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THE ACTION OF THE TOXIN OF *PRYMNESIUM PARVUM* CARTER ON THE GUINEA-PIG ILEUM

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The ichthyotoxin from *Prymnesium parvum* caused a slow contraction of the guinea-pig isolated ileum preparation, followed by decreased sensitivity to acetylcholine and to 5-hydroxytryptamine. These effects were reversed by washing. Administration of toxin was followed by insensitivity to further toxin. It was possible that a cholinergic transmitter participated in the contractions of the gut as this action was reduced both by atropine and by morphine. Illumination with visible light or warming a solution of toxin destroyed its antiacetylcholine activity before abolishing its stimulant action on gut. The crude extract of *Prymnesium parvum* may contain a mixture of active substances with different pharmacological actions.

In brackish fish ponds, sudden outbreaks of mass piscine mortality can follow release of a toxin from the phytoflagellate *Prymnesium parvum* (Otterstroem & Steemann-Nielsen, 1939; Reich & Aschner, 1947). This ichthyotoxin seriously interferes with the commercial breeding of carp and other fish (Reich & Aschner, 1946). In the ponds, the toxin kills only gill-breathing forms such as fish or tadpoles, leaving lung-breathing species, such as adult frogs, unaffected (Shilo & Aschner, 1953). Recently Bergmann, Parnas & Reich (1963) reported that fish were killed more rapidly when they received the toxin by intraperitoneal injection than when they absorbed it from the water in which they swam. As the symptoms accompanying death were identical, the gills probably served only as an easy portal of entry for the toxin.

Preliminary experiments showed that, in addition to fish, various vertebrate species, such as frogs, cats and rabbits, were killed by intravenous or intraperitoneal injection of the toxin probably by actions on the circulation and the central and peripheral nervous system. The work described here was undertaken to find out whether the toxin acted on the guinea-pig isolated ileum and rat isolated uterus preparations and whether analysis of such effects would yield information on the mechanism of action.

METHODS

Culture of the phytoflagellate. The strain of *Prymnesium parvum* used was derived from an axenic (pure) culture isolated from pond water by Reich & Kahn (1954).

To dilute sea water (1:20, v/v) was added 0.03% dehydrated liver infusion (Oxoid, London) and the *p*H of the solution was adjusted to 8.4 by means of N-sodium hydroxide solution

(Reich & Rotberg, 1958). This culture medium was sterilized at 1.5 atmospheres pressure of steam for 20 min. Erlenmeyer flasks, holding 50 ml. of culture medium, were inoculated with 5 ml. of a 15 day old culture of *Prymnesium*. The flasks were incubated for 21 days in a chamber maintained at 20° C and subjected to 12 hr alternating periods of illumination and darkness, a 40 W fluorescent lamp approximately 20 cm from the cultures serving as the light source (Reich & Parnas, 1962).

Isolation of crude ichthyotoxin. All manipulations with the toxin were now performed in the dark at 4° C. At the end of the 21 day period of growth, the cells were sedimented by centrifugation at 3,400 revs/min for 10 min. They were then extracted with cold acetone to remove lipids and pigments, the suspension was centrifuged and the acetone supernatant fluid discarded. Subsequently, the cells were treated for 2 min with methanol in a Teflon homogenizer. The product was centrifuged and the supernatant fluid concentrated *in vacuo* at 0° C. The crude toxin, obtained as a yellowish powder, was kept in stoppered vessels at −18° C. The yield was about 15 mg/l. of culture medium.

The toxin from fifty Erlenmeyer flasks was pooled and used as a standard. This powder had an LD50 value of 100 μg/g when tested by intraperitoneal injection into the minnow, *Gambusia affinis*, of 300 mg mean body weight (Bergmann *et al.*, 1963). As a control, some toxin solution was inactivated either by heating to 50° C for 10 min or by exposing at a temperature of 5° C for 3 hr to the light of a 500 W tungsten filament lamp, placed at a distance of 20 cm (Parnas, Reich & Bergmann, 1962).

A solution of the toxin (2 mg/ml.) in 0.9% saline was made and any insoluble material was removed by centrifugation. Between experiments, this clear solution preserved its activity for at least 1 month when kept at −18° C.

Isolated smooth muscle preparations. Guinea-pigs were killed by a blow on the neck and the small intestine was excised immediately. Pieces, 3 cm long, of mid-ileum were suspended at 37° C in an organ-bath of 50 ml. capacity, containing Tyrode solution bubbled with filtered air. The contractions of the gut were recorded by an isotonic frontal writing lever on a kymograph, using a four-fold magnification.

