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THE PARTIAL PURIFICATION AND BIOASSAY OF A TOXIN PRESENT IN EXTRACTS OF THE SEA ANEMONE, *Tealia felina* (L.)

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1 Column chromatography with Agarose A50m followed by Sephadex G100 was used to separate a fraction (extract II) in the molecular weight range 12,000 to 14,000 daltons from saline extracts of the sea anemone, *Tealia felina*.

2 Extract II inhibited histamine-induced contractions of the guinea-pig ileum and produced haemolysis of human blood, effects on which bioassays were based.

3 The potency of extracts was assayed. A standard unit of activity (= AU) was defined such that 100 AU produced 90% inhibition of histamine-induced contractions of the guinea-pig ileum after 30 to 35 min exposure.

4 The relationship between activity of the extracts measured on the ileum and their haemolytic activity was studied, providing a second assay method based on the latter property.

5 Based on values from both methods of assay, the calculated yield in AU at the end of the separation procedure was 0.53 AU for each AU present in the original extract. In crude extract there were 5.0 AU/mg dry weight and 36.7 AU/mg protein, and after separation (extract II) there were 11.2 AU/mg dry weight and 312.2 AU/mg protein.

6 The acute LD_{50} values determined in mice (i.v.) were: for crude extract 124 mg/kg, for extract I, 76 mg/kg and for extract II, 69 mg/kg.

7 Extract II (0.18 to 0.72 AU/ml) produced a slowly developing contraction of guinea-pig ileum. Indomethacin (2.8×10^{-5} M) substantially reduced this response.

8 Extract II (0.03 AU/ml) reduced the contractile response of the guinea-pig ileum to acetylcholine by $39 \pm 8\%$, $n = 6$, and the response to histamine by $26 \pm 6.6\%$, $n = 6$. The response to 5-hydroxytryptamine (5-HT) was not reduced by 0.08 AU/ml of extract II, a concentration that actually increased the contractile response to KCl by $32 \pm 11.2\%$, $n = 7$.

9 It is proposed that for future work on the extract a new AU should be used. This AU is defined such that 50 AU produce 50% inhibition of histamine-induced contractions of the guinea-pig ileum after 30 to 35 min exposure.

Introduction

The toxicity of extracts from different species of Cnidaria varies widely, from the relatively innocuous *Stomolophus meleagris* to the highly poisonous *Chironex fleckeri* (Burnett & Calton, 1977). The sessile sea anemones mainly yield extracts of low toxicity, although successive intravenous injections of such extracts into a dog can cause death by anaphylactic shock (Richet 1902). Histamine, 5-hydroxytryptamine, tetramethylammonium, homarine and a non-dialysable substance, probably a protein, were found in sea anemone extracts (Mathias, Ross & Schachter, 1960); the protein stimulated frog rectus abdominis and sartorius muscles and was thought to be the main toxic component of the extracts. In the subsequent research on nematocyst toxins and on tentacular extracts (Baslow, 1969; Burnett & Calton, 1977) at least 19 species have been studied. Martin (1963) found that extracts of three species of *Tealia* were toxic to mice. Extracts of *T. felina* have a wide spectrum of pharmacological activity (Elliott & Sheardown, 1980). In the present paper the preparation of the partially purified extract is described together with two methods of biological assay and some of its pharmacological actions.

Methods

Preparation of the extract

Sea anemones, *Tealia felina*, were obtained from suppliers in Aberdeen, Plymouth and Swansea. On arri-

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val they were placed in artificial sea water for at least 24 h to eliminate contamination with compounds in natural sea water and to allow time for undigested food and adherent debris to be removed. The animals were killed by immersion in liquid nitrogen and freeze dried at −20°C for 4 to 5 days. The dried specimens were ground to a powder and passed through a 500 μm mesh; 20 to 200 mg of the powder was homogenized with 1 ml 0.9% w/v NaCl solution (saline). The homogenate was centrifuged at 12000 *g* for 45 min at 4°C and the supernatant was removed to be made up to the original volume with saline. This crude extract was applied to a Biogel A50 m agarose column (160 mm × 25 mm), the eluant being saline at pH 6.1. Visual inspection of the eluant showed first an opaque white fraction followed by a clear olive green fraction. Tests showed maximum pharmacological activity in the green fraction and the immediately succeeding colourless fraction, both of which were combined and concentrated back to the volume of the sample originally applied to the column, by dialysis against polyethylene glycol 6000. This concentrated solution, extract I, was next applied to a Sephadex G-100 column (300 mm × 44 mm) and eluted with saline at pH 6.1. Maximum pharmacological activity was found in a fraction, kav = 0.6 relative elution volume 2.2, which was collected and concentrated as before, forming extract II. The active product appeared to bind to some sites on the Sephadex and it was found that yields rose with repeated use of the column, presumably due to saturation of the binding sites. Sodium estimations were by flame photometry and protein estimations by the Biuret method (Gornall, Bardawill & David, 1949).

