FACILITATION IN SEA ANEMONES

II. TESTS ON EXTRACTS

By D. M. ROSS,* *From the Zoological Laboratory, Cambridge*

(*Received* 23 *May* 1945)

(With Four Text-figures)

INTRODUCTION

In previous papers (Ross & Pantin, 1940; Ross, 1945), the view was advanced that a chemical substance, the 'facilitator', is released at the nerve endings in certain muscles in the sea anemone, *Calliactis parasitica*, and carries out the process of neuromuscular facilitation described by Pantin (1935*a*, *b*, *c*, *d*). The object of the experiments described in this paper was to find out if a substance exists in these animals possessing the properties of the hypothetical 'facilitator'.

The experiments are of two types: (1) attempts to detect a substance with facilitating properties in the fluid bathing stimulated anemones; (2) attempts to detect a substance with facilitating properties in extracts of anemones.

TESTS ON SEA WATER BATHING STIMULATED ANEMONES

A number of experiments of the first type were carried out on *Calliactis parasitica* and *Metridium senile*. The procedure was to maintain a number of anemones in a state of continual activity over a period and test the effect of the sea water in which the active animals were bathed on a fresh anemone.

In a typical experiment six anemones were placed in a jar containing about 400 c.c. of sea water and made to contract repeatedly by mechanical stimulation for 12 hr. A seventh anemone was placed in the jar at the same time and stimulated electrically about once every half hour and its response recorded on a smoked drum. No change was observed in the response of this test animal; its size and form remained perfectly normal and there was no tendency to respond to single stimuli. The same result was obtained when the sea water was tested on another animal in another jar not containing the stimulated animals.

All experiments of this type, involving tests on the sea water in which a number of anemones had been stimulated for varying periods, gave completely negative results. It can be concluded, therefore, that if a 'facilitator' does exist, it is not liberated into the surrounding sea water under the conditions of these experiments in sufficient quantities to affect another animal.

TESTS ON EXTRACTS OF SEA ANEMONES

In the preparation of tissue extracts for pharmacological tests of this kind a number of factors need to be considered. Most substances with neuromuscular effects, e.g. acetylcholine and adrenaline, are extremely unstable and the risk of destruction during the extraction is very great. Therefore, drastic methods must be avoided and temperatures kept as low as possible. It is also necessary for the extract to be as natural as possible and free from foreign substances which adversely affect the animal on which the effect of the extract is to be observed.

After a number of trials ethyl alcohol was found to be the most satisfactory agent for preparing anemone extracts. Attempts to use the trichloracetic and phosphotungstic acid methods were not successful as such extracts had strong depressant effects on the test animals. With alcohol the precipitation of the protein is practically complete and the alcohol can be removed afterwards at low temperatures by distillation *in vacuo*. Moreover, tests showed that small quantities of alcohol (up to 2%) in the sea water have no effect on the anemones, so that any traces that remain will not affect the result. An account of the method follows.

Ten large sea anemones (*Calliactis parasitica* or *Metridium senile*) were ground up in a mincing machine and extracted immediately in 300–400 c.c. cold ethyl alcohol (95%). After standing for 24 hr. or more in the cold, the solid matter, consisting of the insoluble parts of the tissues and the precipitated protein, was removed by filtering. The alcoholic solution was then distilled *in vacuo* until the alcohol had been removed. This caused certain constituents of the extract such as lipoids to settle out and these were removed by centrifuging or filtering. The fluid that is left is then a water extract, usually about 50 c.c. in volume, containing only those substances that are soluble both in water and alcohol.

* 1851 Exhibition Scholar from Canada, 1937–40.

