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Received October 25, 1965. P.S.E.B.M., 1966, v121.

Anticoagulant from the Sea Anemone *Rhodactis howesii*.* (30966)

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The toxic extracts from the sea anemone *Rhodactis howesii* seem to affect the central nervous system of vertebrates(1,2). As a side effect, acute hemorrhages occur in cats, rabbits and mice when the crude extracts are given intravenously. This side effect suggested that an anticoagulant and/or vasculotoxic factor may be present in the extracts. Experiments designed to test the first possibility demonstrated occurrence of an anticoagulant in *R. howesii*.

Material and methods. The anemones, collected in Samoa and stored frozen, were thawed and homogenized at 5°C with 4 times their volume of water. The homogenate was centrifuged at 10,000 *g* for 10 minutes. The supernatant was dialyzed successively against 0.15 M sodium chloride adjusted with HCl to pH 2.0, then 0.15 M sodium chloride adjusted with NaOH to pH 10.5, and finally 0.15 M sodium chloride with 0.01 M sodium phosphate of pH 7.0. The non-dialyzable solution was frozen and thawed. An insoluble fraction which appeared after the thawing was removed by centrifugation. The supernatant was used for the experiments.

To estimate the organic matter in this solution aliquots were exhaustively dialyzed against distilled deionized water, then freeze-dried and ashed. They averaged 7 mg of combustible material per ml. This weight is the estimated total organic matter per ml of extract.

The anticoagulant properties of the anemone extracts were studied by the plasma recalcification method. Citrated, outdated human plasma obtained from a blood bank was used. The reactions were done in 8 mm I.D. glass tubes. The reaction mixture consisted of 200 μl plasma and 600 μl buffer containing 0.15 M sodium chloride and 0.01 M sodium phosphate at pH 7.0 per tube. For fastest coagulation at least 8 μl of 0.4 M calcium chloride was necessary, but 10 μl of that solution was used routinely. In the experimental tubes the anemone extract was added before the calcium chloride solution. Dilution of the plasma was kept constant by reducing the amount of buffer by the amount of extract to be added. The tubes with the reaction mixture were incubated at 25° to 26°C and checked for clotting every minute for the first half hour and every 5 minutes thereafter. The time elapsed from the addition of the calcium chloride until clotting occurred was recorded. Controls which contained no anemone extract were run with each series. The clotting time of the controls varied from 8 to 11 minutes depending on the series. The anticoagulant potency was estimated from the ratio of the mean clotting time of the sample with anemone extract to the mean clotting time of the controls. Duplicate samples were run for each condition and showed acceptable agreement.

Results. The anemone extract effect on plasma clotting time and how it compares with heparin is shown in Table I. The extract of *R. howesii* has anticoagulant effect such that about a 10-times prolongation of the clotting time is caused by 700 μg of dry anemone material as compared with 3.3 μg of heparin. The dose-effect relation of anemone extract does not significantly differ from that of heparin.

Some of the properties of the extract were then studied with respect to the anticoagulant factor. The extract did not digest gela-

* Supported in part by Office of Naval Research, Contract NONR No. 3656(24).

TABLE I. Prolongation of *in vitro* Clotting Time by Heparin and *R. howesii* Extract.

Preparation Name	μg	Mean clotting time of sample / Mean clotting time of control
Heparin*	2.0	4.2
	2.5	6.6
	3.0	8.2
	3.3‡	10.0‡
	3.5	12.0
R. howesii material†	280	2.4
	560	7.0
	700‡	10.0‡
	840	14.0

* Commercial heparin sodium solution of the Upjohn Co. containing 1,000 U.S.P. units of heparin per ml which was assumed to correspond to 10 mg of heparin per ml of solution.

† Calculated weight of the organic material contained in the volumes of extract actually used.

‡ By interpolation.

tine and fibrin clots. Thus the activity of the trivial proteolytic enzymes which can be demonstrated with these substrates can be ruled out as a possible cause of the anticoagulant activity. Heating of the extract for 10 minutes on the boiling water bath and autoclaving it at 120°C under 15 lb pressure did not decrease its anticoagulant potency.

An acetone precipitate obtained at room temperature from the anemone extract was washed with acetone-chloroform and with chloroform, air dried and redissolved in the original volume of buffer. The anticoagulant activity of the reconstituted solution was found to be about 75% of that of the original extract. The difference may be due to mechanical losses and possible aggregate formation during the operations. The findings indicate that the anticoagulant principle was not inactivated by the organic solvents.