The measurement of antihistaminic action on the guinea-pig ileum

The ileum was suspended in Tyrode solution containing atropine sulphate (2.9×10^{-6} M) in a 20 ml bath; the solution was maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. Contractions of the ileum were measured isotonically. A concentration-response curve to histamine was determined from which two concentrations were selected which gave approximately 25 and 75% maximal responses. These concentrations were applied alternately at 5 min intervals until consistent responses were obtained and the bathing fluid was then exchanged for atropinized Tyrode solution containing the extract to be assayed; this solution was used for the rest of the experiment. The extract produced a gradual reduction in the amplitude of the responses to histamine. The activity was measured by comparing the mean amplitude of the control responses with those obtained at 30 and 35 min after application of the extract.

The measurement of the haemolytic activity of the extract

Human blood, obtained from a blood bank, was diluted 100 fold with saline. To 10 ml of this diluted blood was added a sample of the extract in a volume not exceeding 0.8 ml; its NaCl content was 1.2%. A volume of saline equal to the sample volume was added to 10 ml of diluted blood as a necessary control because of the haemolysis present in old blood bank samples. To determine a value for 100% haemolysis the blood was diluted 100 fold with distilled water instead of saline. The suspensions were left for 30 min at room temperature and then centrifuged for 10 min. The supernatants were removed and bubbled with carbon monoxide and their optical densities at 571 nm were measured. The percentage maximum haemolysis produced by the sample was calculated as follows:

$$\frac{(\text{test haemolysis} - \text{control haemolysis})}{(100\% \text{ haemolysis} - \text{control haemolysis})} \times 100$$

Determination of 24 h LD_{50} in mice

Male albino CD-1 mice (20 to 24 g) were randomly assigned to 15 groups of 8 animals. Each group was injected intravenously with one of the following: 300, 400, 500, 600 or 700 AU/kg of crude extract, extract I or II. The maximum volume injected was 0.2 ml. The groups were returned to their home cages and given free access to food and water. The mortality in each group was assessed 24 h after the administration of the extract; percentage mortalities were converted to probits and plotted against the $\log_{10}$ of the dose of the extract. Regression lines were fitted by the method of least squares and confidence limits for the LD_{50} values were calculated by the method of Litchfield & Wilcoxon (1949).

The effects of extract II on the guinea-pig ileum

The ileum was set up for isotonic recording as described for the histamine assay. In contrast to the assay experiments, aliquots of extract II were added directly to the organ bath instead of being made up in the reservoir of Tyrode solution. Unless otherwise specified, atropine was not added to the Tyrode solution used in these experiments. Extract II produced a slow contraction of the ileum. The dose of extract which produced a response which was approximately 50% of the maximum response to the extract was given at intervals of 1 h. After either two or three control responses had been obtained, an antagonist was added to the bathing solution and after 1 h the selected dose of extract was tested in its presence. If the antagonist exerted an inhibitory effect on the

extract-evoked contraction the preparation was washed and the previously effective dose of the extract retested.

The effect of extract II on the responses of the guinea-pig ileum and of the rat fundus strip to spasmogens

Guinea-pig ileum was set up as described in the previous section. Rat fundus strip (Vane, 1957) was set up in a similar manner except that Krebs-Hensleit solution was used instead of Tyrode solution. For studies on the histamine blocking action of the extract, atropine (2.9×10^{-6} M) was added to the Tyrode solution and when acetylcholine (ACh) was studied mepyramine (2.8×10^{-6} M) was added. When ACh and histamine were used in the same experiment no antagonist was added. The experiments with 5-hydroxytryptamine (5-HT) and with KCl were carried out in the presence of both atropine (2.9×10^{-6} M) and mepyramine (2.8×10^{-6} M).