The isolated uterus of the rat was set up under similar conditions.

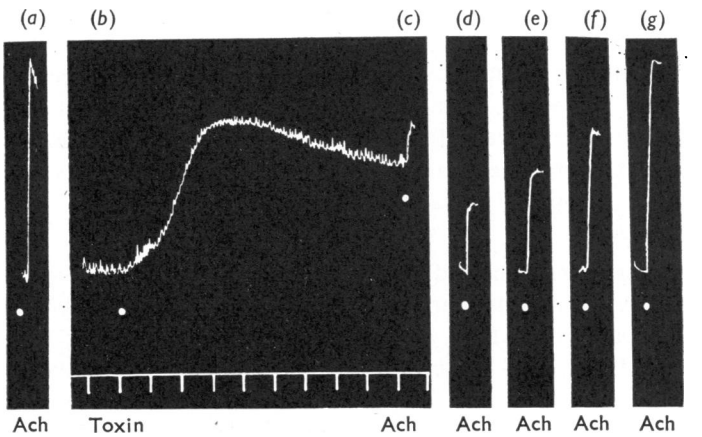

Fig. 1. Dual effect of ichthyotoxin on the guinea-pig ileum. Acetylcholine (Ach) was added in a concentration of 4 ng/ml. Between records, three washings. The organ-bath volume was 50 ml. Time marks in minutes. (*a*), acetylcholine; in (*b*), toxin was added in a concentration of 10 ng/ml. Note slow contraction reaching its maximum after 4 min, followed by partial relaxation; at (*c*) 10 min later without washing, addition of acetylcholine with 80% inhibition of response; (*d*) to (*g*), show stepwise recovery of sensitivity to acetylcholine. (*d*), after 3 min; (*e*), 7 min; (*f*), 10 min; and (*g*), 15 min. Note that in (*d*) the response to acetylcholine is still strongly depressed.

RESULTS

The effect of ichthyotoxin on the guinea-pig isolated ileum preparation. Addition of toxin (20 to 50 ng/ml.) to the fluid bathing the guinea-pig isolated ileum preparation caused a slow and prolonged contraction (Fig. 1, *b*), which reached its maximum within 4 to 5 min and was then followed by a slow decline. After 10 min of incubation, the response to acetylcholine was greatly decreased (Fig. 1, *c*), but the sensitivity to acetylcholine gradually returned with repeated washings (Fig. 1, *d* to *g*).

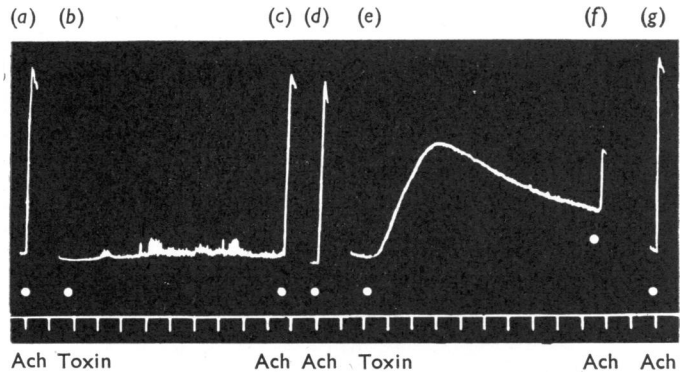

Fig. 2. Photo-inactivation abolished the effect of ichthyotoxin on the guinea-pig ileum preparation. Acetylcholine (Ach), was added in a concentration of 20 ng/ml. (*a*), acetylcholine; (*b*), 100 ng/ml. of toxin after illumination for 4 hr, showing lack of contraction; (*c*), without washing, addition of acetylcholine, which produced its full effect; (*d*), acetylcholine; (*e*), 100 ng/ml. of standard toxin caused contraction with subsequent slow decline; at (*f*), after 10 min of incubation, the response to acetylcholine shows 65% inhibition; (*g*), 18 min later, after repeated washing, there was recovery of sensitivity to acetylcholine. Time in minutes; organ-bath volume of 50 ml.

