While it has been materialized by recent study that extracts with certain hormonal activity are obtainable from the parotid gland of several mammalian vertebrates (Ogata and Ito, 1944), in invertebrates very little is learned about the functions of salivary glands. According to the morphological views, salivary glands were observable in Cephalopoda and Gastropoda but not in Scaphopoda (Takatsuki, 1939), and biochemical studies on the posterior gland of Cephalopoda have been made by a number of workers (Henze, 1929; Taki, 1935; Ungar, 1937). In Gastropoda the biological functions of the gland are not published up to the present time.

This paper deals with some chemical properties of the extract which was obtained from the salivary gland of Dolabella scopola, a marine Gastropod Mollusc.

MATERIALS AND METHODS

Freshly excised salivary gland was collected from 500 animals in breeding season and homogenized in a glass blender with 100 ml of 0.1 M phosphate buffer (pH 8.00), and centrifuged at 4000 r.p.m. for 30 mins. The supernatant was shaken twice with each 50 ml of chloroform in a separatory funnel for each 30 mins and adjusted to pH 4.5 with 0.1 N HCl. After standing in refrigerator for 24 hrs, resultant residue was discarded and the supernatant was collected to be dialyzed at 15°C against running tap water. The dialysate was frozen dried in vacuo, and then dissolved in 10 ml of distilled water and re-dialyzed after being adjusted to pH 4.5. The aqueous solution was frozen dried and 662.5 mg of the crude extract was obtained in yellowish hygroscopic powder. Further fractionation was made by means of electrophoretic procedure in the following way: The crude extract was dissolved in 0.1 M phosphate buffer (pH 8.00, p 0.14) and dialyzed against 0.05 M phosphate buffer at 5°C for 20 hrs, then the electrophoresis was carried at 10 mA and 110 volt for 60 min with the Tiselius's apparatus. Four peaks observed in the electrogram as shown in Fig. 1. Each fraction, which was named F-1, F-2, F-3 and F-4 in accordance with the mobility on electrogram was separated in preparative cells and collected to be frozen dried in vacuo. From 500 mg of the crude extract, 150 mg of F-1, 94 mg of F-2, 87 mg of F-3 and 14 mg of F-4 were obtained in white lyophilized powder.

EXPERIMENTAL RESULTS

Physico-chemical experiments The physico-chemical properties of the crude extract and above mentioned four fractions were examined by means of spectrophotometric, polarographic, electrophoretic and chromatographic analysis.
The ultraviolet absorption maxima in phosphate buffer (pH 8.00) were observed at 268.5 m\textmu in crude extract and 252 m\textmu in F-2 but the other fractions were lack of maxima.

On the polarogram, characteristic waves were obtained both of the crude extract and purified fractions in 0.1 N ammonium hydroxide and 0.1 N ammonium chloride buffer solution in the presence of cobalt ions (6). The polarogram consisted from two waves having half wave potentials at -1.35, -1.55 volt in cobaltous solution and -1.40, -1.60 volt in cobaltic solution. The polarographic wave-height in cobaltic solution was higher than that of cobaltous one.

For the paper electrophoresis the specimen was carried out on filter paper (Toyo no. 51, 12×25 cm) with 0.1 ml of veronal buffer solution (pH 8.0, \( \mu 0.1 \)) for 10 hrs at 600 volt and 0.2 mA per cm. After electrophoresis paper was dried and stained with B.P.B. ninhydrin and P.A.S. and dipped in trichloroethylene solution of vinylchloride for the purpose of increasing of transmittance of the paper (Takikawa, 1956). The photoelectrogram was recorded with Coleman nephocolorimeter by the use of Corning filter no. 4010 (525 m\textmu). Three peaks were observed in the crude extract with ninhydrin and B.P.B. in positive side, and two in positive and three in negative side with P.A.S.. F-2 showed one symmetrical peak as given in Fig. 2. Then the hydrolysate of the extract was examined in the following way: 10 mg of F-2 was hydrolyzed by heating with 1 ml of 6 N HC1 at 100°C for 40 hrs. and the acid was removed by evaporation and the residue was dissolved in 0.1 ml of distilled water. This hydrolysate was submitted to paper electrophoresis by the use of borate buffer (pH 9.3) for 6 hrs. at 300 volt and 0.3 mA per cm. For the development of color, anilin hydrogen phthalate, which was prepared by dissolving of 93 mg of anilin and 160 mg of phthalic acid in 10 ml of n-butanol, and the mixture solution of stannous chloride and urea in H2SO4 were used. Brown color was appeared with anilin hydrogen phthalate, and blue colored spot was found in the sample which was collected at Misaki, and red one at Shimoda with sulfuric acid solution of urea containing stannous chloride in the position of corresponding to D-glucose and D-fructose.