A concentration-response curve was obtained with the selected agonist. Two concentrations of agonist giving approximately 25 and 75% maximum responses were selected and given alternately at 5 min intervals until three pairs of consistent control responses were obtained. The bathing solution was then changed for one containing extract II and further pairs of responses elicited in the continued presence of the extract. The amplitude of the response to the lower concentration of the agonist, after 30 min exposure to the extract, and of the higher concentration after 35 min, were expressed as percentages of the corresponding control responses. When 5-HT was tested on the rat fundus strip preparation, the same procedure was adopted except that 5-HT was added at 15 min intervals. The responses after 30 and 45 min exposure to the extract were used to calculate the percentage change in 5-HT responses produced by the extract. This procedure was varied when ACh and histamine were tested on the same preparation. The drugs were given alternately in concentrations that produced responses of 40% of their respective maxima and the subsequent effect of the extract was measured after 30 min for ACh and 35 min for histamine.

Drugs and solutions

The composition of the Tyrode solution was (in mM): NaCl 140, KCl 2.7, $NaHCO_3$ 12, $MgCl_26H_2O$ 0.5, $NaH_2PO_42H_2O$ 0.3, $CaCl_26H_2O$ 0.9 and glucose 5.5; that of the Krebs-Hensleit solution was: NaCl 118, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_47H_2O$ 1, $CaCl_26H_2O$ 3 and glucose 11: that of the artificial seawater was: NaCl 400, KCl 9, $MgCl_26H_2O$ 53, $Na_2SO_410H_2O$ 28, $NaHCO_3$ 2 and $CaCl_26H_2O$ 10.

The drugs used were, acetylcholine chloride (BDH), atropine sulphate (BDH), histamine acid phosphate (Evans), 5-hydroxytryptamine creatine sulphate (BDH), indomethacin (Sigma), mepyramine maleate (May & Baker), methysergide bimaleate, (Sandoz). The indomethacin was dissolved in a minimum quantity of 0.1 N NaOH and made up to the desired volume in saline. This stock solution was used within 3 h.

Results

Assays

The crude extract reduced histamine-induced contractions of the guinea-pig ileum. The extracts prepared on different occasions varied in their histamine blocking activity, no doubt reflecting differences in the efficiency of extraction and the use of several different batches of *T. felina* and possibly variations in sensitivity of ilea to histamine. To standardize extracts, an arbitrary unit of biological activity was defined. A sample of extract was added to a litre of Tyrode solution and a guinea-pig ileum preparation was exposed to the solution for 35 min. If the solution produced 90% inhibition of the response of the ileum to histamine then the sample was said to contain 100 units of activity (activity unit = AU). No reference is made in this definition to the volume of the sample, but when necessary the activity of a sample could be expressed in units per ml. A solution of extract was prepared which produced about 90% inhibition of the histamine-induced contraction; to samples of this were added known volumes of Tyrode solution to give a series of dilutions, each of which was then assayed for histamine blocking activity on a separate piece of ileum taken from one guinea-pig. Seven such complete experiments were done on different samples of crude extract and the results obtained from 52 separate ileum preparations are shown in Figure 1. The dilution-response relationship was apparently sigmoidal and a straight line regression was fitted to the points between 30 and 80 AU by calculation, using the method of least squares. By extrapolation from this graph estimates could be made of the potency of new extracts.

The extract also had haemolytic activity and this formed the basis of a second assay. A series of dilutions with saline was made of an extract of known potency (calculated in AU). These dilutions were then assayed for haemolytic activity. Figure 1 shows the relationship between AU and percentage maximal haemolysis, a straight line regression being fitted to the points between 5 and 40 AU by calculation, using the method of least squares.

The two assays were used to monitor the progress of the extract purification outlined in the methods. Table 1 shows the activity of the extract in AU at

