to obtain large sediment cores (4 m by 15 cm by 15 cm). Cores from the flanks of the brine pools, above the hot-water zones, contained brightly colored sediments comprising alternating black, brown, red, orange, and yellow layers (Fig. 3). The layers are dominantly of amorphous iron oxides in various states of oxidation and goethite, with minor amounts of montmorillonite and foraminiferal debris; the amorphous material does not give an x-ray-diffraction pattern. Microscopically the amorphous iron material appears as yellow-red 1- to 10-μ spherulites that are opaque under crossed polarizers. The spherulites become slightly birefringent with depth in the cores, and x-ray-diffraction patterns indicate goethite. These indications suggest that the amorphous material is crystallizing with time to goethite. Goethite comprises 70 percent of the dried salt-free sediment at the bottom of the 4-m core.

Our preliminary interpretation of these data is that the hot salty waters and their associated heavy metals are being forcibly discharged periodically from *Atlantis II* Deep. Isotope data suggest that the water essentially derives from the Red Sea (*11*). The source of the heat and some of the heavy metals is probably associated with some local geothermal event. As hot waters meet the Red Sea waters above the present brine-pool levels and contact oxygenated waters, the metals dissolved in the brine are oxidized to varying degrees depending on the available supply of oxygen, as has been suggested (*1*). Bottom currents then may sweep away part of the water, the remainder being stratified in the three known deeps. This situation would result in the most recent discharge filling the deeps with amorphous iron oxides, while adjacent, more-elevated areas would add another thin layer to their accumulations of colored iron oxides. Box cores collected near the hot-brine areas indicate that several of these discharges have occurred within the last several thousand years.

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Glycerol Excretion by Symbiotic Algae from Corals and Tridacna and Its Control by the Host

Abstract. *Zooxanthellae isolated from reef corals and* Tridacna crocea *incorporate labeled carbon dioxide photosynthetically. In the presence of some component of the host tissue, up to 40 percent of the labeled algal photosynthate is liberated primarily as glycerol. Excretion of glycerol by the algae* in situ *and its control and utilization by the host may represent a mechanism by which zooxanthellae contribute to productivity of coral reefs.*

All reef-building corals possess unicellular algae (zooxanthellae) in their gastrodermal cells. These symbionts accelerate the calcification process in corals (*1*), but knowledge of their contribution to the organic productivity of reef corals has remained inconclusive (*2*). This report describes the production of soluble extracellular organic material by zooxanthellae from reef corals and the reef-dwelling bivalve mollusk *Tridacna crocea*. Data show that the algae liberate soluble organic material in vitro, principally as glycerol. It is interesting that excretion occurs in significant amounts only in the presence of some component of host tissue.

Algae were isolated from the reef coral *Pocillopora damicornis* by crushing 100-g (fresh weight) portions of freshly collected coral in an aluminum foil envelope and then vigorously shaking the coarsely fragmented slurry in a flask of sea water (100 ml) for several minutes. The abrasive action yielded a brownish supernatant that was decanted through several layers of surgical gauze, leaving behind the skeletal material and a small fraction of nonrecoverable algae and animal tissue. The brownish supernatant containing the algae and the homogenized animal tissue was treated in either of two ways: (i) centrifuged (2 minutes at 3000 rev/min) to yield about 1 ml of wet packed algae which were then washed virtually free of animal tissue by additional centrifugations, or (ii) divided into 4-ml portions, containing about 0.1 ml of wet packed algae, and centrifuged; the homogenate was removed, the algae were washed with sea water, and then the original homogenate (4 ml) was added back to the tube. The first treatment was used in experiments in which algae were incubated in sea water only; the second in experiments in which algae were incubated in the original homogenate containing animal tissue.