Many extracts of *Calliactis* and *Metridium* were prepared by the alcohol method. The action of these extracts was tested on both these animals at dilutions varying from 1 : 10 to 1 : 500 parts of extract in sea water. As in the experiments with drugs, the responses of the test animals were recorded at the beginning of each experiment. Then a quantity of the extract was run into the sea water from a pipette and mixed well. After that the response to stimulation and the behaviour of the animal were observed closely. The methods for stimulating the test animal and recording the responses were the same as those used in earlier work on this subject. Stimuli were provided by condenser discharges delivered through Ag/AgCl electrodes and the contractions of the sphincter of *Calliactis* or the longitudinal mesenteric muscles of *Metridium* were recorded on a smoked drum.

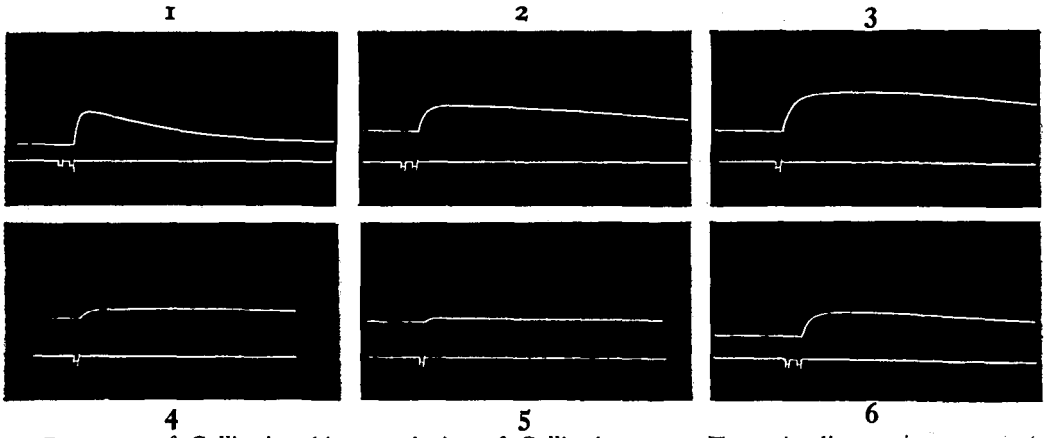

Fig. 1. Responses of *Calliactis* sphincter. Action of *Calliactis* extract. Two stimuli at 1 in 0·5 sec. (1) in natural sea water, (2) after 4 min. in extract (1 part in 100 parts sea water). One stimulus (3) after 5 min., (4) after 9 min., and (5) after 10 min. in extract; two stimuli (6) at 1 in 0·5 sec. after 17 min. in extract. (Note reappearance of response on second stimulus.)

The most definite and consistent property of the anemone extracts is the ability to cause responses to single stimuli. These appeared in a large number of experiments soon after the extract was introduced. About fifty tests were carried out in all and responses to single stimuli were observed in about twenty-five of these. Figs. 1–3 show some of the contractions obtained from *Calliactis* sphincter and the mesenteric retractor muscles of *Metridium* in these tests. It can be seen that the size of the responses to single stimuli varies considerably. Sometimes they are very small, but at times they equal and even exceed the size of the normal response to two stimuli close together.

There can be no doubt that these are truly responses to single impulses and not additional responses due to 'after-discharge' (Pantin, 1935*c*), i.e. the setting up of more than one impulse by one stimulus. If 'after-discharge' were the cause of the effect, a series of stimuli would be expected to give rise to many additional contractions. However, the rule that each effective stimulus causes a single response continues to operate even when the responses to single stimuli are most in evidence. Furthermore, a close examination of the records shows that the latent period between the application of the stimulus and the beginning of the contraction is the same in these responses as in the normal response to the second stimulus. This would not happen if the animal were responding to an additional impulse arriving some time after the first.

When the extract is first introduced into the sea water both *Calliactis* and *Metridium* usually respond with a quick contraction. This contraction only lasts for a short time, less than 1 min. as a rule, and when relaxation is complete the effect of the extract on the recorded response of the anemone can be observed without further contractions of this kind. It is unlikely that this spontaneous contraction is due to an effect by the extract on the muscles themselves. It begins with a twitching of the tentacles which grows in intensity until closure of the animal takes place. This is the usual form of the response to peripheral stimulation. Moreover, if the extract were acting directly on the muscles so as to cause this contraction, the effect would not be expected to be of such short duration or its recovery so complete.