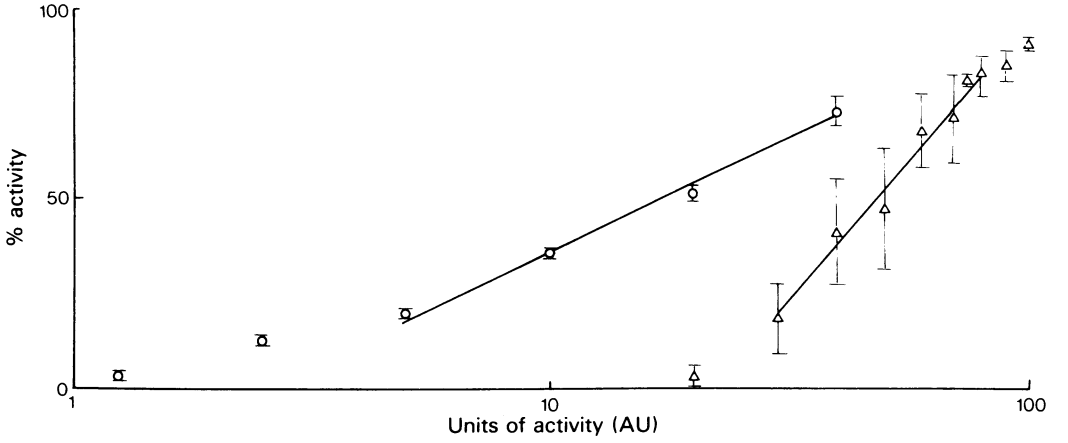

Figure 1 The relation between AU, inhibition of histamine-induced contractures and haemolysis. Extract was diluted to give solutions with different numbers of AU, these solutions were assayed for histamine inhibition (△) (n = 52) and for haemolysis (○) (n = 38). For definition of AU see text. The regressions were for % histamine inhibition y = 146 log x − 196 (correlation coefficient 0.52) and for % haemolysis y = 59.4 log x − 23.9 (correlation coefficient 0.97). Note that AU refers to the number of units and not to their final concentrations in the assay test solutions, which were different for the two assays (see methods).

three stages in the preparation. Dry weights and protein estimations are also given which permit a rough estimate to be made of the degree of concentration and of the yield achieved. The yield was 0.53 AU for each AU present in the original crude extract. To compare the precision of the two assays, a batch of crude extract was rapidly processed to give extract II and the potency was compared by each assay method. By the histamine inhibition method, extract II had 3.02 times the potency of the crude extract whilst with the haemolysis technique the relative potency was 3.06, the index of precision (Ghosh, 1971) being 0.130 and 0.007 respectively.

Stability of the extracts

The extracts and the dried sea anemone powder from which they were prepared were stored at −25°C. Repeated thawing and freezing of the extract led to rapid loss of activity; consequently the extract was stored in aliquots of 1 or 5 ml so that unused thawed extract could be discarded without excessive wastage. The extract was stable for some hours at room temperature but lost activity rapidly when stirred. For this reason the extract was only diluted in Tyrode solution immediately before assay. It was difficult to assess the long-term stability of the extracts since the assessment itself would depend on having a stable standard extract against which comparison could be made; nevertheless the assay techniques indicated that extracts lost activity in storage. Whenever possible, 'new' preparations of extract were obtained before the old ones were discarded so that a comparison could be made. This was not always possible due to the unavailability of *Tealia felina* at certain seasons.

Table 1 Dry weight, protein content and activity of the extracts

	*Dry weight** (mg/ml)	*Protein* (mg/ml)	*AU*** (per mg dry wt.) Histamine	Haemolysis
Crude extract	64.5	8.80	4.5	5.5
	n = 3	n = 2	n = 3	n = 2
Extract I	30.2	0.90	6.0	6.2
	n = 3	n = 3	n = 4	n = 2
Extract II	15.4	0.55	11.5	10.8
	n = 3	n = 3	n = 4	n = 2

* After deduction of NaCl content determined by flame photometry.

** Units of activity: Histamine = activity determined by inhibition of histamine-induced contractions of guinea-pig ileum; Haemolysis = activity determined by haemolysis of human blood. As the separation and assay procedures were developed slowly, not all the procedures were carried out on all the samples, n = numbers of measurements.

Toxicity

Table 2 shows the LD_{50} values obtained with the various extracts; the results are expressed in terms of AU/kg and also as mg dry weight of the extract per kg. Some mice died within 5 min of intravenous injection; respiration became laboured, with occasional gasps, and brief coordinated clonic convulsions occurred immediately before death. The remaining mice died between 1 and 24 h after administration of the extract. They became immobile and cold to touch and when disturbed they shook their heads from side to side: death was preceded by severe coordinated clonic convulsions. Four mice, which survived 500 AU/kg of extract I, had a mean oesophageal temperature of 28.6°C at 24 h after administration of the extract. The crude extract, but not extract I or II, produced a fine tremor of the fore and hind limbs immediately following intravenous injection. A group of 5 mice given 750 AU/kg of crude extract intraperitoneally showed immobility, hypothermia and piloerection, but they survived for 5 h at which time the experiment was terminated.

The action of the extract II on the guinea-pig ileum preparation

The extract at a concentration of 0.18 to 0.72 AU/ml produced a slowly developing contraction of the ileum. The contraction had a latency of 15 to 30 s, reached a maximum in 4 min and then declined slowly over a period of 15 min in the continued presence of the extract. Superimposed on the slow contraction there were sometimes rapid twitch-like contractions. If the preparation was washed after 4 min exposure, recovery occurred and further contractions could be elicited with the extract, but the responses varied in amplitude. Unless a dose interval of 1 h was used, tachyphylaxis was marked.

