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ON THE VENOM OF THE LESSER WEEVERFISH, *TRACHINUS VIPERA*

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Recently Russell & Emery (1960) have described the physiological effects of the venom of the weeverfish, *Trachinus draco* Linnaeus and *T. vipera* Cuvier & Valenciennes. They also amplify the earlier clinical findings of the effects of the stings of these fish and describe the venom apparatus in detail. Although a chemical analysis of the venom was performed they made no attempt to carry this further than elementary analysis and percentages of protein, carbohydrate and fat, so that we have no indication of the nature of the toxic agents in the venom. The nature and intensity of the pain experienced after a weever sting suggested to me that this might be caused by 5-hydroxy-tryptamine, which Armstrong, Dry, Keele & Markham (1953) have shown to be one of the most potent of pain-producing substances. This investigation was designed to test this supposition and to gain some idea of the chemical nature of the toxins present in weever venom.

PREPARATION OF VENOM EXTRACTS

All extracts were prepared from *Trachinus vipera* using only the dorsal fins, not the opercular spines. The live fish was placed in a small dish of sea water ($10 \times 7 \times 2$ cm deep) and harried with a small fragment of expanded polyurethane sponge, held in forceps, until the fish attacked. As soon as it sank its dorsal spines into the sponge this was held still for about 15 sec and then withdrawn. The fragment of sponge, which measured $6 \times 6 \times 6$ mm, was then rinsed out in three changes of 1 ml. each of distilled water, with repeated squeezing with forceps. The same fish could be used again after an interval of about 3 days.

The 3 ml. of extract from each fish could either be used fresh or on occasion it was concentrated under reduced pressure at 40° C. Five extracts were taken to dryness after dialysis and found to represent between 0·5 and 2·1 mg of non-dialysable material per animal per extraction. In what follows 1 unit of venom is taken as the amount of venom which can be extracted by one application of the sponge and presumably represents the amount of venom which the fish injects when it stings.

In most experiments 'blanks' were prepared by dipping a piece of the sponge into sea water and then extracting it in the same way as the sponge containing venom. Such a blank never had any effect and could never be shown to contain anything but a little sea water.

Many samples were dialysed against distilled water in a micro-dialysis apparatus, using cellophane as the semi-permeable membrane.

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THE LETHAL EFFECT OF THE VENOM

The LD 50 of the venom was determined upon *Gobius ruthensparri*, a small fish weighing about 2 g. The whole venom and the non-dialysable fraction were made up into serial dilutions and injected into the gobies which each received 0·01 ml. Four gobies received each dosage level, and deaths were recorded 30 min later. For whole venom the LD 50 was 0·0032 unit with 5% fiducial limits of 0·0173 and 0·0011 unit. For the non-dialysable fraction the LD 50 was 0·0027 unit with 5% fiducial limits of 0·0155 and 0·0009 unit. Slightly less of the non-dialysable fraction was thus required to kill a goby than of the whole venom, but the difference was not significant. A number of attempts were made to kill a goby with the dialysable fraction, but none succeeded: no gobies died within 24 h of the administration of this fraction. We may conclude that the major part of the lethal effect of the venom is contained in the non-dialysable fraction.

The amount of venom obtained from a single weever at one time is apparently 300 times the amount needed to kill the average goby within half an hour. At the higher dosages therefore it is not surprising that death often occurred within 90 sec of the injection. The fish threshed violently for a moment and then turned over on to the back and ceased to respire. Twitches continued for a minute or so and then ceased. The fish usually died with the fins and operculum fully extended. In the laboratory I have seen a blenny (*Blennius pholis*), weighing 10·7 g, attack a weever only half its length and attempt to swallow it. The blenny died within 2 min while the weever was unharmed.

THE PAINFUL EFFECT OF THE VENOM

The sting of the weever is characterized by the extreme pain which it produces. The immediate pain appears to be sometimes more severe than that of any other venomous sting. Pain must be assessed upon the human volunteer and to this end I injected various fractions under the skin of my forearm. Each injection was made in 0·025 ml of fluid. The fine needle (no. 30) was inserted under the epidermis and left for 15 sec until the pricking sensation had quite subsided. Then the test fluid was injected.

