protein and is not secreted, or if it is secreted it is inactive in saliva due either to extensive degradation or to the presence of an inhibitor.

Covalent binding of [^{32}P]-8-N_3-cAMP with specific proteins of rat parotid saliva after stimulation with α-adrenergic and cholinergic agonists is $< 40\%$ (19% with methoxamine; 36.5% with methacholine) of that found in isoproterenol-stimulated saliva (table 2 and fig. 2b). The presence of R subunits at much higher levels in saliva after β-adrenergic stimulation suggests that they are released during exocytosis. Their presence at lower levels in saliva after α-adrenergic and cholinergic stimulation, which cause low but measurable levels of exocytosis, supports such a mechanism.

Protein secretion by the rat parotid gland is hormonally regulated via β-adrenergic receptors[18]. Receptor reactions which activate adenylate cyclase result in a rapid increase in levels of cyclic AMP, and activation of cA-PK. Subsequent phosphorylative modification of specific intracellular proteins is one of the steps which leads to exocytotic release of the stored secretory granule content. We have previously shown[17] that cA-PK holoenzyme and R_I subunits are associated with particulate cytoplasmic components (both microsomal and secretory granule cell fractions) in rat parotid cells. Association of cA-PK with the secretory granules would position the enzyme at an optimal site for phosphorylation of cytoplasmic or membrane proteins involved in exocytosis. At present it is not clear whether the R subunits are loosely associated with the granule membrane, or whether they are granule 'content' protein. The apparent increase of R_I in stimulated saliva (fig. 2a, channel 5) and in granules of in vivo stimulated animals[17], may be related to its physical properties of interchain disulfide bonding[19] and involvement in the cyclic AMP binding sites in protein kinase activation[20]. The R_I subunit may also be involved in hormonally stimulated enzyme translocation within the responsive cell and represent an intracellular mechanism of action in exocytosis.

The presence of R subunits in saliva provides an easily obtainable source of this protein for secretion or binding studies, and for purification for immunological and molecular biology studies. The physiologic function of the R subunit in saliva, however, remains enigmatic. Cyclic AMP has repeatedly been shown to be present in saliva[21-23]; to what extent it is in the free form compared to a protein-bound form to the R subunit has not been clearly demonstrated. The release of the R subunits along with secretory proteins may be a mechanism for 'unloading' intracellular cyclic AMP. The presence of R subunits in saliva may have important implications for the growth and metabolism of the oral microbial flora by serving to regulate the levels of cyclic AMP in saliva, or may reflect changes in the regulation of secretory responses in disease processes.

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Antibacterial activity in the egg mass of a sea hare

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Summary. The eggs of a sea hare, *Aplysia kurodai*, contained antibacterial factors which probably play a role in the defense of eggs against bacterial infection. The active factors were composed of several heat-labile proteins, unrelated to lysozyme, and were produced in the albumen gland.

Key words. Sea hare eggs; *Aplysia kurodai;* antibacterial activity.

Sea hares belong to the subclass Opisthobranchia of the gastropod molluscs. In recent years, they have attracted the interest of many workers investigating chemical defense mechanisms[1,2]. The eggs of sea hares are laid in gelatinous strings. There does not appear to be bacterial infection causing the eggs to deteriorate before they hatch as swimming veliger larvae. In addition, the egg mass seems to be rejected as a food source by carnivorous fish. These observation led us to look for physiologically active components in the egg mass of *Aplysia kurodai,* and we found potent agglutinins which could ag-

glutinate both animal erythrocytes and marine bacteria[3]. We describe here the in vitro antibacterial activity in the egg mass of *A. kurodai*.

As shown in table 1, a 0.85% saline extract of egg mass showed antibacterial activity against both Gram-positive and Gram-negative bacteria of both terrestrial and marine origin. In the paper disc-diffusion assay, there was no significant difference in the inhibitory activity against the various bacterial species tested. The area of inhibition was totally devoid of bacteria for 24 h, but a number of bacterial colonies was observed in the area when the plate was kept for 24 h longer.

The effect of the active factors on a growing culture of *Bacillus subtilis*, measured by the turbidometric assay, is shown in figure 1. The growth of the bacteria was depressed for 24 h after addition of the agents. After this period of depression, the bacteria started to grow at the normal growth rate. When the sample was added to an actively growing culture, it stopped further growth for at least 20 h. However, there was no apparent subsequent decrease in turbidity which might have been expected if cell lysis were occurring. A similar time-course of inhibitory action was also observed for other bacterial species tested. A turbidometric assay was rapid and reproducible, and was adapted for quantitative estimation of antibacterial activity. Briefly, a mixture of 0.2 ml bacterial suspension ($\sim 1 \times 10^7$ cells), 0.5 ml test solution, and 4.0 ml peptone water was incubated at 27 °C until control tubes containing Tris buffer (0.05 M, pH 8.0) reached an optical density of ~ 0.25 at 660 nm. The concentration of sample solution which was required for a 50% decrease in optical density compared to the control was calculated from the dose-response curve.