Considerable variation occurred between preparations in their response to the extract and some preparations were virtually insensitive. Owing to the tachyphylaxis and the variation, further study of the direct effects of the extract was difficult and incomplete. Preliminary experiments had shown that a response which was half of the maximal response to the extract could be obtained with 0.36 AU/ml of extract II. The actions of some pharmacological antagonists were tested against this dose of extract as described in the methods section (Table 3). Only indomethacin (2.8×10^{-5} M) inhibited the extract-evoked contraction; indomethacin had no cholinolytic action at this concentration. The inhibitory action of indomethacin could be reversed by washing for 1 h (Figure 2). Mepyramine (2.8×10^{-6} M) invariably caused an increase in the extract-induced contractions.

The antagonist actions of extract II on the guinea-pig ileum and rat fundus preparations

Histamine The extract reduced the contractile response of the guinea-pig ileum to histamine. This reduction was maximal after 30 to 35 min exposure to the extract (Figure 3, 4) and was sustained at the same level for at least 2 h of continuous exposure to the extract. For this reason the % inhibition produced by the extracts was measured after 30 to 35 min exposures in the assay procedure. There appeared to be no difference between any of the extracts as regards the rate of onset of histamine blockade, when solutions of similar activity were compared. Only a partial recovery could be obtained (Figure 5) even with prolonged washing. Attempts to overcome the inhibitory effects of the extract by successively doubling the doses of his-

Table 2 The acute toxicity of extracts of *Tealia felina*, given intravenously to mice

	Crude extract	*Extract I*	*Extract II*
LD_{50} AU/kg	580 (472–716)	458 (344–579)	756 (614–943)
LD_{50} mg/kg	124 (101–153)	76 (57–96)	69 (56–86)

The values in parentheses are the 95% confidence limits for the LD_{50}s.

Table 3 The mean % change in the amplitude of the contractile response of guinea-pig ileum to 0.36 AU/ml of extract II produced by some pharmacological antagonists

Drug	*Concentration* (M)	% *Change* ± *s.e.*	n
Atropine	2.9×10^{-6}	+4.9 ± 1.0%	5
Methysergide	2.5×10^{-5}	+16.4 ± 4.9%	5
Mepyramine	2.8×10^{-6}	+53.3 ± 14.1%	5
Indomethacin	2.8×10^{-5}	−74.8 ± 11.1%	7

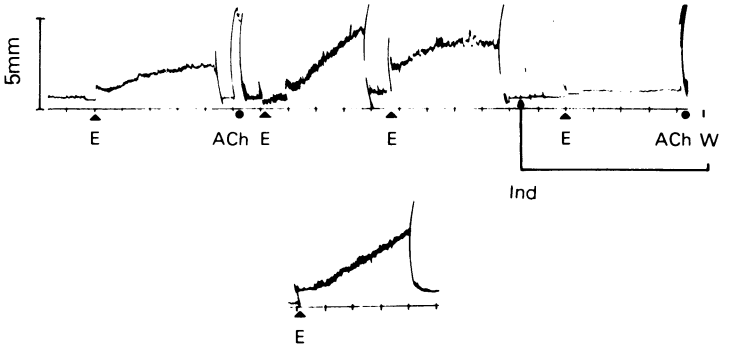

Figure 2 The action of indomethacin on slow contractions of guinea-pig ileum elicited by extract II. Isotonic contractions of the guinea-pig ileum in response to 0.36 AU/ml extract II (E) and 10 ng/ml acetylcholine (ACh). Indomethacin 2.8×10^{-5} M (Ind) added to the bathing solution. W = wash for 1 h. Indomethacin markedly reduced the response to extract II; washing out the indomethacin restored the response to extract II (lower trace). Note that the doses of extract II were applied at intervals of 1 h. Time marker 1 min.