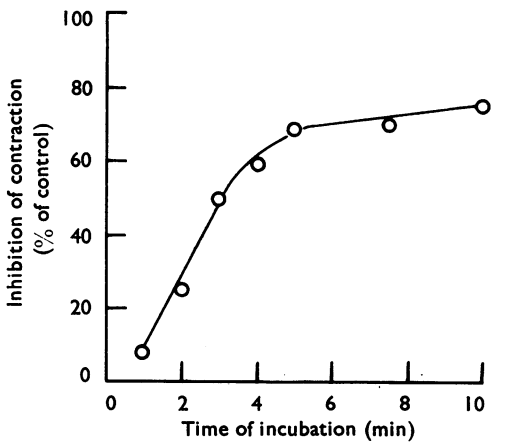

Fig. 3. Influence of time of exposure to toxin on the response to acetylcholine. Pieces of guinea-pig intestine were incubated with 200 ng/ml. of toxin. To restore the original tone of the smooth muscle, the toxin was washed out once after the period indicated on the abscissa, and the amplitude of contraction induced by subsequent application of 10 ng/ml. of acetylcholine was measured and used to calculate the percentage inhibition of the control. Note that the inhibition approached its maximal value after about 5 min.

The actions described above did not occur if the toxin had been subjected to strong illumination for 4 hr (Fig. 2), which also abolished toxicity towards fish (Parnas *et al.*, 1962). Likewise, heat inactivation abolished both the toxicity to fish and the effects on the gut.

Nature of the antagonistic effect of ichthyotoxin against smooth muscle stimulants. Fig. 3 shows the decrease of the response of the ileum to a test dose of 10 ng/ml. of acetylcholine after different periods of exposure to 200 ng/ml. of toxin. For each point on the curve, a fresh piece of intestine from the same animal was used. After 1 min of contact with the toxin, the contraction of the ileum induced by acetylcholine was only slightly depressed, but the reduction increased gradually and progressively and approached its maximal value (about 70%) after about 5 min. Therefore, the diminution in response to various smooth muscle stimulants was always measured after 10 min of incubation.

The experiment illustrated in Fig. 4 demonstrates that exposure of the gut to 160 ng/ml. of toxin greatly reduced the response to 4 ng/ml. of acetylcholine

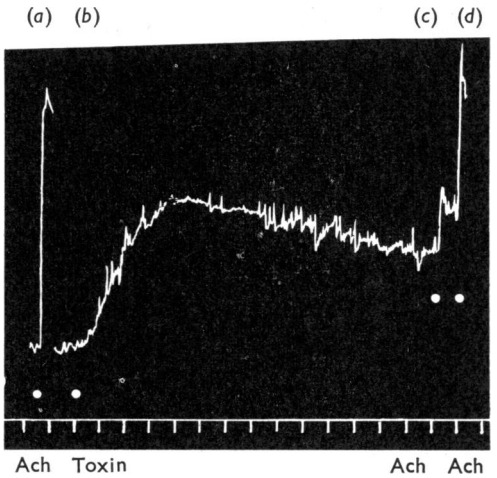

Fig. 4. Antagonistic action of ichthyotoxin and acetylcholine (Ach) on the guinea-pig ileum preparation. (*a*), 4 ng/ml. of acetylcholine; (*b*), 160 ng/ml. of toxin; at (*c*), 10 min later without washing, 4 ng/ml. of acetylcholine, with strong reduction of the amplitude of contraction; at (*d*), without washing, 40 ng/ml. of acetylcholine caused a strong contraction. Time in minutes; organ-bath volume of 50 ml.

(Fig. 4, *c*), while the contraction evoked by 40 ng/ml. of acetylcholine was still of considerable magnitude (Fig. 4, *d*), which indicates that an increase of acetylcholine concentration could overcome the decrease in sensitivity due to toxin.

A linear relationship existed between the percentage reduction of the amplitude of response to a standard concentration of acetylcholine and the log of the concentration of toxin. Fig. 5 illustrates an experiment in which 6 ng/ml. of acetylcholine were added after 10 min of incubation of the tissue with various doses of toxin.

Previous treatment with toxin reduced the responses to histamine (10 ng/ml., Fig. 6, *c*) 5-hydroxytryptamine (0.4 μg/ml., Fig. 8, *c*), nicotine (0.8 μg/ml.) and

bradykinin (10 ng/ml.). The toxin also blocked its own action in contracting gut. In Fig. 7, after the contraction to the first application of 160 ng/ml. of toxin had declined (after about 15 min), a second application of the same dose was without effect.