Responses to single stimuli may occur at any time between 1 and 15 min. after the introduction of the extract. Unlike the single shock responses caused by the effective drugs, this effect is seldom preceded by a period of enhanced responses. But like the drug effects, the responses to single stimuli occur suddenly; there is no transition from small to larger responses as the effect develops. The responses to single stimuli soon disappear. Usually after a few minutes the response occurs only after two stimuli as in the untreated animal. After that the extract exerts a depressant action and the response becomes steadily slower and smaller as it does with certain ions and drugs.

Fig. 1 shows the effect of *Calliactis* extract on the response of *Calliactis* at a concentration of 1 part extract in 100 parts sea water. In this experiment the response was unchanged after 4 min. but after 5, 9 and 10 min. responses to single stimuli were recorded. The effect gradually disappeared, the responses to single stimuli becoming smaller and smaller until they vanished altogether after 17 min. This concentration (1 : 100) of *Calliactis* extract proved most effective in causing the effect. Stronger solutions (1 : 10, 1 : 25, 1 : 50) all caused responses to single stimuli, but these were less than half the size of the response of the untreated animal to two stimuli at 1 sec. intervals. At the highest concentrations the extract had marked depressant effects which almost abolished the response after 1 hr., and to a single stimulus 2 min. after the extract was introduced (Fig. 2 B), but the effect only lasted for 3 min.

Extracts of *Metridium* are equally effective in causing responses to single stimuli. Fig. 3 shows the effects of *Metridium* extracts at concentrations of 1 : 50 and 1 : 250. The stronger dose caused the effect within 2 min., but it only lasted 2 min. before it disappeared. At a dilution of 1 : 250 the responses to single stimuli lasted much longer, and although the normal response to the second stimulus predominated after 20 min., single stimuli caused occasional responses up to 50 min. after the extract was introduced.

Apart from the depressant effects which set in after long exposure, the responses to single stimuli are the only consistent effects of the extracts. In