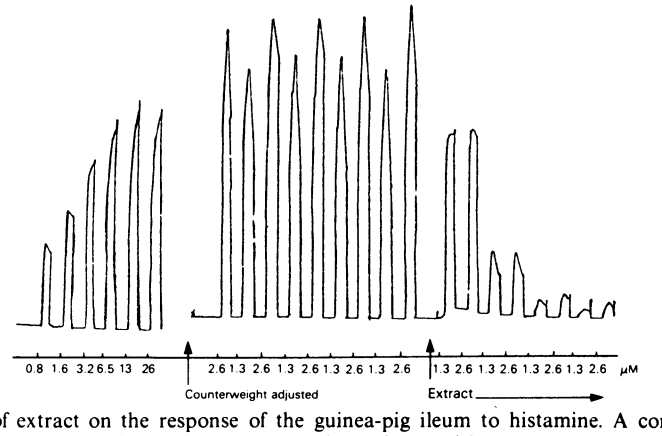

Figure 3 The effect of extract on the response of the guinea-pig ileum to histamine. A concentration-response curve to histamine was determined from which concentrations of 1.3 and 2.6 μM were selected and given alternately at 5 min intervals. At 'Extract' the bathing solution was replaced by Tyrode solution containing 0.04 mg dry weight of crude extract/ml. Atropine (2.9×10^{-6} M) was present in the Tyrode solution throughout the experiment. Lever magnification × 6.8. This extract Tyrode solution contained more than 100 AU/l (inhibition of histamine responses in excess of 90%) and would be further diluted for assay on another preparation.

tamine were unsuccessful. The first increased dose of histamine resulted sometimes in a slightly larger response but thereafter increased doses produced diminishing responses as though the tissue were desensitized. The relationship between extract dilution and histamine antagonism is described in the assay section of the paper.

Acetylcholine Preliminary experiments showed that extract II reduced the amplitude of ACh-evoked contractions of the guinea-pig ileum. In comparisons on the same piece of ileum, the extract more effectively blocked ACh than histamine; Figure 6 shows a typical experiment in which ACh and histamine were given alternately. The concentrations were chosen to give similar percentage maximal responses; the ACh response was 38% of its own maximum and the histamine response 36% of its own maximum. Extract II (0.03 AU/ml) reduced the ACh response by 35% and the histamine response by 22%.

In six experiments of this type with an extract II concentration of 0.03 AU/ml the mean % reduction of ACh-evoked responses was 39 ± 8.0% and the corresponding value for histamine was 26 ± 6.6%.

KCl Extract II (0.08 AU/ml) increased KCl-evoked contractions of the guinea-pig ileum by a mean 31.6 ± 11.2% ($n = 7$).

5-Hydroxytryptamine Although it appeared that extract II did not reduce 5-HT-induced ileal contractions, the 5-HT responses were variable and little

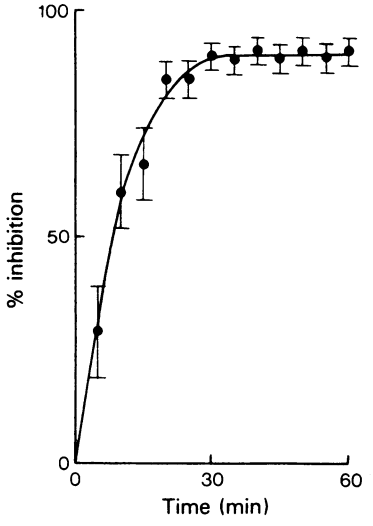

Figure 4 The development of inhibition of the contractile responses of the guinea-pig ileum to histamine. The results of 10 experiments with crude extract of the type illustrated in Figure 3 have been averaged, bars show s.e. mean. Note that maximal inhibition was obtained within 30 min of application of the extract at zero time. The extract concentration was 0.04 mg dry weight/ml.

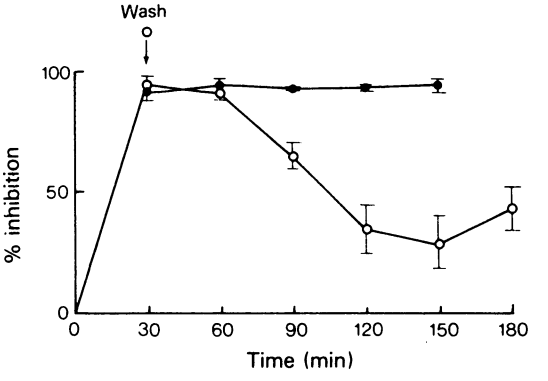

Figure 5 Maintenance of extract-induced inhibition of response of guinea-pig ileum to histamine and the effect of washing. Percentage inhibition was measured of the responses of the guinea-pig ileum to histamine by the extract applied at zero time. In the continued presence of the extract, (n = 5) inhibition was maintained for at least 150 min (●); if the preparation was washed (n = 5, ○) after 30 min exposure to the extract there was partial recovery, but it was never complete, even after washing for 3 h with a total volume of 360 ml Tyrode solution. Extract concentration, 0.04 mg dry wt. of crude extract/ml.

reliance could be placed on the results. Consistent responses to 5-HT were obtained on the rat fundus preparation and extract II (0.08 AU/ml) produced a slight but statistically insignificant increase in the responses to 5-HT, the mean being 5.8% ± 2.5% (n = 5). It should be noted that the extract II concentration of 0.08 AU/ml used in the experiments with KCl and 5-HT would be expected to produce about 80% inhibition of histamine-evoked responses in the guinea-pig ileum.