Specimens of *T. crocea* were dissected free of the valves; the distal portion of the mantle where algae reside was excised, cut into pieces, and about 50 g (fresh weight) was placed in 50 ml of sea water in a Waring Blendor (with a serological head) and homogenized for 1 minute at high speed. The homogenate was filtered through gauze, leaving behind small pieces of muscle and connective tissue and a small fraction of the algae, and the filtrate was treated in the two ways mentioned above. All suspensions were incubated in stoppered test tubes with 20 to 100 μc of $Na_2C^{14}O_3$ for periods ranging from 1 minute to 4 hours in daylight in

the shade [400 to 600 ft-ca (4400 to 6600 lu/m^2)] with temperature controlled at 25°C. Occasionally the tubes were shaken to keep the cells in suspension. After incubation the suspensions were centrifuged for 5 minutes (3000 rev/min), and the supernatants were drawn off and saved. Cells were rinsed with 0.5 ml of sea water, centrifuged again, and the supernatants combined to known volume. For assay of total radioactivity in the medium a sample was placed on a planchet, acidified with a drop of 1*N* HCl to remove unused $Na_2C^{14}O_3$, and evaporated to dryness with an infrared lamp. The dried residue was assayed with a thin end-window counter (Lionel-Anton 1007T), and the result was corrected for self-absorption (calculated from standards in sea water) and background. The pellet of cells was resuspended in hot 80 percent ethanol, and a sample was assayed for radioactivity as described above. Excretion is expressed as the percentage of the total carbon fixed (cells plus medium) that was detected in the original medium (see legend, Table 1). For analysis of labeled extracellular products, the original incubation medium was evaporated (40°C, in a vacuum) to dryness and the residue was extracted with dry absolute ethanol. This salt-free ethanolic extract contained about 85 to 95 percent of the radioactivity in the original medium. The ethanolic solution was chromatographed in two dimensions and radioautographed with x-ray film by the procedures of Benson *et al.* (*3*). Total radioactivity of a spot on a paper chromatogram was determined from the sum of square centimeter areas of the spot counted separately with a thin end-window counter. The amount of an individual compound in the medium is expressed as a percentage of the total radioactivity (sum of all radioactive spots) assayed after development of the chromatogram. Ethanolic extracts of the algae were chromatographed, by the same method, for determination of intracellular photosynthetic products.

Results are summarized in Table 1. Algae from either *Tridacna* or *Pocillopora,* washed free of animal tissue, suspended in sea water, and incubated in the light with $Na_2C^{14}O_3$ fixed substantial amounts of labeled carbon, but excreted very little soluble radioactive organic photosynthate (Table 1, A). Algae from either host, that were washed and then incubated with their own host animal tissue homogenate as it occurred in untreated suspensions, excreted up to 40 percent of their total photosynthate (Table 1, B). Table 2 shows that photosynthesis and excretion of C^{14} by zooxanthellae increased during a 4-hour incubation in host homogenate. Excretion was sustained at 33 to 50 percent of the total carbon fixed and did not appear to be a short-term transient phenomenon. In all cases, radiochromatographic analysis indicated that in incubations of short duration (about 1 to 30 minutes) the major extracellular product, usually accounting for more than 90 percent of the products in the medium, was glycerol (*4*). Additionally, but only during longer incubations (2 to 4 hours), glycolic acid was liberated and in some cases comprised nearly half of the excreted material. The appearance of glycolate during long incubations of algae in stoppered test tubes is not surprising, since the decreased carbon dioxide tension which ensues is known to stimulate glycolate excretion (*5*). Glucose and a ninhydrin-positive substance were invariably detected in the medium and comprised less than 10 percent of the carbon excreted by algae from *Pocillopora* and *Tridacna.* The major intracellular carbohydrate was glucose, an unusual finding for a "brown" alga, the majority of which, as free-living algae, manufacture mannitol instead (*6*). Other intracellular products included photosynthetic intermediates and lipid. Since all of the water-soluble intracellular compounds were not detected in the medium, I conclude that the appearance of glycerol was selective and not the result of cell lysis. Change in *p*H was ruled out as a cause of excretion, since glycerol appeared as an extracellular product after 80-second photosynthesis, during which time there was no detectable change in *p*H from the initial control value of *p*H 8.0. Enrichment with nitrate and phosphate (10 and 1 μg-atom/liter, respectively),

Table 1. Percentage excretion of C^{14}-labeled photosynthate by zooxanthellae from *Pocillopora damicornis* and *Tridacna crocea* incubated under different conditions. Incubation times varied from 1 to 4 hours. Treatment: A, algae washed and incubated in sea water (control); B, algae incubated in host homogenate; C, algae incubated in boiled tissue homogenate; D, *Tridacna* algae in *Pocillopora* homogenate; E, *Pocillopora* algae in *Tridacna* homogenate; and F, effect of darkness after initial fixation in light. Results are given as ($\bar{X}$) ± standard deviation; *N* is the number of assays.

