<td>3.</td>
<td>100</td>
<td>Good</td>
<td>2</td>
<td>Good</td>
<td>3</td>
</tr>
<tr>
<td>4.</td>
<td>90</td>
<td>Excellent</td>
<td>1</td>
<td>Good</td>
<td>2</td>
</tr>
<tr>
<td>5.</td>
<td>80</td>
<td>Good</td>
<td>3</td>
<td>Excellent</td>
<td>1</td>
</tr>
<tr>
<td>6.</td>
<td>70</td>
<td>Sparse</td>
<td>8</td>
<td>Fair</td>
<td>7</td>
</tr>
<tr>
<td>7.</td>
<td>60</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>50</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>120</td>
<td>Fair</td>
<td>4</td>
<td>Good</td>
<td>5</td>
</tr>
<tr>
<td>10.</td>
<td>90</td>
<td>Fair</td>
<td>5</td>
<td>Fair</td>
<td>8</td>
</tr>
<tr>
<td>11.</td>
<td>70</td>
<td>Sparse</td>
<td>9</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>50</td>
<td>None</td>
<td>9</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Control</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>
dye and the results can be graded but no firm conclusions can be reached from a small sample such as this. The study does indicate that newly settled polyps of *P. damicornis* can stand altered environmental conditions of some types better than can the adult polyps of the same species.

Figure 35  Skeleton of newly settled polyp of *P. damicornis* showing alizarin deposition that occurred when seawater was diluted to 70% normal. (x36)
VI ALIZARIN USEFULNESS IN CORAL STUDIES

Alizarin can serve as a marker to delineate the pattern of calcium carbonate by reef corals. These studies show that this dye is deposited in the same proportion as 45Ca in normal seawater. It is assumed that the visual pattern of alizarin deposition is an accurate measure of calcium carbonate deposited during the time the organism was in contact with the dye.

Time-lapse studies of living coral show that they are not static and that all visible surfaces are in constant motion. Some of this activity consists of a rhythmical contraction and expansion of the polyp, although the coenosarc also displays constant movement. The function of this may be to aid circulation of water which would thus bring a renewed supply of seawater supersaturated with calcium carbonate to the areas of skeleton formation.

These studies have shown that a coral polyp can form a skeleton under conditions in which available calcium is reduced to about one third of normal seawater. Cloud (1965) reports that skeletal construction can take place in molluscs even where waters are barely saturated or actually undersaturated with calcium carbonate. The accumulation of calcium carbonate by corals is probably also an active process and in this way resembles some other marine animals. The coral animal is extremely successful in construction of a skeleton and undoubtedly the supersaturation of the surrounding waters favor this.
There are a number of factors which control the polymorphic crystallization in calcareous organisms. Pytkowicz (1965) lists as inhibitors of inorganic calcium carbonate nucleation, magnesium, organic molecules, phosphates and carbon dioxide. Coral tissues apparently selectively transport calcium and carbonate and deposit them into a confined space between the skeleton and the calicoblastic surface. In doing so the natural inhibitors can be held back. The materials in solution most probably pass from the gastrovascular cavity and through the gastrodermis where the zooxanthellae can utilize phosphates and carbon dioxide, thus abetting the process. The reduced carbon is transferred from the zooxanthellae to the corals and can be incorporated into the skeleton by mechanisms not understood, though some of it is lost to the animal (Johannes et al. 1970).

Walton (1965) in discussing skeletal growth confirms that supersaturation is required before an orderly growth of a crystal can occur on a preexisting crystal or on a suitable nucleus. It has never been demonstrated that a coral actually does create a condition of supersaturation although it presumably does so. Whether it does so by direct transfer of materials into a solution or if this is accomplished by reabsorption of excess water is unknown. Alizarin probably accompanies the calcium in this process. It has not been determined if the two are in chemical combination at that
time. Alizarin being a colored substance affords a means to visualize certain phases of these processes but the exact mechanisms of these processes are still obscure.
VII SUMMARY

The studies here reported were performed using the reef coral *Pocillopora damicornis* either in plastic aquaria with running seawater or in a laboratory with constant temperature, humidity and lighting. Several other hermatypic and one ahermatypic coral found in Kaneohe Bay, Oahu, Hawaii were also used. Spectrophotometric and pH determinations were made with standard equipment but calcium determinations by titrimetric methods necessitated changes in technique which are described.