Discussion

When a pharmacologically active product of unknown composition is being purified, a biological assay is essential to quantify progress. Simpson (1970) noted that an extract of *T. felina* reduced histamine evoked contractions of the guinea-pig ileum and this formed the basis of our first assay. Haemolytic activity has been found in extracts from the sea anemone, *Rhodactis howesii* (Martin, 1966), *Aiptasia pallida* (Hessinger & Lenhoff, 1973), *Stoichactis helianthis* (Devlin, 1974; Bernheimer & Avigad, 1976) and also

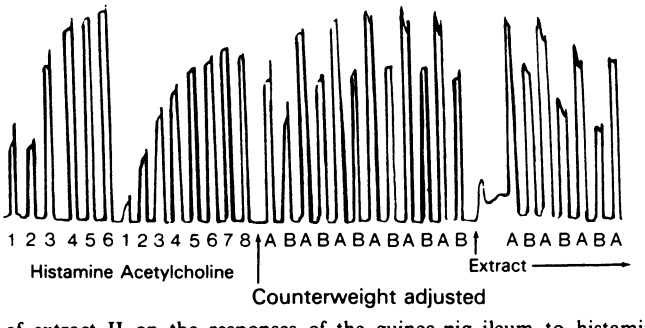

Figure 6 The action of extract II on the responses of the guinea-pig ileum to histamine and acetylcholine. Histamine, 1 = 28, 2 = 14, 3 = 56, 4 = 112, 5 = 224, 6 = 448 ng/ml. Acetylcholine, 1 = 6, 2 = 12, 3 = 24, 4 = 48, 5 = 96, 6 = 192, 7 = 384, 8 = 768 ng/ml. A = 28 ng/ml histamine, B = 12 ng/ml ACh. All doses at 5 min intervals. At 'extract' normal Tyrode solution was replaced by one containing 0.03 AU/ml extract II. The ACh response was reduced by 35% and the histamine response by 22%. Lever magnification ×8.5.

in extracts from the scyphozoan *Chrysaora quinquecirrha* (Burnett & Calton, 1976) and the cubomedusan *Chironex fleckerii* (Endean & Henderson, 1969; Freeman & Turner, 1969). Hessinger & Lenhoff (1973) described an assay of the nematocyst toxin of *Aiptasia pallida* based on measurements of the rate of haemolysis. The present assay is simpler, requiring only measurement of the haemolysis produced by 30 min exposure to two samples of the extract. The haemolysis assay is preferable to the ileum assay as it is cheaper, quicker and does not require the use of animals.

It is not yet possible to prepare an extract which is stable for more than a few weeks, consequently the same preparation cannot be used to compare a 'standard' extract with other extracts prepared some months later. This is why a large number of experiments were done to establish the standard curves for the two assays ($n = 52$ for the ileum assay and $n = 38$ for the haemolysis assay). When it was possible to compare extract II with crude extract directly, without using the standard curves, the haemolysis assay gave a better index of precision than the ileum assay. Although the curves are probably sigmoidal, logit transformation of the response data did not improve the fit of data to a straight line regression. Consequently, straight line regressions were fitted to the middle regions only of the sigmoidal curves. When extract solutions were assayed their concentrations were adjusted so that they gave results in this linear region of the dose-response curves.

When the ileum assay was originally developed 100 AUs were defined as producing 90% inhibition of histamine responses under specified conditions. The use of 90% inhibition was dictated by practical considerations and by the observation that the responses to the extract of different ileal preparations were less variable when a high rather than a low concentration of extract was used. However, it appears more satisfactory to base the activity units scale on a point in the linear region of the sigmoid concentration-response curve rather than at the upper end of the curve. The AU is therefore redefined so that 50 AU applied under standard conditions to the ileal preparation produce a 50% inhibition of the response to histamine. This AU will be used in future work on the purification of the extract but a new concentration-response curve based on this unit must be established before it can be used. The values for activity given in this paper are on the scale 100 AU equivalent to 90% inhibition; 50 AU measured on the latter scale gave 53% inhibition (Figure 1) so that the difference between the two AU scales in this region is small.