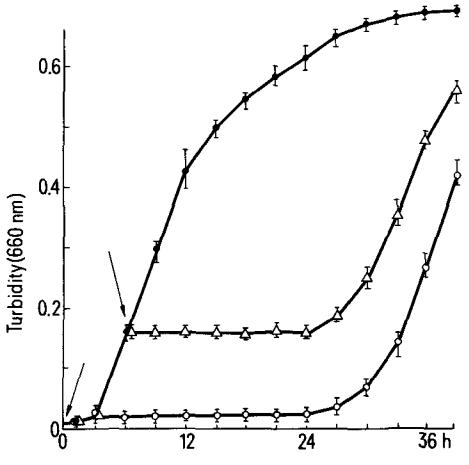

Figure 1. Effect of antibacterial factors on a growing culture of *Bacillus subtilis;* turbidometric assay. × 500 µl of *Aplysia kurodai* egg extract (200 µg protein) was added to a mixture of 4.0 ml of peptone water and 0.2 ml of bacterial suspension at 0 h (○—○) or 6 h (△—△). Tris buffer (0.05 M, pH 8.0) was used as control (●—●). The time of addition was indicated by the arrow. The results have been corrected for optical density change at 660 nm due to dilution effect of the sample solution.

Antibacterial factors in the crude extract of the egg mass could be separated from the agglutinins by chromatography on acid-treated Sepharose 4B. They were not adsorbed by acid-treated agarose gels whereas agglutinins binding specifically to D-galactose were adsorbed, as shown in figure 2. Ultrafiltration revealed that the antibacterial factors in the *Aplysia* egg mass had a molecular weight greater than 50,000 daltons. The filtrate did not show any bactericidal activity against *B. subtilis*. Size-exclusion chromatography on TSK-G3000SW (Toyo Soda Manufacturing Co.) showed that the active factors were composed of several different molecular species of proteins.

The concentration of the partially purified antibacterial factors which was required for the 50% decrease in optical density varied with the bacterial species tested (table 1). *B. subtilis* and *Aeromonas salmonicida* were found to be highly susceptible, and gave values as low as 50–70 µg protein. *Escherichia coli* was less sensitive and the concentration giving a 50% decrease was 10 times greater than that for *B. subtilis*.

The activity of the antibacterial factors was reduced to nearly a half of the original activity by heating at 45 °C for 30 min, and was lost completely at 50 °C. Active factors were found to be

Table 1. Antibacterial activity of *Aplysia kurodai* egg mass

	Paper disc diffusion assay*	Turbidometric assay**
Gram-negative bacteria:		
Pseudomonas aeruginosa	+	160
Escherichia coli	+	500
Salmonella pullorum		120
Aeromonas salmonicida		70
Vibrio sp.	++	180
Gram-positive bacteria:		
Bacillus subtilis	++	50
Micrococcus luteus	++	100

* +; inhibition zones < 16 mm, ++; zones > 16 mm; ** Amount of protein (µg) required for 50% decrease in optical density at 660 nm.

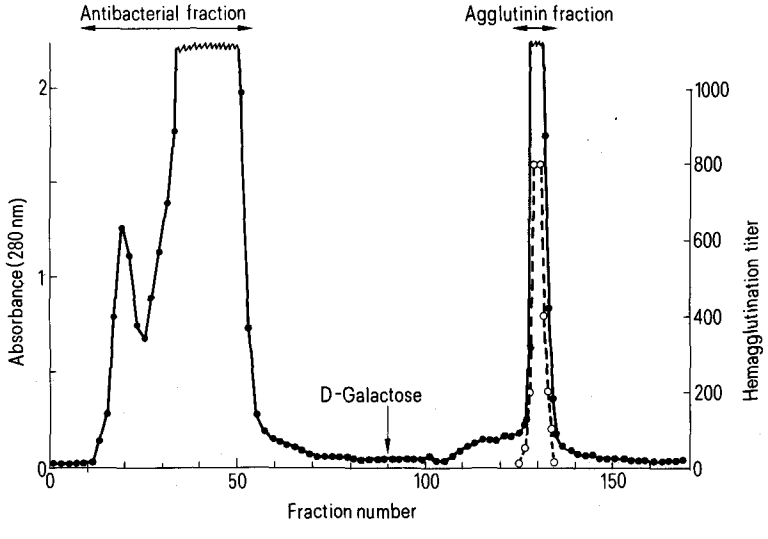

Figure 2. Chromatography of *Aplysia kurodai* egg extract on acid-treated Sepharose 4B. *Aplysia* egg extract was applied to a column of acid-treated Sepharose 4B. The column was eluted with 0.05 M Tris buffer (pH 8.0) containing 0.15 M NaCl. The agglutinins were eluted with 0.2 M D-galactose in the buffer. Fractions were analyzed for UV absorption at 280 nm (●—●) and for hemagglutinating activity against rabbit erythrocytes (○- - - -○).