2. The purpose of these studies was to evaluate the use of a hydroquinone dye, alizarin red S, which is at times deposited with calcium through biological activity. The dye has been used as an indicator and at the pH of seawater it imparts a magenta color. It is readily oxidized to colorless breakdown products. Alizarin forms salts with earthy metallic cations but it is not understood why it is incorporated with calcium by living animals.

3. Experiments were performed with varying amounts of alizarin in seawater using newly settled coral polyps, small colonies of *P. damicornis* and comparable fragments of a large colony to determine the optimum concentration to use for testing coral growth. The dye deposition was more marked in newly settled than in adult coral polyps. Visible incorporation occurred with 1 ppm of dye in seawater for 24 hr. Alizarin deposition was widespread and most intense when
applied continuously in running seawater at 10 ppm.

4. Alizarin is toxic in large amounts to all living organisms on which it has been tested. Alizarin at 10 ppm in running seawater caused a mild stress to adult corals so they released mature planulae and withdrew their tentacles. Time-lapse studies on coral planulae and newly settled polyps revealed no behavioral changes. Serpulid worms found on many slides showed lessened deposition of calcium when alizarin was incorporated as compared to deposition in comparable periods without the dye.

5. Alizarin has low solubility in seawater estimated to be about 15 ppm. A method was devised so skeletally deposited alizarin could be redissolved from the corals with 10% EDTA solution adjusted to the pH of seawater. Spectrophotometric measurements showed an absorbance peak at 548 μm which corresponds to the magenta color of alizarin in seawater or in coral. Quantitative spectrophotometric measurements of the alizarin deposited by corals can be compared among specimens.

6. Simultaneous uptake studies employing $^{45}$CaCl$_2$ and alizarin were performed on small heads of _P. damicornis_ and indicated a high correlation between these two methods of calcium measurement. Statistical regression analysis of the two methods gave $r = .987; p < .01$. Alizarin is an accurate measure of calcium deposited by reef corals although due to its mildly toxic effect there is a slightly reduced
deposition of calcium carbonate when 10 ppm of alizarin are present in seawater.

7. Specimens of *P. damicornis* and 10 other species of corals found in Kaneohe Bay were subjected to alizarin 10 ppm simultaneously for 24 hr in an aquarium with running seawater. The pattern of alizarin deposition was irregular and did not follow polypary boundaries. Most deposition of the dye occurred on the most elevated surfaces. Deposition was not uniform. Apparently some parts of a coral colony may be depositing alizarin during a test period while comparable areas on the same colony may be inactive. Specimens were ranked according to amount of alizarin visualized. Growth rates could then be estimated and compared and in this series *P. damicornis* appeared to have grown the most rapidly. Estimates of coral growth using this method differ from established methods in which linear growth and weight increases are measured; however, it is a valuable method for estimating growth during short periods.

8. Various sized colonies of *P. damicornis* and various sized polyps of *Fungia scutaria* were subjected to alizarin in running seawater. These studies indicate that initial growth was rapid with a leveling out so that in medium sized colonies or polyps, growth proceeds regularly. The radius growth is nearly constant with time probably until the corals reach a maximum size.

9. In light-dark experiments heads of coral were split into two pieces and half was preconditioned in total darkness
for 48 hr. Both halves were subjected to alizarin during the following 48 hr. The dye uptake in the halves subjected to dark almost equalled and at times exceeded the uptake of the halves subjected to light. It was also noted that the deposition of alizarin was often most marked in areas far removed from zooxanthellae concentrations. This was especially noticeable in *P. damicornis* where the algae concentrated in the center of the stalks whereas growth is most rapid at the tips of the columns. In *Fungia scutaria* maximum uptake may occur on the aboral surface which has not been exposed to light. The area of maximal algal density is often 5 cm distant from the area of maximum calcification.