For the paper chromatography F-2 was placed on two pieces of filter paper (Toyo no. 51, 2×40 cm) in 10% aqueous solution and developed for 24 hrs. at 20°C with a mixture of N-butanol, acetic acid and water (4 : 1 : 5). After development in one dimensional ascending method a piece of the paper was sprayed with 0.2 N AgNO3-5 N NH4OH mixture (1 : 1) and heated in oven for 5 mins. at 100°C, and the other strip was treated with 0.2% solution of ninhydrin in N-butanol after being dried at room temperature. The purple colored spot was found on the start line of paper with ninhydrin. The brown colored spot was not found with alkaline silvernitrate. The aqueous solution of F-2 (electrophoretic purity: 98.2%) was hydrolyzed by heating with 6 N HC1 at 100°C for 40 hrs. and the hydrolysate was developed with the above mentioned solvent. Pale pink color with triphenly tetrazoliumchloride aqueous solution, dark brown with alkaline silver nitrate solution and brown with anilin hydrogen phthalate appeared at the spot (Rf 0.09), which was corresponded to D-glucose and D-fructose. For the detection of amino acids the chromatogram was sprayed with 0.2% solution of ninhydrin in N-butanol and heated for 10 mins. at 100°C. As the results, leucine,
isoleucine, phenylalanine, valine, tyrosine, alanine, glutamic acid, threonine, glycine, arginine, histidine, lysine and cystine observed at the position of corresponding to each standard amino acid on the photochromatogram, and also tryptophane was detected from the alkaline hydrolysate. Further identification was proceeded by the method of dinitrophenyl derivatives of amino acids in the following manner: Ten mg of the dried hydrolysate of F-2 was dissolved in phosphate buffer (pH 9.0) and added 2 mg of dinitrofluorobenzene. After shaking at 40°C for 90 mins. the excess of dinitrofluorobenzene was removed by extraction with ether. The solution was acidified with 6 N HC1 up to pH 3.0, and the dinitrophenyl derivatives of amino acids were extracted into ether. The ether extract and the aqueous solution were both together applied on filter paper strip for the qualitative paper chromatography, which was developed with water saturated N-butanol by the ascending procedure. As the result, dinitrophenyl derivatives of amino acids which were in just agreement with the above mentioned result with the exception of cystine, were detected from the photochromatogram.

![Fig. 1. Electrophoretic pattern of the crude extract in phosphate buffer (pH 8.0, μ 0.14) at 110 volt and 10 mA](image)

**Fig. 1.** Electrophoretic pattern of the crude extract in phosphate buffer (pH 8.0, μ 0.14) at 110 volt and 10 mA.

![Fig. 2. Photoelectrogram of the crude extract and fraction no. 2. in veronal buffer solution (pH 8.0, μ 0.1) at 600 volt and 0.2 mA. per cm. 1; crude extract stained with ninhydrin; 2, crude extract stained with P.A.S.; 3, fraction no. 2 stained with ninhydrin; 4, fraction no. 2 stained with P.A.S.](image)

**Fig. 2.** Photoelectrogram of the crude extract and fraction no. 2. in veronal buffer solution (pH 8.0, μ 0.1) at 600 volt and 0.2 mA. per cm. 1; crude extract stained with ninhydrin; 2, crude extract stained with P.A.S.; 3, fraction no. 2 stained with ninhydrin; 4, fraction no. 2 stained with P.A.S.

