

## ACETYLCHOLINE RELEASE EVOKED BY SINGLE OR A FEW NERVE IMPULSES IN THE ELECTRIC ORGAN OF *TORPEDO*

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### SUMMARY

1. The acetylcholine (ACh) store in the *Torpedo* electric organ was partially labelled with choline and acetate at the same molar concentration but with different isotopes. Under these conditions the two precursors were incorporated into ACh in a ratio 1 to 1.

2. After a single electrical stimulus, or a brief burst of stimuli, the compound electroplaque potential (e.p.p.) was recorded and the radioactive choline and/or acetate counted in the perfusion fluid, providing a sensitive assay for ACh release in the absence of anticholinesterase drugs.

3. The so-called depression of transmission was found to be due to progressive impairment of ACh release in the successive impulses evoked by repeated stimuli.

4. In a pair of impulses separated by 50 ms interval, less ACh was released by the second than by the first impulse; this explained why the size of the second e.p.p. was depressed, using a direct measurement of ACh.

5. In repetitive stimulations of longer duration, the maximum rate of release declined as the activity was prolonged. Thus the tissue progressively lost its ability to ensure release at high frequencies.

6. An unexpected finding was that anticholinesterases like eserine or pre-treatment with fluostigmine (DFP) greatly reduced ACh release even by a single impulse.

7. Evoked ACh release and e.p.p. amplitude were both maximum between 10 and 20 °C. At higher temperatures, the evoked release decreased as the spontaneous release increased.

8. Changes in external  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  produced similar changes in the e.p.p. and evoked ACh release. The dose-response curve for Ca dependency of ACh release was very steep with a Hill's coefficient of 3.2.

9. With a single stimulus in the presence of 4-aminopyridine, there was a dramatic enlargement of the e.p.p. and a still larger potentiation of the evoked ACh release.

10. It has been possible with this approach to avoid the inconveniences often encountered in similar studies, i.e. repetitive stimulation, low Ca solutions and cholinesterase inhibition. This permitted a good correlation between electrophysiological and biochemical estimates of transmitter release even by a single nerve impulse.

## INTRODUCTION

Direct measurements of acetylcholine (ACh) release are by necessity performed in the presence of inhibitors of cholinesterase. The ACh released at synapses is then analysed in the medium perfusing the tissue, following repetitive stimulation of the afferent nerve. On the other hand, there is an indirect way to analyse ACh release. It is to record with electrophysiological techniques the potentials generated by ACh at the post-synaptic membrane.

However, it has proven difficult to correlate the results of biochemical and biophysical experiments for several reasons. First, the anticholinesterase drugs used in the former biochemical experiments are expected to modify the dynamics of ACh metabolism since they inhibit the hydrolysis of the transmitter and consequently inhibit the recycling of choline and acetate. Moreover, repetitive stimulation by itself brings the synapse into a non-equilibrium state, making it difficult to determine whether changes occur due to a direct alteration of the process of release or to some indirect modification of the synaptic kinetics. Finally, the most refined electrophysiological experiments have been done under conditions in which the amount of ACh released was too small to be analysed by direct methods.

We have tried to overcome these difficulties by comparing the amount of ACh released in parallel with the electrophysiological events with only one or a few nerve impulses. The experiments were performed with the electric organ of the fish *Torpedo marmorata*. This tissue has a profuse, purely cholinergic innervation (Feldberg, Fessard & Nachmansohn, 1940; Feldberg & Fessard, 1942). The electric organ is composed of a large number of prisms which are arranged side by side; each prism consists of approximately 500 superposed electroplaques which are embryologically derived from muscle cells. However, unlike normal muscle fibres, the electroplaques are totally devoid of contractile material and are not able to generate propagated and regenerative action potentials. This has been demonstrated by the voltage to current relationships which are linear in both the resting and the active states (Bennet *et al.* 1961; Dunant, 1976). Thus the electrical discharge of the *Torpedo* is the summation of the uncomplicated 'end-plate' potentials (e.p.p.s) generated by each electroplaque. This is of course a favourable condition to analyse the electrophysiological correlates of ACh release.

The direct measurement of ACh release in this organ is facilitated since both external acetate and choline are utilized to the same extent for the synthesis of ACh (Israël & Tuček, 1974). Thanks to this property, it has been possible to incorporate two precursors labelled with different radioisotopes into ACh. On stimulation, a part of the released choline and acetate escaped in the perfusing medium and allowed a sensitive assay for ACh release in the absence of any anticholinesterase drug.

Some of the results published here have been presented as short notes (Eder, Hirt & Dunant, 1976; Dunant & Eder, 1977).