$$\left(\% = \frac{\text{count min}^{-1}\text{ of medium}}{\text{count min}^{-1}\text{ of medium + cells}} \times 100\right).$$

Treatment	Excretion (%) by algae from			
	Pocillopora		*Tridacna*	
	$\bar{X}$ ± S.D.	(*N*)	$\bar{X}$ ± S.D.	(*N*)
A*	3.6 ± 1.7	34	2.0 ± 0.8	15
B	38.0 ± 8.2	18	37.4 ± 10.5	18
C	0.5, 2.0	2	8.6, 5.2	2
D			29.3, 4.8	2
E	22.2, 22.5	2		
F	4.6 ± 0.9	8	Not tested	

* Material detected in the medium in control experiments was not sufficient for chromatographic analysis.

Table 2. Photosynthesis and excretion of C^{14} by zooxanthellae from *Pocillopora damicornis* and *Tridacna crocea* incubated in 4 ml of host homogenate and assayed at 1-hour intervals.

Time (hr)	Source	*Pocillopora*		*Tridaena*	
		C^{14} activity (count min^{-1})	Excretion (%)	C^{14} activity (count min^{-1})	Excretion (%)
1	Medium + cells	9,423		43,262	
	Medium	3,989	42.3	14,484	33.5
2	Medium + cells	15,989		48,429	
	Medium	6,748	42.1	19,148	39.5
3	Medium + cells	21,964		49,061	
	Medium	7,778	35.5	20,092	41.7
4	Medium + cells	24,238		56,059	
	Medium	11,941	49.1	21,523	38.2

Table 3. Photosynthesis and excretion of C^{14} by zooxanthellae from *Pocillopora damicornis* and *Tridacna crocea* incubated for 1 hour with varying amounts of host tissue homogenate. The concentration of animal tissue in the original homogenized preparation was taken as 100 percent. Concentration was decreased by centrifuging the algae, withdrawing the desired amount of supernatant that contained the animal tissue, adding sea water to adjust the total volume to 4 ml, and resuspending the algae.

Homogenate (ml)	Source	*Pocillopora*		*Tridacna*	
		C^{14} activity (count min^{-1})	Excretion (%)	C^{14} activity (count min^{-1})	Excretion (%)
0	Medium + cells	241,670		255,890	
	Medium	29,120	12.0	4,720	1.8
0.25	Medium + cells	457,260		340,330	
	Medium	38,560	8.4	9,240	2.7
0.50	Medium + cells	395,620		314,840	
	Medium	62,520	15.8	14,840	4.7
1.0	Medium + cells	321,640		428,940	
	Medium	73,440	22.8	57,720	13.4
2.0	Medium + cells	364,330		410,980	
	Medium	103,880	28.5	88,760	21.6
4.0	Medium + cells	311,390		443,380	
	Medium	114,640	36.8	208,000	46.9

variation of algal cell concentration, or darkness after an initial fixation period in the light (Table 1, F) did not stimulate the appearance of soluble extracellular products in control suspensions. In the absence of algae, the animal homogenate alone neither fixed appreciable labeled carbon nor caused labeled glycerol to appear in the suspension.

The primary effect of animal tissue homogenate in stimulating excretion of glycerol and the chemical nature of the stimulant or stimulants has not yet been thoroughly investigated, but some preliminary observations were made. Table 3 shows that excretion of C^{14} by zooxanthellae increases both in percentage and in total counts excreted, as the concentration of host homogenate increases. Effect of the concentration of host homogenate on photosynthetic fixation is difficult to assess from data given here. Experiments now in progress suggest that host tissue seems to stimulate photosynthesis, possibly indirectly through shading to yield more favorable light intensities incident on the algae, or directly by chemical catalysis. Boiling the homogenate for 3 minutes abolishes its stimulatory property (Table 1, C). Zooxanthellae from *Tridacna* responded to treatment with animal tissue homogenate from *Pocillopora* and vice versa (Table 1, D and E). Frequent handling of the homogenate or delaying its application often resulted in loss of potency. A survey now underway in this laboratory indicates that zooxanthellae from a variety of other reef corals (*Acropora* sp. and *Heliopora sp.*), a xeniid soft coral, a temperate intertidal sea anemone, and a subtropical subtidal zoanthid all excrete substantial quantities of glycerol when allowed to photosynthesize $Na_2C^{14}O_3$ in the presence of their host tissue homogenate.