resistant to treatment with trypsin (2 mg/ml, 60 min, 25 °C). Egg mass extract did not lyse freeze-dried *Micrococcus luteus* cells. The absence of lytic properties was also shown by the fact that the addition of antibacterial factors to a bacterial culture did not cause a reduction of turbidity. *Aplysia* antimicrobial factors are quite different from the lysozymes found in many species of marine invertebrates[4,5] in the following characteristics; their heat-lability, high molecular weight, and lack of lytic properties. These properties of the agents are similar to those of the antibacterial factor against Gram-negative bacteria found in the coelomic fluid of the marine annelid *Glycera dibranchiata*[6,7]. The extract of *Aplysia* egg mass also possesses antitumor activity against various murine tumor cell lines both in vivo and in vitro. Details will be presented elsewhere.

Table 2 summarizes the distribution of antibacterial factors against *B. subtilis* and agglutinins in the various tissues of *A. kurodai*. Albumen gland showed potent antimicrobial activity but no hemagglutinating activity. 2 out of 4 mucous gland samples showed weak antibacterial activity, probably due to contamination with albumen gland. Ovo-testis showed high hemagglutinating activity but no antibacterial activity. Hypobranchial gland also gave weak agglutinating activity. On the other hand, hemolymph caused stimulation of bacterial growth compared to the control. Neither antibacterial nor hemagglutinating activity was observed in extracts of gill, mantle, foot, or digestive gland. These data suggest that antibacterial factors are produced in the albumen gland of *A. kurodai* and that each egg is coated with antibacterially active albumen before passing down the oviduct to the gonapore. It is of interest from the comparative physiological point of view that the albumen glands of certain snails contain agglutinins[8] and protease inhibitors[9], whereas that of *Aplysia* does not possess agglutinins but does produce antimicrobial proteins.

Agglutinins in the egg mass are unlikely to play a significant role in the defense of the egg mass after laying, since antibacterial factors freed from agglutinins showed an increased specific activity in comparison with the crude extract. It might be possible, however, that agglutinins participate in the defense system of the egg mass by creating an unfavorable environment for bacteria.

Table 2. Distribution of antibacterial factors and agglutinins in *Aplysia kurodai*

	Antibacterial activity*	Hemagglutinating activity**
Egg mass	++	2^8
Albumen gland	++	–
	++	–
	++	–
	++	–
Mucous gland	–	–
	–	–
	+	–
	+	–
Gill	–	–
Mantle	–	–
Foot	–	–
Ovo-testis	–	2^{11}
Digestive gland	–	–
Hypobranchial gland	–	2^4
Hemolymph	–	2^6

* ++, decrease in optical density > 70%; +, 70–50%; ** Agglutinating titer against rabbit erythrocytes.

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On the binding of steroid sulfates to albumin[1]

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Summary. ^{3}H-Labeled steroid sulfates, sulfate of estrone (E_1S) or dehydroepiandrosterone (DHAS), were dialyzed against delipidated human serum albumin or human plasma in the presence of increasing amounts of competing non-labeled sulfates (DHAS or E_1S). The apparent equilibrium constants (K) of the tracers did not measurably change at concentrations of the non-radioactive sulfates below 10^{-5} mol/l. At higher concentrations, K decreased gradually. The apparent equilibrium constant of ^{3}H-E_1S was diminished by plasma in a similar fashion. It may be concluded that albumin possesses one strong, non-specific binding site. This site, however, does not seem to be utilized for the binding of E_1S in vivo, because of its preferential occupation by other ligands. This may be true for other steroid sulfates as well, depending on their relative abundance in plasma.
Key words. Serum albumin, human; steroid sulfates; steroid binding site.

Steroid sulfates are present in human blood primarily as complexes with albumin[2]. No straightforward information is available on the avidity of albumin binding in vivo. Equilibrium constants estimated from in vitro experiments vary in dependence on the concentration of the sulfate (Scatchard plots are not linear, but concavely curvilinear[2-4]).

It may be deduced from the structure of albumin[5] that the strength of binding can change gradually in the process of occupation of various binding regions, and that the binding potency may even change because of the alterations of the tertiary albumin structure due to binding[6].

In spite of this flexibility of the albumin molecule it may be speculated that – in analogy with other compounds (thyroxin[7], coumarin[8], palmitic acid[9]) – there exists a single strong binding site for steroid sulfates. The aim of the present study was to elucidate whether or not this is the case and if so, whether or not this strong binding site is employed for the binding of steroid sulfates in vivo.