## METHODS

*Materials*

The fish *Torpedo marmorata* were supplied by the Station de Biologie Marine, Arcachon, France. Most of them were females of 30–60 cm length. They were kept alive in sea water at

about 15°C. The radioactive substances were obtained from the Radiochemical Centre, Amer-sham, U.K. They were [ $1-^{14}\text{C}$ ]acetate, specific radioactivity 60.2 mCi/mmol; [ $^3\text{H}$ ]acetate, 5 Ci/mmol; [methyl- $^{14}\text{C}$ ]choline chloride, 52 mCi/mmol; [methyl- $^3\text{H}$ ]choline chloride, 6.4 Ci/mmol; [ $^3\text{H}$ ]acetylcholine, 250 mCi/mmol. Fluostigmine (DFP, diisopropylfluorophosphate) was obtained from Fluka, Switzerland and acetylcholine perchlorate, physostigmine (eserine) and 4-amino-pyridine from Merck, Germany.

#### *Preparation and labelling of the tissue*

The *Torpedo* was anaesthetized by tricaine methane sulphonate (MS 222, Sandoz, Switzerland) at a concentration of 1 g/3 l sea water. Controls showed that this brief anaesthesia is rapidly reversible and has no effect on nerve-electroplaque transmission. Slices of electric organ were excised and small fragments of tissue composed of one or two intact prisms of electroplaques were carefully dissected. In some cases, they were dissected with their afferent nerve branch to allow stimulation via the nerve. All the fragments used in a given experiment were approximately the same size, weighing 200–400 mg. They were kept in an elasmobranch physiological saline medium containing (mM): NaCl, 280; KCl, 7; CaCl<sub>2</sub>, 4.4; MgCl<sub>2</sub>, 1.3; NaHCO<sub>3</sub>, 5; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; urea, 300 and glucose 5.5. This medium was oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>; its pH was kept between 7.1 and 7.4. It was shown by electrophysiological, morphological and biochemical controls that the properties of the tissue were not altered by incubation in this saline for more than 40 h. Unless mentioned, the experiments were done at room temperature.

The tissue ACh was labelled by adding the two radioactive precursors to the saline medium. In a typical experiment we used [ $^3\text{H}$ ]choline (5  $\mu\text{Ci/ml}$ ) and [ $^{14}\text{C}$ ]acetate (0.5  $\mu\text{Ci/ml}$ ) at the same molar concentration (8.4  $\mu\text{M}$ ). In other experiments we have used the converse condition, that is [ $^3\text{H}$ ]acetate and [ $^{14}\text{C}$ ]choline. When the aim of the experiment was just to compare the amount of transmitter released under normal and modified conditions, only one radioactive precursor was used. The incubation with the radioactive precursors lasted for 4–6 h and the medium was stirred by continuous bubbling. In typical experiments, sixteen to twenty fragments (total weight 3–5 g) were incubated in 80 ml. Only a small part of the ACh content was labelled under this condition (see Table 1). The labelling could have been improved by electrical stimulation during this period but this was not done because of the fatigability of the tissue and also since the homogeneity of the labelling seemed better at rest. After the incubation, the fragments were washed for three successive 20 min intervals in 100 ml of the saline in the absence of the radioactive precursors. They were then kept overnight in a large volume of saline (300 ml) at 7°C to reduce the rate of ACh metabolism.

#### *Superfusion, stimulation and electrical recording*

The labelled fragments of *Torpedo* electric organ were gently placed on a small piece of nylon cloth between two stimulating platinum electrodes which were situated parallel to the prisms. The recording electrodes were made of stainless-steel wires inserted at the dorsal and ventral ends of the prism. The tissue was superfused by a regular and continuous flow of the saline medium. A 'field' stimulus of 60–110 V and 0.5–0.8 ms was applied to the tissue, resulting in a well synchronized compound e.p.p. ranging from 0.2 to several volts according to the size of the fragment. This response was always separated from the stimulus artifact by a latency of 2–3 ms (Fessard, 1947). In a few control experiments, the prisms were stimulated via their afferent nerve; the stimulating electrodes were a pair of Ag-AgCl<sub>2</sub> electrodes on which the nerve branch lay transversally. The intensity and duration of the stimulus needed for maximal response were much less when the nerve trunk was stimulated. The electrophysiological response was similar except for the latency which was greater due to the conduction delay in the nerve.

After the initial test of the electrophysiological response, the prisms were left at rest under continuous superfusion with the saline for 90 min. This time was sufficient to allow complete exchange of the extracellular space by diffusion, even for rather thick prisms (0.4–0.7 cm in diameter) excised from large *Torpedoes* (see Dunant, Gautron, Israël, Lesbats & Manaranche, 1972). When the effects of drugs were to be tested, they were added to the saline medium during that time, except for fluostigmine (DFP) or other irreversible drugs which were added before the tissue was mounted on the electrodes and the excess drug was washed out later.