McLaughlin *et al.* (*7*) examined the medium from axenic cultures of zooxanthellae from the jellyfish *Cassiopeia* and found only extracellular insoluble material, which has not been identified. If the phenomenon I have described is a general one, then appreciable soluble extracellular products would not be expected to appear in axenic cultures in the absence of some constituent of the host. Kawaguti (*8*), using electron microscopy, has observed "scale-like" substances adjacent to the cell envelopes of coral zooxanthellae. He interprets these as products of the algae that may serve as nutritive substances for the host coral. Results of both of these studies indicate that zooxanthellae may liberate insoluble material. Hellebust (*9*) studied excretion of photoassimilated radiocarbon in 22 species of free-living marine phytoplankton. Most of the species liberated only 3 to 6 percent of their photosynthate during the log phase of growth. Glycerol was excreted in small amounts by free-living representatives of the Bacillariophyceae, Chlorophyceae, and Cyanophyceae. Free-living Dinokontae, the group with which zooanthellae have been allied (*10*), did not excrete glycerol.

The present findings have twofold significance. First, the facts show that, under appropriate conditions, coral zooxanthellae liberate appreciable amounts of a soluble product of photosynthesis. This property offers a means by which some of the free energy of photosynthesis can be made available to the host coral animal and possibly to the entire reef community. This interpretation is consistent with the radioautographic demonstration of the translocation of labeled carbon from zooxanthellae to coral and *Tridacna* tissue (*11*). This type of host-symbiont metabolic interaction is now well established for a variety of freshwater algae–invertebrate associations; algae from ciliates, sponges, and coelenterates liberate a major portion of their photosynthate as glucose or maltose (*12*). In the case of freshwater coelenterates, actual utilization by the host of translocated carbon has been demonstrated experimentally (*13*). Second, the stimulatory property of host tissue reveals an unsuspected role of the host in regulation of specific metabolic activities of symbionts. Since glycerol is in the control algae as an intracellular photosynthetic product, stimulatory action of host tissue may be to alter the permeability of algal cells to glycerol, possibly by activation of a glycerol permease. Wainwright (*14*, p. 181) has hypothesized that "in *Pocillopora* a function of zooxanthellae is the contribution . . . of glucose which is incorporated into the skeleton of the coral as the monomer of chitin." Acquisition of nitrogenous compounds from the zooxanthellae may also be an important supplement in nutrient-poor tropical waters.

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Progesterone: Its Possible Role in the Biosynthesis of Cardenolides in Digitalis lanata

Abstract. *The incorporation of progestrone-7α-^{3}H and pregnenolone-7α-^{3}H into digitoxigenin, gitoxigenin, and digoxigenin in isolated, surviving leaves of* Digitalis lanata *was demonstrated. In addition, the conversion of pregnenolone to progesterone in the same system was proved. The results tend to indicate that progesterone is as good a precursor of cardenolides as pregnenolone. It is suggested that the biosynthesis of cardenolides might proceed through the intermediacy of progesterone.*

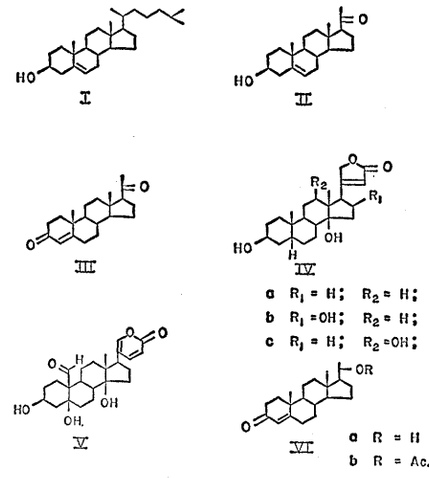
The transformation of cholesterol (I) to pregnenolone (II) in *Digitalis purpurea* (*1*) and in *Haplopappus heterophyllus* (*2*) has been described. The role of pregnenolone in the formation of steroid hormones from cholesterol in animals is well documented (*3*, p. 125). Thus pregnenolone (or its analog) may have an analogous role in the biosynthesis of certain plant sterols, such as cardenolides, bufadienolides, and others. This is supported by the observation that carbons No. 22 and No. 23 of cardenolides do not originate from mevalonic acid (MVA) (*4*), and that pregnenolone may be a precursor of digitoxigenin (IVa) (*5*) and bufadienolides (hellebrin V) in plants (*6*).