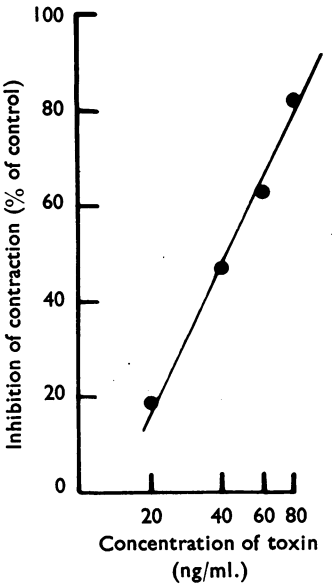

Fig. 5. Inhibition of contractions of the guinea-pig ileum induced by acetylcholine (ordinate) as a function of toxin concentration (abscissa). Each point in the graph was determined with a fresh piece of ileum from the same animal. The gut was incubated for 10 min with the toxin before application of 4 ng/ml. of acetylcholine.

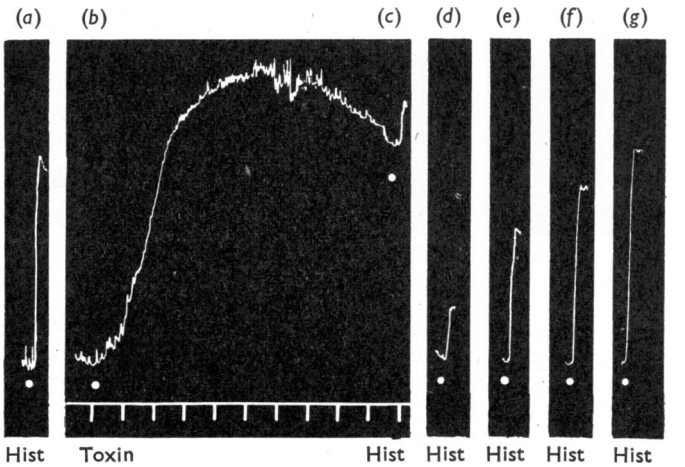

Fig. 6. Antagonism between ichthyotoxin and histamine on guinea-pig ileum preparation. Histamine (Hist) was added in a concentration of 10 ng/ml.; between records three washings; time in minutes; organ-bath volume of 50 ml. (*a*), histamine; (*b*), 40 ng/ml. of toxin; (*c*) after 10 min of incubation without washing, addition of histamine, with 80% inhibition; (*d*) to (*g*), gradual return of the response to histamine by repeated washing during 30 min.

The slow contraction of the guinea-pig ileum preparation, following the application of ichthyotoxin, might have arisen from the gradual release of an active substance. The following experiments were designed to examine whether acetylcholine might be such a substance.

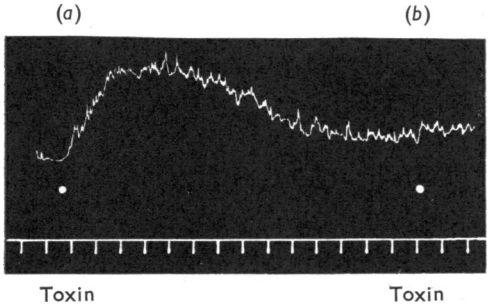

Fig. 7. Guinea-pig ileum preparation. Ichthyotoxin prevented the contraction induced by a second dose of toxin. Time in minutes; organ-bath volume of 50 ml. At (*a*), 160 ng/ml. of toxin; at (*b*), 10 min later without washing, same dose of toxin was given.

After 10 min of exposure of the isolated ileum to 160 ng/ml. of toxin, the response to 5-hydroxytryptamine was reduced to about 40% of the amplitude of contraction in the absence of toxin (Fig. 8, *a* and *c*). On the other hand, even prolonged incubation of the gut with 12 ng/ml. of atropine diminished the contraction induced by 5-hydroxytryptamine less than one-third (Fig. 8, *a* and *e*). After the same dose of atropine, the contraction induced by toxin was considerably reduced in height (Fig. 8, *h*), while 5-hydroxytryptamine, applied 10 min later, evoked a response