**Biological experiment** To examine the effect on the calcium and sodium level in rat serum, 20 rats of approximately 200g in body weight were fed with fixed food for 15 days and after fasting for 24 hrs. the solution of crude extract in 0.1M phosphate buffer was subcutaneously injected in dosages of 30, 20, 5 and 3 mg per kg of body weight. At fourth and eighth hr. after the injection, the blood was drawn and the amount of total sodium and calcium in rat serum was determined with flame photometrically by the use of Beckman DU spectrophotometer,
calcium was measured at 554.7 mÌ and sodium at 589 mÌ. The percentage of maximum decrease against the initial level before injection was taken as the decrease rate. In the case of administration of 20 mg per kg, arithmetic average of the maximum changes of calcium level was 12.5% at fourth hr. and 19.0% at eighth hr. after the injection, and in the dosage of 30 mg per kg 21.5% at fourth hr., and 31.6% at eighth hr. after the administration. No serious change in the sodium level was observed in rat serum. The control group of rats was injected with the equal volume of phosphate buffer. On the contrary, the administration of 3 and 5 mg per kg caused rather slight increase of the calcium level at eighth hr. after injection. And also when F-2 was subcutaneously injected in the dosage of 3 mg per kg, which was prepared from 20 mg of the crude extract by means of electrophoretic procedure, the calcium level increased to 25.0% at fourth hr. and 41.0% at eighth hr. after the injection. No apparent increases were observed in the other fraction. The results were given in Fig. 3.

The effect of this principle on the number of circulating leucocytes of rabbits was examined. Male and female rabbits of approximately 2 kg in body weight were employed. The number of leucocytes was counted by the use of haemacytometer with Türk's solution. The blood was taken just before the injection and at 2nd, 4th, 6th, 8th and 10th hr. after injection and three samples for each measurement were prepared. As shown in Fig. 4, intravenous injection of F-2, F-3 and crude extract in the dosage of 3 mg per kg of the body weight caused a transitory decrease of leucocytes followed by its successive increase as likely as parotin obtained from bovine parotied gland.

Morphological observation Two elongated salivary glands of approximately 3 to 5 cm length were observed symmetrically about the alimentary canal just behind
the rhinophores, which were compact bodies of a light yellow color. The left gland lies more posterior and longer than the right. Both of the glands passed through the nerve ring and discharged into the buccal pauch and attached by connective tissue to the side of the crop. The microscopic finding of the gland was given in Fig. 5. The glandular portion was consisted from a simple arrangement of spheroid, which was stained in light orange with Azan and dark blue particles were found in the periphery. Capillary blood vessels were seen around the spheroid, in which small granules were observed in Sudan III preparation. The contents of spheroids were stained clear purple with P.A.S. and scarcely mucicarmin.

Fig. 5. Photomicrograph of the salivary gland stained with Sudan III. \((\times600)\)
DISCUSSION

It has been reported that the anterior salivary gland secretes only mucus and the posterior gland excretes some biological amines, which were concerned with the color-change in Cepholapoda (Taki, 1935). Tyramine was isolated from the posterior gland and whole blood of Sepia officinalis and Octopus by Henze (1929). Recently, Erspamer (1954) succeeded in isolating octopamine (p-hydroxyphenyl ethanolamine) from the posterior gland of Octopus vulgaris. While Fleig (1910) reported that the salivary gland of Octopus contained a poison substance and not digestive enzyme, amineoxydase was found by Blaschko (1937) and hyaluronidase by Romanini (1952) in the posterior gland. Bacq and Chiretti (1953) indicated the internal secretion of monophenolic amine during nerve excitation in the posterior gland of Octopus vulgaris.

From the results of the present work, though the amine or amine derivatives were not extractable, it may be concluded that the salivary gland of Dolabella contained appreciable amounts of the biologically active protein, which was consisted from fourteen kinds of amino acid and one mono-saccharide. Since the biological effect upon the number of leucocytes was likely to that of parotin from bovine salivary gland, and the microscopic finding was learned to be in fair agreement with those of the paper electrophoresis in staining ability, it is supposed that the isolated fraction would be secreted out of the salivary gland. Though the physico-chemical properties and the result of biological experiment on calcium level in rat serum were different from those of parotin, it is of much interests that the extract from invertebrate source has a biological activity on vertebrates. Whether this extract is the salivary gland hormone or not, it cannot be clarified by the present experiments, further biological studies being under the way toward elucidation of this problem.

SUMMARY

A protein extract was isolated from the salivary gland of Dolabella scopola, a marine opisthobranch mollusc. The extract was a kind of glycoprotein or glycoprotein-like substance, which was consisted from fourteen kinds of amino acid and one sugar. It caused an increase of calcium level in rat serum on the intravenous administration and a transitory decrease of the number of leucocytes in rabbit followed by its successive increase.

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