At the time required, a single stimulus (or a brief burst of stimuli) was applied to the prisms;

the e.p.p. was recorded on an oscilloscope and photographed. Samples of the saline medium which superfused the tissue were taken before and after the stimulation. The radioactivities in these samples were then counted by liquid scintillation spectrography.

The experiment was then repeated for several series of four to eight samples. In the successive series the new samples were washed for a slightly longer period of time, but this did not result in a decrease of the radioactivity released. From one series to the next, the places where the samples were submitted to a given condition were changed to avoid any systematic effect of time or position.

#### *Miniature electroplaque potentials*

In one experiment, the frequency of miniature electroplaque potentials (m.e.p.p.s) was analysed as a function of temperature. The m.e.p.p.s of the electric organ of *Torpedo* resemble the classical ones recorded at various neuromuscular junctions (Miledi, Molinoff & Potter, 1971). They are abolished by curare and their time course is prolonged when the cholinesterase is inhibited (Dunant, 1973). Because of the extremely low resistance of the electroplaque membranes (see Bennet *et al.* 1961), the m.e.p.p.s can hardly be recorded intracellularly in the *Torpedo* electric organ. We have done extracellular recording in the following way. A glass micro-electrode of approximately 1 M $\Omega$  filled with 1 M-NaCl or the physiological medium was inserted into a prism placed with the ventral (innervated) surface of electroplaques upward. After a transient and sometimes large occurrence of m.e.p.p.s, the frequency decreased to the low value of about 15/min and remained stable for very long periods of time. This was only an apparent frequency since, even when the m.e.p.p.s with rapid rising times were selected, their amplitude distribution was usually skewed toward the background and it was not known how many could have been lost in the noise. The temperature was recorded in the preparation with a thermistor and when the steady state was obtained the temperature was slowly changed and the apparent frequency of the m.e.p.p.s measured.

#### *Extraction and measurement of ACh*

In a few experiments the radioactive and non-radioactive ACh of the tissue was compared to the released transmitter. The tissue fragment to be analysed was left for 1 h in 4 ml of trichloroacetic acid (TCA) at 5% (w/v). After homogenization and centrifugation, the TCA was extracted by water-saturated ether from the supernatant. For the measurement of radioactive ACh, a sample was chromatographed on high-voltage electrophoresis (4500 V, 70–100 mA for 20 min) in formic acid–acetic acid buffer, pH 1.9. The migrated ACh was identified by iodine vapours, eluted in water and counted in Instagel (Packard). The non-radioactive ACh was measured in the same TCA extract by a radiochemical method (Dunant & Hirt, 1976) which also served for testing the activity of acetylcholinesterase. When the extract contained radioactivity, the unlabelled ACh was estimated by the conventional frog rectus bio-assay.

## RESULTS

### *Transmitter released by one, five, ten and twenty stimuli*

Fig. 1 shows a typical experiment in which the relationship between the amount of ACh released and the number of impulses was determined. Several fragments containing one or a few prisms of electroplaques were prepared as indicated in the methods section. They were incubated in [<sup>3</sup>H]acetate, washed overnight and mounted on the recording and stimulating electrodes. After a 90 min period of rest under continuous superfusion with the saline medium, some pieces were stimulated with a single shock and the others with a brief burst of one second duration containing five, ten or twenty stimuli.

As shown in the records of Fig. 1, the successive e.p.p.s recorded in the brief bursts were of decreasing amplitude. This 'fatigue' or depression of the electrical response was more pronounced at the higher stimulation frequencies.