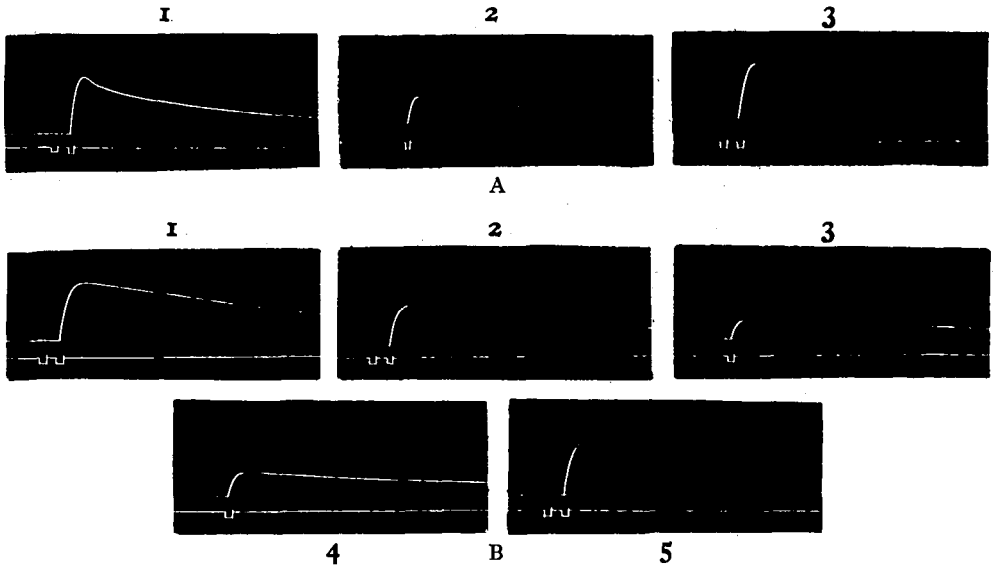

Fig. 2. Responses of mesenteric retractor system of *Metridium*. A. Action of *Calliactis* extract (1 part extract in 25 parts sea water). (1) Two stimuli at 1 in 1 sec. in natural sea water; (2) one stimulus after 1 min. in extract; (3) two stimuli at 1 in 1 sec. after 3 min. in extract. B. Action of *Calliactis* extract (1 part extract in 50 parts sea water). Two stimuli at 1 in 1 sec. (1) in natural sea water, and (2) after 1 min. in extract; one stimulus (3) after 2 min. in extract, (4) after 3 min. in extract; (5) two stimuli at 1 in 1 sec. after 5 min. in extract.

this may account for the poor effects obtained. At dilutions above 1 : 100 the effect becomes more and more difficult to demonstrate and it could not be obtained at dilutions of 1 : 500 and over.

Metridium proved to be a more satisfactory test animal than *Calliactis*. The responses to single stimuli appear more rapidly and as a rule are larger than those obtained from *Calliactis*. Possibly this is because the active substance in the extract can reach the mesenteric muscles of *Metridium* more easily than the sphincter of *Calliactis*. Fig. 2 A shows the effect of a *Calliactis* extract diluted 25 times in sea water on *Metridium*. A response to a single stimulus occurred 1 min. after the extract was introduced, but 2 min. later the animal reverted to normal again. At a dilution of 1 : 50 *Metridium* responded occasional experiments, however, the response was enhanced considerably. This only happened in three out of fifty tests and cannot be regarded as an essential feature of the extract effect. An example of enhancement caused by an extract is shown in Fig. 4. In this case the size of the response was more than doubled by *Calliactis* extract at a dilution of 1 : 20. Unlike the enhancement observed with certain drugs, this effect did not precede but followed the responses to single stimuli, occurring 30 min. after the experiment began. The absence of the marked enhancing action, which was a consistent feature of the effects of those drugs causing responses to single stimuli, suggests that the action of the extract is fundamentally different from the action of the drugs.

e records that have been described show that a substance capable of causing responses to single stimuli exists in sea anemones. It does not follow from these experiments, however, that this substance is the hypothetical 'facilitator', whose existence was postulated in preceding papers. There is no evidence so far which associates the active substance in the extracts with the excitation which should cause the 'facilitator' to be released. All the extracts were prepared from animals which were chopped up into small pieces, a process which must excite what remains of the neuromuscular system intensely. But it is a considerable advance to have demonstrated the existence of a substance with this property in the anemone extracts and opens the way for further investigation.