When Ringer's solution was injected in this way it produced no detectable effect, though larger volumes (0·1 ml or over) produced a slight transient pain. Distilled water produced slight immediate pain, lasting about 30–40 sec. Whole fresh venom produced a slight pain at first, then after about 15 sec a dose of 0·01 unit caused a sharp stabbing pain which lasted for about 2 min before subsiding to an itch lasting about 15 min. This dosage of venom was also followed by a rise in pulse (mean of ten observations: 12% rise) and some respiratory distress. The non-dialysable fraction of the venom produced little pain in the same dosage, merely such pain as could be ascribed to distilled

water, but the injection was followed by a rise in pulse and by respiratory distress. Three times the dose produced a rise in pulse of 30% (two observations) with respiratory distress but no more pain. The dialysable fraction of the venom produced a stabbing pain after a delay of some 15–20 sec, with much the same characteristics as that produced by the whole venom, but without the effects on pulse and respiration. All fractions produced some swelling and discoloration of the skin, rather like a gnat bite, starting a few minutes after the injection. This is presumably a result of histamine release.

An injection of 0·025 ml. of an aqueous solution of 5-hydroxytryptamine containing $2{\cdot}5 \times 10^{-8}$ g in this volume (10^{-6} g/ml.) produced pain like that of the dialysable fraction of the venom, with the same delay of 15–20 sec. It was not, however, followed by the flare and weal which appeared after all venom injections.

We may conclude that the systemic effects of the venom are a result of the non-dialysable fraction, while the pain is a consequence of some constituent of the dialysable fraction of the venom. Both fractions possess a histamine releasing effect.

FRACTIONATION OF THE EXTRACT

The dialysable fraction

The dialysable fraction was de-ionized on an ion-exchange column and then concentrated by distillation under reduced pressure at 40° C. It was then chromatogrammed on paper in *n*-butanol:acetic acid:water: (4:1:5). Two spots were revealed, one of which was readily identified as 5-hydroxytryptamine. The other material did not appear to be an indole derivative, but gave a violet colour fading to brown with ninhydrin. When these materials were eluted and injected under the skin the 5-hydroxytryptamine produced the characteristic pain, while the other material produced no pain but the weal and flare of a histamine releaser. The two substances together produced the same effect upon injection as the unfractionated dialysable material.

We may conclude that the chief pain-producing substance of the weeverfish sting is 5-hydroxytryptamine and that it is associated with a substance of low molecular weight which acts as a histamine releaser.

The non-dialysable fraction

Paper chromatography of this material left the whole fraction at the origin and nothing could be found elsewhere on the paper. The material was therefore subjected to paper electrophoresis in a hanging strip apparatus using barbiturate buffer pH 8·6, $\mu = 0{\cdot}075$, 16 h runs on Whatman 3 MM paper with a current of 8 mA. Dextran and human serum were used as markers. The paper strips were then dried in the oven and stained either with bromophenol blue, to reveal the proteins, or by the periodic acid-Schiff

reaction for polysaccharides. By the former staining method two barely separated spots were revealed at the level of blood albumin, while the latter method revealed a mucopolysaccharide at the level of the dextran. The non-dialysable fraction of the weeverfish venom thus appears to consist of two albumins and a mucopolysaccharide. Elution of the albumins as one extract and the polysaccharide as another yielded solutions which could be injected into gobies. Not enough material was available for a full-scale assay but both fractions were lethal in doses roughly corresponding to the lethal dose of the unfractionated venom.

BIOLOGICAL ASSAY OF 5-HYDROXYTRYPTAMINE

The dialysable fraction of the venom was assayed for its content of 5-hydroxytryptamine by its action upon the heart of the prawn *Palaemon serratus*. The prawn is prepared for the assay by injecting 2·5 μg of reserpine. It may be used for assay of 5-hydroxytryptamine 2–8 h after this injection. For the assay the prawn is observed, as it swims in a dish of seawater, by the use of magnifying spectacles. In this way the heart can be seen through the transparent shell and the time for 100 beats taken with a stop-watch, or, more simply, a metronome may be synchronized with the beat. It is then injected with a standard preparation of 5-hydroxytryptamine and the heart-beat counted 1, 5, 10 and 20 min after the injection. Half an hour after the first injection it may be similarly injected with the solution under test and then at half an hour intervals with varying concentrations of the test and standard materials. In this way the concentration of test substance may be matched to the concentration of standard required to produce the same acceleration in heart-beat. All injections are made in 0·01 ml. The method will be described more fully in forthcoming paper devoted to the endocrine control of heart-beat in prawns.