The determination of the LD_{50} values of the extracts confirmed that the two assay techniques can be used to monitor progress in the isolation of the anemone toxin. With the small numbers of mice used the 95% confidence limits of the LD_{50} values were large. Nevertheless, it appears that these values expressed as mg/kg get smaller with purification but expressed in AU/kg they remain constant, or increase, with purification. If the AU is a good measure to use in the separation procedure then the LD_{50} for different crude extracts and for any active fraction should remain constant when expressed in AU/kg.

The typical features prior to death in the toxicity tests were immobility, hypothermia and piloerection, with terminal convulsions. The hypothermia may be related to the immobility but further experiments are required to elucidate this. The tremor which occurred transiently following intravenous injection of crude extract may be due to the presence of quaternary ammonium compounds which are known to be present in some sea-anemone extracts (Mathias *et al.*, 1960).

The results in Table 1 indicate that during column chromatography, the fraction with both haemolytic and histamine blocking activity moved as a single fraction, with a molecular weight in the range 12,000 to 14,000 daltons. Further studies will be required to determine whether these two actions are produced by a single compound. Burnett & Calton (1977) reviewed attempts to isolate the toxic components from nematocyst or tentacle extracts in five venomous *Cnidaria*. A constant feature was the affinity of the toxins for the support media used in chromatography; this was also noted in our experiments and made separation difficult. We have recently noted that yields may be considerably augmented if columns are pretreated with serum albumin which appears to saturate sites on the support media which otherwise bind the toxin.

Nematocyst toxins have molecular weights in the range 10,000 to 30,000 daltons, which is consistent with the present results, although fractions with molecular weights of *c.* 70,000 and *c.* 250,000 have also been found toxic. Multiply-active fractions are often found but these may result from the toxin aggregating with several different polypeptides during the separation (Burnett & Calton, 1977). There is considerable discrepancy between the protein content of extract II determined by the Biuret method and the dry weight (Table 1), which may suggest the presence of a non-polypeptide constituent. Extract II contains a high molecular weight carbohydrate, but not in a sufficient quantity to account for the discrepancy.

The main purpose of this paper was to describe how we purified and quantified the biologically active component in the sea-anemone extract. As one of the assay procedures involved histamine-induced contractions of the guinea-pig ileum it was deemed advisable to explore the reactions of this tissue to the extract in more detail. It was noted that extract II produced a slowly developing contraction of the ileum, an effect unrelated to the histamine blocking action of the extract, since some preparations in which the extract

blocked histamine responses did not contract to extract II. That the contraction was reduced by indomethacin might suggest the presence of a kinin, but this seems unlikely in view of the estimated molecular weight of the active fraction. However, a compound with kinin-like activity in generating prostaglandins may be present in the extract. Burnett, Calton, Meir & Kaplan (1975) have suggested that a kinin-like substance might be present in *Chironex fleckerii* and some other Cnidaria.

The crude extract probably contains tetramethylammonium (Mathias *et al.*, 1960); in our experiments its spasmogenic effect, unlike that of extract II, was reduced by atropine, which explains why atropine was included in the Tyrode solution when carrying out the histamine assay experiments. In the absence of atropine the large sustained contractions produced by the crude extract made it difficult to measure histamine-evoked contractions. We are unable to explain why mepyramine augmented the slow contraction produced by the extract. In view of the variable response of the ileum to the extract it is probably more significant that the response was not inhibited by mepyramine. This implies that the slow contraction produced by extract II is not due to histamine release.

The histamine blocking action of the extract was slow in onset and could only be partially reversed by prolonged washing with extract-free Tyrode solution. In view of its probable high molecular weight, the compound may only penetrate slowly to its site of action. However, the slow onset of action could merely reflect the low concentration of the active compound present in the extracts. Typically, extracts for assay were diluted to give considerably less than 90% block of histamine in 35 min (=100 AU/l), although occasionally with extracts which were more active than expected, 90% block was attained in 10 min.

Extract II was relatively non-selective as an antagonist since responses to ACh and histamine were reduced to approximately the same extent. The antagonism of histamine is probably of a non-competitive type since it was not surmounted by increasing the histamine concentration. Since the extract did not reduce smooth muscle responses to either KCl of 5-HT, a generally toxic or a local anaesthetic action seems excluded. However, it is premature to speculate on the actions of extract II on smooth muscle since it may contain more than one active compound; further progress must await refinements in the separation techniques.

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