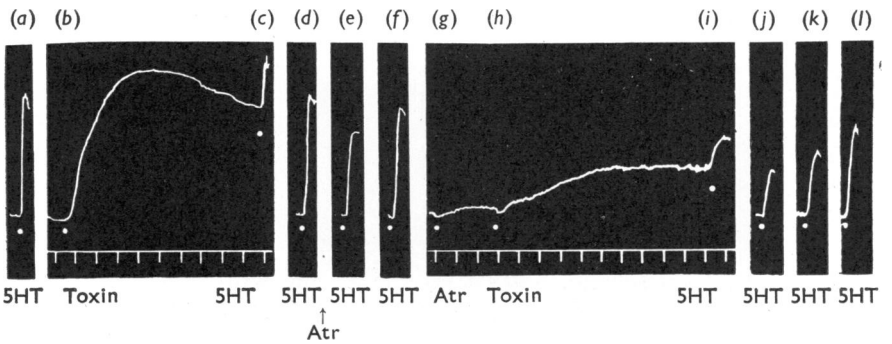

Fig. 8. Influence of atropine (Atr) on the actions of ichthyotoxin and of 5-hydroxytryptamine (5HT) on the guinea-pig ileum preparation. 5-Hydroxytryptamine was added in a concentration of 0.4 μg/ml., and toxin, 160 ng/ml.; time in minutes; organ-bath volume of 50 ml. (*a*), control response to 5-hydroxytryptamine; (*b*), toxin; (*c*), after 10 min without washing, 5-hydroxytryptamine, with a 60% reduction of the height of contraction; (*d*), after twenty washings (during 20 min), restoration of response to 5-hydroxytryptamine; (*e*), 5-hydroxytryptamine, after 12 ng/ml. of atropine, gives a 30% reduction of amplitude of contraction; (*f*), recovery of response to 5-hydroxytryptamine after three washings; (*g*) atropine; (*h*), after 3 min without washing, toxin, with a 70% reduction in the height of the contraction induced by toxin; (*i*), 10 min later without washing, 5-hydroxytryptamine shows a 73% inhibition of contraction;] (*j*) to (*l*), gradual recovery of the response to 5-hydroxytryptamine after repeated washings.

(Fig. 8, *i*) only about one-quarter of the control. The sensitivity to 5-hydroxytryptamine returned gradually after repeated washings (Fig. 8, *j* to *l*).

Morphine, in a concentration of 20 to 60 ng/ml., reduced the height of the contraction induced by toxin by about half (Fig. 9, *b* and *f*), but had little effect on the contractions caused by acetylcholine (Fig. 9, *c* and *g*).

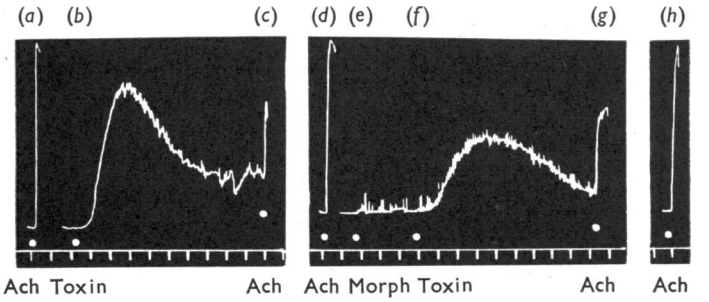

Fig. 9. The effect of ichthyotoxin on the guinea-pig intestine was modified by morphine (Morph). Acetylcholine (Ach) was added in a concentration of 4 ng/ml., and toxin, 50 ng/ml.; between records, three washings; time in minutes; organ-bath volume of 50 ml. (*a*), control response to acetylcholine; (*b*), toxin; (*c*) after 10 min incubation without washing, acetylcholine, with a 60% reduction of the height of contraction; (*d*) the response to acetylcholine was restored by repeated washing; (*e*), 60 ng/ml. of morphine; (*f*), 2 min later, toxin, with the height of the contraction induced by toxin decreased by about half; (*g*) the response to acetylcholine was similar to that in (*c*) in the absence of morphine; (*h*), recovery of sensitivity to acetylcholine after repeated washing.

The antagonistic effect of the toxin against various smooth muscle stimulants was abolished by 60 min of exposure to light, while destruction of the contractile action of the toxin required illumination for 4 hr. Similarly, incubation of a toxin solution at 20° C for 24 hr eliminated the antiacetylcholine activity, while the contractile effect of the toxin on the gut remained. The latter action was destroyed by heating to 50° C for 10 min. Fig. 10 shows the responses to three applications