By this method 1 unit of venom was shown to contain from 2 to 10 μg of 5-hydroxytryptamine. This would imply that 0·1–2% of the dry weight of the venom is accounted for by this substance, an amount in excess of any other biological source. The highest level recorded is 0·2–0·4% in the venom of scorpions (Adam & Weiss, 1956, 1958, 1959).

ASSAY OF ANTICOAGULANT ACTIVITY

The assay was carried out by a method based on that of *The British Pharmacopeia* (1948), but using citrated human blood, instead of the fresh cat's blood of the standard method. The whole venom was assayed against a standard preparation of heparin in which 0·0077 mg represented 1 i.u. The standard was made up at a concentration of 2·5 i.u./ml. (i.e. 0·019 mg/ml.) while the venom extract was later shown to contain 0·52 mg/ml. of non-dialysable material. Two sets of three 5 ml. stoppered test-tubes received respectively

0·1, 0·2 and 0·3 ml. of the standard, while two more sets received the same volumes of the venom extract. To each tube was added 0·5 ml. citrated human blood. To each tube was then added 0·2 ml. of 100 mM $CaCl_2$ solution. The tubes were stoppered and mixed by inverting so that the mixture covered completely the inside walls, and were then incubated for 2 h at 27° C. At the end of this period the four control tubes as well as all six containing venom had clotted completely; the two tubes with 0·3 ml. of heparin solution had clotted, while the remaining four heparinized tubes showed lesser degrees of clotting.

We may conclude that 156 μg of venom has much less anticoagulant activity than 1·9 μg of heparin. It is thus a reasonable assertion that the mucopolysaccharide of the venom is not a heparin-like material, a conclusion consonant with the lack of sulphur in the sample of venom analysed by Russell & Emery (1960), and with their clinical finding that the amount of bleeding from the puncture wound appears to be about what one might expect from a similar non-venomous injury. The polysaccharide in the weever venom thus does not subserve any anticoagulant function, such as is often found in mucopolysaccharides in other venoms.

HYDROLYSIS OF THE NON-DIALYSABLE FRACTION

A sample of the non-dialysable fraction of the venom was mixed with an equal volume of concentrated HCl, sealed into a glass tube and heated at 100° C for 6 h. The hydrolysate was dried *in vacuo* over P_2O_5 and KOH and the residue taken up in water and subjected to paper chromatography against glucose, galactosamine and glucosamine using six different solvent mixtures. The chromatograms were sprayed with silver nitrate or with the Elson and Morgan reagent (Kent & Whitehouse, 1955). An amino sugar behaving like glucosamine or galactosamine was detected but no glucose. It was not possible to distinguish between glucosamine and galactosamine and no attempt was made to determine the amino acids present in the hydrolysate.

The results of this hydrolysis taken together with the results of the electrophoresis experiments would suggest the presence of a neutral mucosubstance, evidently an amino polysaccharide, as one of the constituents of the venom.

DISCUSSION

Five major constituents have been demonstrated as contributing to the toxicity of the weever venom. These are: 5-hydroxytryptamine, which is the material responsible for most of the local pain of the sting; a histamine releaser of low molecular weight, not an indole; two separable albumins and a neutral amino polysaccharide lacking in sulphur, which between them seem to account for the systemic toxicity of the venom.

5-Hydroxytryptamine is commonly found in venoms. Thus Erspamer and his co-workers (see Erspamer, 1954) found it in large amounts in molluscan salivary glands, subserving a venomous function. They also found it in the skin of toads where it may act as a distasteful agent, though hardly as a true venom for stinging. Adam & Weiss (1956, 1958, 1959) and Welsh & Moorehead (1960) found it in the venom and venom glands of scorpions; Jacques & Schachter (1954), Erspamer (1954) and Welsh & Moorehead (1960) found large amounts in the venom of wasps, and Welsh (1960) suggests that it is probably a constituent of coelenterate stings. Collier & Chesher (1956) found it in nettle stings. Erspamer early showed that 5-hydroxytryptamine is widely distributed in vertebrates and several phyla of invertebrates and Welsh & Moorehead (1960) indicate that it is probably universally present in the nervous sytem of animals. I am unable, however, to find any previous report of the presence of 5-hydroxytryptamine in a vertebrate venom.