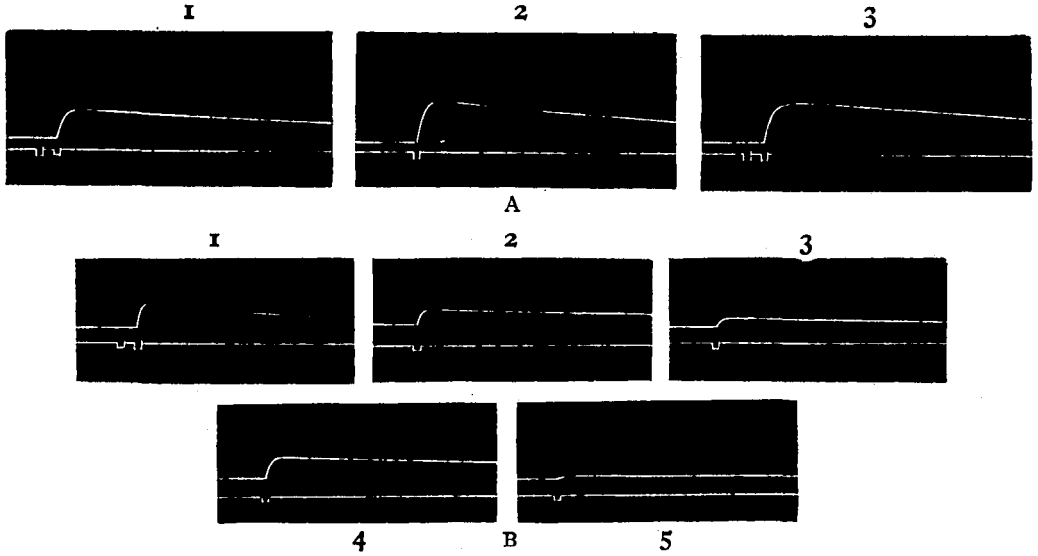

Fig. 3. Responses of mesenteric retractor system of *Metridium*. A. Action of *Metridium* extract (one part in 50 parts sea water). (1) Two stimuli at 1 in 1 sec. in natural sea water; (2) one stimulus after 2 min. in extract; (3) two stimuli after 4 min. in extract. B. Action of *Metridium* extract (1 part in 250 parts sea water). (1) two stimuli at 1 in 1 sec. in natural sea water; one stimulus (2) after 5 min., (3) after 20 min., (4) after 40 min. and (5) after 50 min. in extract.

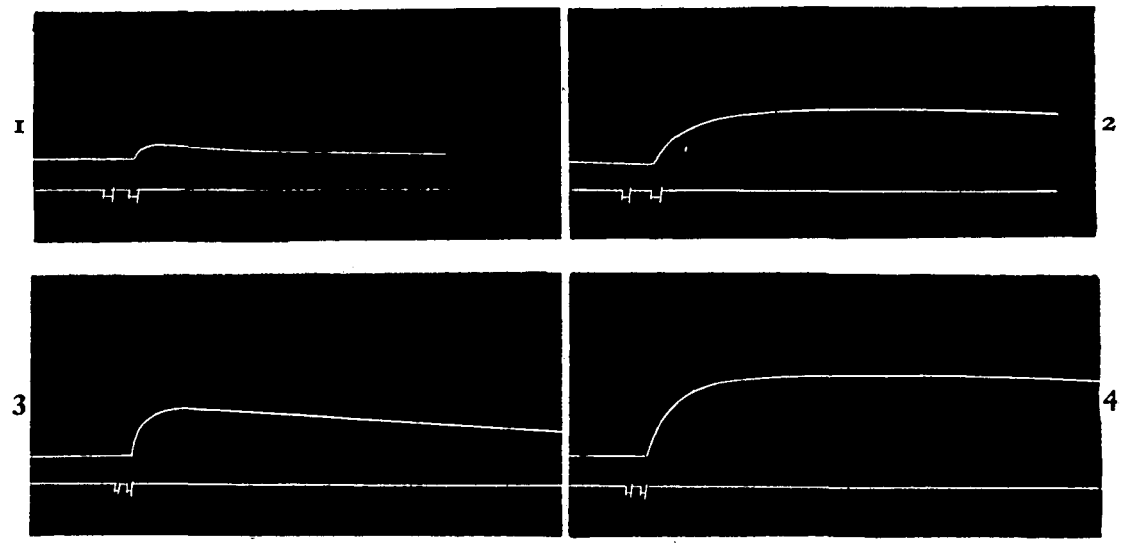

Fig. 4. Responses of *Calliactis* sphincter. Increase in size of response with extract of *Calliactis* (1 part extract in 20 parts sea water). Two stimuli at 1 in 1 sec. (1) in natural sea water, (2) after 34 min. in extract; two stimuli at 1 in 0·5 sec. (3) in natural sea water, (4) after 41 min. in extract.

DISCUSSION

The hypothesis was advanced in previous papers that there are two processes in neuromuscular transmission in sea anemones: (1) a process of sensitization or facilitation, carried out by a 'facilitator' released at the nerve endings; (2) a process of excitation of the muscle which can only occur when facilitation has been set up. Up to a certain point, the action of drugs and ions agreed closely with the predictions of this hypothesis. Under the appropriate conditions they may enhance the size of the responses and sensitize the muscles to respond to single stimuli, whilst they never directly cause contractions of the muscle (Ross & Pantin, 1940; Ross, 1945).