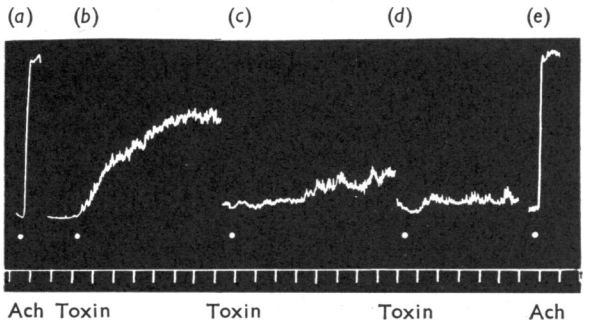

Fig. 10. Decline of contractions of the guinea-pig ileum upon successive applications of ichthyotoxin. The toxin was incubated at 20° C for 24 hr, a procedure which abolishes the antiacetylcholine effect. Acetylcholine (Ach) was added in a concentration of 4 ng/ml., and toxin, 100 ng/ml.; between applications, three washings; time in minutes; organ-bath volume of 50 ml. (*a*) and (*e*), acetylcholine; (*b*), (*c*) and (*d*), repeated applications of toxin. Note that while the contractions induced by the partly inactivated toxin declined in height on each successive occasion, the final response to acetylcholine was undiminished. Compare with Fig. 1, *a* and *d*.

of a toxin which had lost its antiacetylcholine effect after thermal treatment, yet retained its ability to contract the gut. The amplitude of the successive contractions became gradually smaller.

The action of ichthyotoxin on the rat uterus preparation. Even after exposure for 30 min to concentrations of toxin which were 100-times higher than those required to block completely the response of the gut to acetylcholine, no reduction in spontaneous movement or in the responses to acetylcholine or to oxytocin was encountered, whether uteri from pregnant or nonpregnant rats were used.

DISCUSSION

In a recent study of nitroparaffins, Bergmann, Chaimovitz & Wind (1962) have shown that a single substance may affect the function of the ileum in a dual way. Short-chain nitroparaffins evoke first contraction of the intestinal muscle by release of the physiological transmitter. Shortly afterwards, when the gut has relaxed, they block the action of smooth muscle stimulants.

The active principle of the ichthyotoxin of *Prymnesium parvum* probably has a high molecular weight for it does not pass through a cellophane membrane (Yariv, 1958). Therefore it is not surprising that its actions on the gut are much slower than those of the nitroparaffins. Thus the contraction of the intestine induced by toxin develops sluggishly. Likewise, the antiacetylcholine effect reaches its maximum only after 5 min of incubation. The present experiments show that these two activities of the toxin exhibit different sensitivities towards illumination and heating.

Various interpretations of the unequal rates of inactivation may be proposed. For example, the antagonistic actions of the toxin against smooth muscle stimulants might be due to more labile groups in a single molecule, which are not essential for the contractile effect. Parnas *et al.* (1962) have shown that the spectral changes in a toxin solution during photoinactivation come to an end within about 90 min. This period is more than adequate to destroy the antiacetylcholine activity of the toxin completely, but leaves part of the excitatory effect, responsible for contraction of the gut, intact. The latter effect is abolished only by much longer illumination.

On the other hand, the crude extract of *Prymnesium* may contain several active principles with diverse biological effects. Preliminary experiments indicated that the piscicidal activity disappeared at about the same rate as the contracting effect on guinea-pig intestinal muscle. Further work is necessary before it can be decided whether the different actions of the ichthyotoxin are due to a single substance or to a mixture of substances.

Current bioassay methods require a few milligrams of purified toxin, quantities which are procured only with considerable effort. Information on the homogeneity of the toxin would help us to decide whether tests with the guinea-pig ileum, which need only a few micrograms of toxin, could be used instead. Separation of the gut-contracting and the antiacetylcholine activities would also permit a fuller understanding of the complex mechanism of the poisonous action of the ichthyotoxin.

It is noteworthy that the toxin has no effect on the rat uterus, whether taken from a pregnant or nonpregnant animal. Either the toxin cannot penetrate to an

appropriate site of action or the contractile mechanism of the rat uterus differs sufficiently from that of guinea-pig ileum to make the former tissue insusceptible to the toxin effect. Our results do not permit us to distinguish between these two possibilities.

This paper is dedicated to Professor O. Krayer, Department of Pharmacology, Harvard Medical School, Boston, Mass., U.S.A., on the occasion of his 65th birthday. The authors thank Mr R. Knafo for preparation of the illustrations. The work forms part of that undertaken by I. Parnas in the Faculty of Science for a Ph.D. of the Hebrew University, Jerusalem, 1963.

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