If it is tentatively assumed that pregnenolone (II) is a key intermediate in the biosynthesis of cardenolides, the question arises as to what is the mechanism of reduction of its C-5 double bond. In animals the saturation of the homo-allylic alcohol (Δ^5-3β-OH) proceeds by way of the oxidation of the 3β-hydroxyl to a 3-ketone and isomerization of the olefinic bond from C-5 to C-4 to yield a Δ^4-3-ketone moiety (*3*, p. 310, references in tables 17–19; *7*). Subsequently, the conjugated double bond is reduced to either a *cis*-(5β-H) or *trans*-(5α-H) junction between rings A and B (*8*; also *3*, p. 406); and this is followed by the reduction of the C-3 ketone.

If a mechanism analogous to that described for animals operates in *Digitalis* plants, two conditions must first be satisfied. The plant should be able to convert pregnenolone (II) to progesterone (III) and to utilize the latter in the biosynthesis of cardenolides (IV). We now offer proof of these transformations in isolated *Digitalis lanata* leaves.

One average-size leaf was cut with its stem from a *Digitalis lanata* plant, and its surface was rinsed with acetone. An acetone solution of pregnenolone-7α-^{3}H (*9*) (9.0×10^8 dpm, disintegrations per minute) was then applied to the surface of the leaf; and, after evaporation of the acetone, the leaf was sprayed with a 10 percent solution of silicon oil DC 200 (*10*) in ligroin (b.p. 60° to 90°C) (*11*). The leaf was then suspended in approximately 40 ml of a modified Hoagland's medium containing the requisite amounts of micrometabolic elements (*12*) and continuously illuminated with light of 12,000 lu/m^2. The nutrient medium was changed at 48-hour intervals, and the experiment was terminated after 10 days. The leaf was rinsed with ethyl acetate to remove the unabsorbed pregnenolone (4.8×10^7 dpm), then macerated with a razor blade. The products were recovered exactly as described by von Euw and Reichstein (*13*). An analogous experiment was carried out with progesterone-7α-^{3}H (9.65×10^8 dpm), and 3.5×10^8 dpm were found in the ethyl acetate washings of the leaf at the end of the experiment (Table 1).

We found 19.2 percent of the radioactivity of pregnenolone and 25.6 percent of that of progesterone in the fractions containing "plant sterols." The bulk of cardenolides (IV) is recovered with chloroform (*14*), and, consequently, we have undertaken the



processing of this extract. The results of both experiments reported here refer to products from this fraction. The glycosides were hydrolyzed by refluxing with a mixture (8 ml) of methanol and of 0.1*N* sulfuric acid (1 : 1), and the aglycones were recovered with ethyl acetate. The aglycones originating from progesterone (III) contained 8.6×10^7 dpm, and those from pregnenolone (II) 12.88×10^7 dpm. The isolation of digitoxigenin (IVa) [systems A, B(6 hours), C], gitoxigenin (IVb) [systems A, B(6 hours), D], and digoxigenin (IVc) [systems A, B(6 hours), B(19 hours)] was carried out on portions of the aglycones by chromatography (*15*). When systems B, C, and D were used the paper was treated as suggested by Bush and Crowshaw (*16*).

Table 1. Radioactivity in the "sterol fractions" recovered from isolated leaves of *Digitalis lanata* after the application of pregnenolone-7α-^{3}H (9.0×10^8 dpm) and progesterone-7α-^{3}H (9.65×10^8 dpm). The counting was carried out in a scintillation counter with 15 ml of scintillating fluid consisting of 4 g of 2,5-diphenyloxazole and 100 mg of *p*-bis-[2-(5-phenyloxazolyl)]-benzene dissolved in 1000 ml of toluene.

Extract	Radioactivity (10^7 dpm)	
	Pregnenolone-7α-^{3}H	Progesterone-7α-^{3}H
Ligroin	2.98	0.48
Chloroform	8.6	14.43
Chloroform-ethanol (2:1)	3.7	0.62
Chloroform-ethanol (3:2)	0.62	0.85
Unabsorbed radioactivity*	4.8	35.0
*Percentage recovery in "sterol fractions"**	19.2	25.6

*The percentage recovery is corrected for the unabsorbed radioactivity present in the ethyl acetate washings of the leaves at the termination of the experiment.