10. Studies were done with adult heads of *P. damicornis* in running seawater with alizarin 10 ppm. Ambient phosphate levels were altered incrementally with logarithmic increases from normal of $2 \times 10^{-7} \text{ M}$ to $2 \times 10^{-4} \text{ M}$. The alizarin uptake was inversely proportional to the ambient phosphate levels. At the higher concentrations pH may have been a more limiting factor than the phosphate.

11. Alizarin was used to mark adult heads of *P. damicornis* as well as newly settled polyps. They were then returned to a seawater table or to an open reef for varying times. A large head of *P. damicornis* showed 1 cm linear growth in 5 winter months.

12. Seawater calcium was bound with a chelating agent, EGTA in amounts estimated to give an incremental series of
ambient calcium from higher than normal levels to none at all. Newly settled polyps of *P. damicornis* were more vigorous in their deposition of alizarin than were polyps of adult colonies at all levels of ambient calcium tested. In newly settled polyps, traces of alizarin were visible in the skeletal septa when available calcium levels had been reduced to one third normal. Individual polyps remained alive for 48 hr when seawater calcium was reduced to one fourth normal values but they did not deposit alizarin at these levels.

13. A laboratory experiment was performed with adult and newly settled polyps of *P. damicornis* at various dilutions of seawater to 50% and increased salinities to 120% of normal. Nearly normal deposition of alizarin occurred with a ten percent alteration in the salinity. The most apparent alizarin deposition occurred at seawater dilutions to 90% of normal with polyps of adult corals and at 80% of normal with newly settled polyps under the conditions of these experiments. Newly settled polyps continued to deposit alizarin at 70% dilution which they did not do at 50% dilution nor did they survive for 48 hr at that dilution.

14. These experiments were a study in the methodology of the use of alizarin as a dye to mark corals under varying conditions to explore growth potential. It is satisfactory and has the advantages of requiring little sophisticated apparatus, the patterns of alizarin deposition can be seen
and photographed, the dye can be measured, it is stable, and specimens can be stored for later study.
General Information on Reef Corals and Description of Some Terms used in this Dissertation Relating to Corals

Stony reef corals are of the Class Anthozoa, Order Madreporaria or Scleractinia. Some corals such as Fungia scutaria are solitary with polyps that in Kaneohe Bay may reach 20 cm in length. Most corals are colonial with small polyps averaging 1-3 mm in diameter. Coral polyps are similar to sea anemones in structure but they secrete a calcium carbonate skeleton composed of almost pure aragonite.

Each polyp is housed in its own skeletal depression, a cup called a calyculus or calyx; plural calyces or calyxes (Webster, 1966). When disturbed, the living polyp can withdraw completely into this cavity. A series of vertical partitions called septa divide the cavity of the calyx into a number of radiating segments. These septa are fused to the wall of the calyx which is called the theca. The septa may number in the hundreds as in F. scutaria where they project toward the center or they may be few and rudimentary as in P. damicornis where they fuse with the theca to form a septo-theca.

Colonial corals may have an expanse of skeleton called coenosteum between the calyces. The sheet of tissue covering the coenosteum is called coenosarc and consists of the same four cell layers as the polyp and a direct extension
of the gastrovascular cavity of the various polyps.

The entire skeleton of each polyp is called a coralite whereas the skeleton of the colony as a whole is called a corallum. Costae are longitudinal ridges on the external surface of the theca and they may or may not connect with the epitheca which is a calcareous second wall or vertical continuation of the basal plate. At times it is indistinguishable from the theca.

The skeleton grows by accretion and it does so slowly, the individual polyps riding higher in their calyces. Eventually each polyp may construct a small horizontal plate or dissepiment between the various adjoining septa to cut off the older lower part of the skeleton. This serves as a bulkhead and it forms a new basal support for the polyp.

Certain reef corals such as Pavona varians reproduce asexually by intratentacular budding. The original calyx may become greatly elongated and form what is called a "brain coral" type of pattern. The ridges between these multi-mouthed valleys are called collines.

Fungia scutaria reproduces asexually by budding a new corallum from a stalk which either formed from a planula larva or from a parent coral. The stalk is called an anthocaulus and the disk shaped polyp is the anthocyathus (Bourne, 1893).
Appendix B

Figure 36. Apparatus used for study of alizarin deposition by reef corals in light-dark experiments.
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