Further tests were made to see whether the anticoagulant affected the clotting time by depriving the system of available calcium. If this were the case the anticoagulant effect should be overcome when excess amounts of calcium are added. Since increased amounts of calcium cause precipitation in the 0.15 M sodium chloride solution containing 0.01 M sodium phosphate, these tests were duplicated in a series with buffer containing only 0.002 M sodium phosphate at pH 7.0. At this concentration of sodium phosphate, no precipitation with excess calcium chloride could be demonstrated by photometric turbidimetry. The amounts of calcium chloride added to the system were varied at 5 different levels, from the minimum required for recalcification up to 6.2 times that amount which was a 2.5×10^{-2} molarity of calcium. At each level, amounts of anemone extract containing from 70 to 700 μg of dry material were assayed for delay of clotting time. It was found that the excess of calcium did not shorten the delay of clotting under any of these conditions. These findings suggest that the action of the anticoagulant is not based on depriving the system of calcium.

Discussion. The chemical nature and biochemical action of the anticoagulant of the sea anemone *R. howesii* are unknown. The heat stable anticoagulant is distinct from the lethal, supposedly neurotropic, toxin which is inactivated at 100° in 10 minutes and from the hemolysin which is inactivated at 56° in 30 minutes(2). Concerning the function of the anticoagulant in the biology of that anemone, and the anatomical structure in which it occurs, one cannot even venture a learned guess. It is also unknown whether this anticoagulant factor is a common feature of all sea anemones or a peculiarity, in kind or quantity, of *R. howesii*(3).

Blood coagulation can be deranged by a great variety of anticoagulants each acting on dissimilar effector sites(4-7). Since trivial calcium deprivation as a possible cause of the anticoagulant action of the *R. howesii* extract can be ruled out, other possible effector sites should be investigated.

Summary. An anticoagulant factor has been demonstrated in the extract of the sea anemone *Rhodactis howesii.* It is not related to the lethal "neuro"- toxin and the hemolysin which occur in that anemone. The factor is non-dialyzable. It is resistant to a pH of 2.0 and 10.5 in the cold, to autoclaving for 2 hours at 120°C under 15 lb pressure, and to treatment with acetone and chloroform. By the plasma recalcification method 700 μg of the total dry organic material of the extract afford a 10-times prolongation of coagulation time as compared with 3.3 μg of heparin. Calcium deprivation as a possible mechanism for the anticoagulant effect has

been ruled out.

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Received November 1, 1965. P.S.E.B.M., 1966, v121.

Exposure of Aquarium Fish to Dimethyl Sulfoxide (DMSO) with Special Reference to Toxicity and Effects on Uptake of Radioactive Dyes.* (30967)

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It has been reported that dimethyl sulfoxide (DMSO) increases cell membrane permeability and thereby enhances and potentiates the activity of drugs and toxins(1-7). It was felt that this property might prove a valuable adjunct to the use of various fluorescein dyes and radioactive substances currently employed as diagnostic and therapeutic agents.

Aquarium fish were utilized as research models since they could be readily exposed to a steady and constant environment of DMSO. They are also well adapted to study the total body uptake of fluorescent dyes and radioactive materials. The glowlight tetra (*Hemigrammus erythrozonus*-Durbin) was most frequently employed. Other species used in smaller numbers included neon tetras (*Paracheirodon innesi*), platys (*Xiphophorus maculatus*), mollies (*Pescilia latipinna*), guppies (*Poecilia reticulata*), zebras (*Brachydanio rerio*) and catfish (*Corydoras paleatus*). The volume of water per aquarium was about 45 ± 1 ilters. The water employed was distilled and maintained at a temperature of 24-25°C and a pH of 6.8 ± 0.2. Aeration was maintained by means of a pump with a delivery rate of 100 ± 2 ml per minute. The aquaria were algae-free. The fish were fed once daily with 100 mg of a commercially available fish food preparation.†

The work was divided into two phases. 1. *Determination of LD_{50} levels.* Initial studies were performed with the glowlight tetra. Groups of 10 fish were exposed to various concentrations of DMSO. A concentration of 1.9% DMSO killed one-half of fish in the aquarium in 48 hours. Fish surviving a concentration of over 1.9% DMSO for 48 hours appeared to suffer no permanent ill effects when removed at the end of this period and placed in water containing no DMSO. They survived their usual 3 to 4 month life expectancy in the laboratory and reproduced normally.

Toxicity in the glow tetra was first manifested by erratic behavior, such as backward swimming and standing on their tails, suggesting disturbances in neurologic function or abnormalities in vision or maintenance of equilibrium. This was followed by progressive anasarca and death.

Histologically, changes were most prominent in the subcutaneous tissues where cellular enlargement, rupture and an increase in interstitial fluid were noted. These changes resemble those described in the human skin (8).

Neon tetras, platys, mollies and guppies had LD_{50} concentrations very similar to that of the glow tetras (1.9% DMSO) (Table I).

* This study was aided by a grant from Syntex Laboratories, Palo Alto, Calif. We are indebted to Dr. P. Feigl for statistical evaluation of the data and to Miss G. Horowitz for technical assistance.

† Biorell, Manufactured by Long Life Fish Food Products, Harrison, N. J.