The wide distribution of 5-hydroxytryptamine in venoms raises the question of the role it may be fulfilling there. The important part played by this substance in the functioning of the vertebrate brain (see the review by Page, 1958) might suggest a 'neurotoxic' role for this constituent of venom, but in vertebrates, at least, this does not seem to be borne out in practice, though in crustaceans it acts as a powerful paralysant. Thus in cephalopods the role played by 5-hydroxytryptamine and by other indole derivatives in the salivary glands seems to be one of paralysing the crustacean prey (see Erspamer, 1954), while Welsh & Moorhead (1960) suggest that in the saliva of carnivorous gastropods it serves to relax the muscles of the molluscan prey. But there is no evidence that weeverfish use their sting in feeding. Indeed, in the laboratory they may be seen to catch their food by pouncing on it from hiding and use their stings only in defence. Similarly, the sting of the nettle seems to be directed chiefly against mammals, which of the terrestrial fauna are the only creatures which are big and clumsy enough to be stung. Welsh & Moorhead (1960) suggest that the most important role of 5-hydroxytryptamine in venoms 'could be the facilitation of the absorption and distribution of the true toxic components through its own action on permeability and circulation'. Although this may well be an important part of its role in venoms (see also Page, 1958) it remains for the moment pure supposition, without direct supporting evidence, and I would incline rather to the view of Adam & Weiss (1959) that the pain produced by this substance upon subcutaneous injection has in itself survival value. Armstrong *et al.* (1953) have shown that 5-hydroxytryptamine is one of the most potent of pain-producing substances, and the work presented in this paper suggests that it is the major if not the only pain-producing substance of the weever venom. I am therefore prepared to regard its pain-producing effect as its primary role in the venom, with an additional possibility that it may also facilitate the absorption of the other toxic components.

The histamine releasing effect of the venom needs little comment. It accounts for the marked swelling which is a feature of the sequelae of a weever sting.

There is no real evidence to suggest that the macromolecular toxic components of the venom (the two separable albumins and the aminopolysaccharide) are in fact separate compounds. Their separation is more likely to be an artifact of the methods of preparation, and in the native venom they probably represent a complex muco-substance of combined polysaccharide-protein nature. The data of Russell & Emery (1960) suggest that carbohydrate accounts for some 30–35% of the dry weight of the venom and the protein for about the same amount. It is perhaps more useful to suggest that some 60% or more of the dry weight of the venom is represented by muco-substances of a toxic nature. The toxicity of this complex appears to be purely neurotoxic. There is no evidence of any haemotoxic effect, either of haemolysis or of action upon blood coagulation. Indeed the absence of sulphur demonstrated by Russell & Emery should be sufficient indication that no anti-coagulation effect should be expected, for Kent & Whitehouse (1955) point out that the anti-coagulation activity of heparin and of other polysaccharides is directly related to the sulphate ester content of the molecule.

SUMMARY

A method is described for obtaining the venom from the dorsal fin of the lesser weever without harming the fish. It is suggested that the amount of venom normally 'injected' into the wound by the weever when it stings is 0·5–0·2 mg dry weight of venom. Some 60% of the dry weight of the venom appears to consist of toxic muco-substances, which have a neurotoxic effect, but are without toxic effect on the blood. In extracts this fraction may be separated into two albumins and an amino polysaccharide, though in the native venom these are probably associated into a single complex muco-substance. When injected subcutaneously this fraction of the venom produces no local pain. The venom also contains about 1–20 μg/mg (dry-weight basis) of 5-hydroxytryptamine which appears to be the origin of the pain of the sting, together with some undetermined histamine releaser (not an indole) of low molecular weight. It is suggested that the chief role of the 5-hydroxytryptamine in the venom is to produce pain around the area of the inflicted wound.

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Note added in proof

Since writing the above Dr F. E. Russell has drawn my attention to an investigation by C. J. D. Zarafonetis & J. P. Kalas (*Amer. J. med. Sci.*, Vol. 240, 1960, pp. 764–68) into the venoms of a number of snakes and other reptiles. They found 5-hydroxytryptamine and other indoles in all the venoms studied and suggested that the presence of these substances 'serves as a common denominator of venoms on a broad biological basis'.