In this paper an attempt is made to determine whether a substance possessing facilitating properties is actually produced in the anemone itself. The experiments show that a substance can be extracted, especially from *Metridium* tissues, which possesses some of the properties of a 'facilitator'. In particular, the presence of extract permits the appearance of a response to a single stimulus and, unlike the corresponding effect with some drugs, it exerts this effect with regularity. It also acts at great dilution, 1 part extract in 250 parts sea water giving a significant response, and acts without much delay, usually after about 5 min., but ranging from 1 to 15 min. in different experiments. These latter features of the extract effect do not correspond with the effects of the drugs, tyramine, tryptamine and 933 F, which caused responses to single stimuli only at relatively high concentrations and after 2–3 hr. It is likely, therefore, that the sensitizing effects of the extract and drugs are due to different substances and they may reflect different modes of action altogether.

The extracts, like the drugs, fail to exhibit all the effects that would be expected of a facilitating substance. The presence of the extract does not delay the decay of facilitation in the expected manner. This point was tested in the same way as it was with tyramine and 933 F. Records were taken of the time required for the facilitating effect of a stimulus to die away in the period before and after responses to single stimuli occurred. In no case did this time exceed the limit of 3 sec. which applies to an anemone in sea water at ordinary temperatures. A parallel failure was found in the action of drugs (Ross, 1945). The extract fails in another respect as well. Unlike the effective drugs, it has no marked effect on the size of the response. Only three out of fifty tests showed any increase in the size of the response and in these cases the increase is not great (Fig. 4), when compared with the enhancement caused by drugs.

We are left, therefore, in the paradoxical situation that both drugs and extract produce some of the effects expected of a facilitating substance, but they neither produce all the expected effects nor do they fail or succeed in the same way. Nevertheless, even though it is not yet possible to account for all the facts, the evidence is clearly against transmission being due to the direct action of some chemical substance on the muscles, and there are sufficient points of agreement to indicate a chemical link in the facilitation process.

SUMMARY

1. Extracts of the sea anemones, *Calliactis parasitica* and *Metridium senile*, have been prepared and their effects on the neuromuscular response of these anemones tested. The presence of extract sensitizes the organism so that a response is given to a single stimulus, whereas normally this occurs only on the second and subsequent stimuli. No other significant effects were observed.

2. The sensitizing effect of the extract differs from the effect of a sensitizing drug like tyramine; it appears more quickly, more regularly, and it is rarely accompanied by an increase in the size of the muscular contraction.

3. The fact that a substance with sensitizing properties has been detected in anemones supports the view that a 'sensitizer 'or 'facilitator' exists and takes part in neuromuscular transmission in these animals. Nevertheless, the extract, like the sensitizing drugs, lacks some of the properties that would be expected of a true 'sensitizer' or 'facilitator'.

The author wishes to make the following acknowledgements: to Dr C. F. A. Pantin, F.R.S., under whose direction the work was carried out; to Prof. J. Gray, F.R.S., for use of the facilities at the Zoological Laboratory, Cambridge; to the late Dr Stanley Kemp, F.R.S., for the use of the facilities at the Marine Biological Laboratory, Plymouth; to Dr F. C. MacIntosh, of the National Institute of Medical Research, for advice on methods.

REFERENCES

PANTIN, C. F. A. (1935*a*). *J. Exp. Biol.* **12**, 119.
PANTIN, C. F. A. (1935*b*). *J. Exp. Biol.* **12**, 139.
PANTIN, C. F. A. (1935*c*). *J. Exp. Biol.* **12**, 156.
PANTIN, C. F. A. (1935*d*). *J. Exp. Biol.* **12**, 389.
ROSS, D. M. (1945). *J. Exp. Biol.* **22**, 21.
ROSS, D. M. & PANTIN, C. F. A. (1940). *J. Exp. Biol.* **17**, 389.