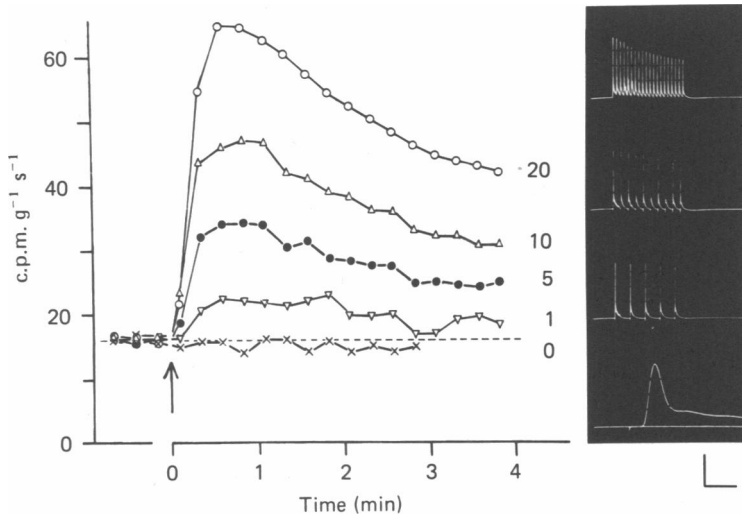


Fig. 1. Overflow of radioactive transmitter and e.p.p.s from excised prisms of the electric organ of *Torpedo marmorata*. The curves are mean values from two different prisms, showing the radioactivity counted in the perfusion medium in absence of any electrical stimulation (X), in response to a single impulse ( $\nabla$ ) or to a short burst containing 5 ( $\bullet$ ), 10 ( $\Delta$ ) or 20 ( $\circ$ ) impulses in 1 s. Arrow indicates the time of stimulation. Labelling by [ $^3\text{H}$ ]acetate, 2  $\mu\text{Ci/ml}$ . In the electrophysiological records, the compound e.p.p.s generated by the same prisms are shown with calibration bars of 2 V and 4 ms for the single response, and of 2 V and 400 ms for the others. Room temperature.

After stimulation, the radioactivity overflowing in the effluent increased significantly over the background level, even in response to a single impulse. As expected, larger releases were observed when the number of stimuli was increased. However the relationship was not linear but rather hyperbolic indicating that the first impulse released more transmitter than the following ones. The curve of Fig. 2 is consistent with an hyperbola which would show saturation for more than 100 impulses in 1 s and half of this maximum for 18 impulses in 1 s. In conclusion, even in such brief bursts occurring after a long period of rest, the rate of release was limited by a maximum value.

The time course of release showed that the radioactivity increased rapidly after the stimulus but returned to the background level only after several minutes. In the experiment of Fig. 1, the decay of radioactivity could be approximated by two exponentials having time constants of 5.5 and 9.8 min respectively, independently of the number of impulses in the stimulation. The prisms utilized were in the form of cylinders of 25 mm length and 6 mm diameter. They were excised from a fish measuring 53 cm. From one experiment to another, the shape and time constants of the decay curve were often different since they were governed by diffusion of the released radioactivity from the more central region of the prisms whose size varied with that of the fish (see also Dunant *et al.* 1972).

When, after stimulation, the prisms were left at rest for 30–60 min and then stimulated again, the release of radioactivity in the second stimulation was identical to that in the first.

*Inhibition of ACh release by anticholinesterase drugs*

Anticholinesterase substances have been widely used in electrophysiological and still more in biochemical investigations of ACh release. As they were not used in the above experiments, we determined whether these drugs could affect the release of radiolabelled transmitter under our conditions.

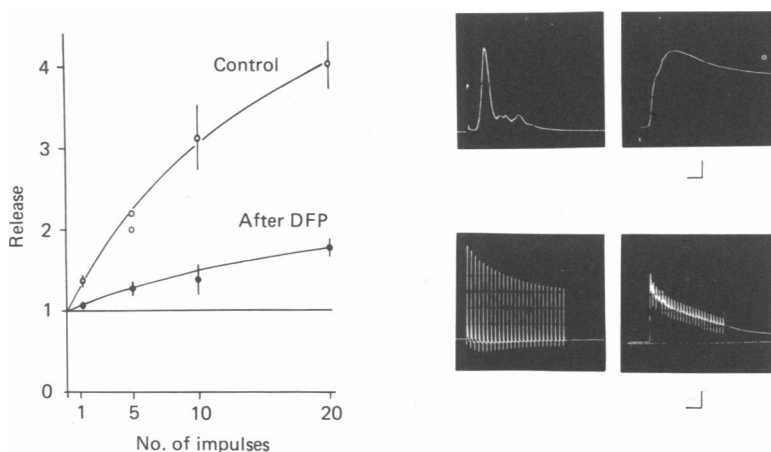


Fig. 2. Effects of cholinesterase inhibition on transmitter release and e.p.p. In the graph, the release of radioactive transmitter measured in different experiments has been expressed as the ratio of the peak of evoked release over the background level. Stimulation, as in Fig. 1 consisted of single impulses, or short bursts of five, ten or twenty impulses in 1 s. The relationship between evoked release and the number of stimuli (○) was not linear but bent towards a maximum rate of release. Pre-treatment with DFP ( $10^{-3}$  M), which irreversibly abolished cholinesterase activity, led to a strong inhibition of evoked ACh release even by a single stimulus (●). The spontaneous release of radioactivity was not modified by the drug. Labelling by [ $^3$ H]acetate. Means  $\pm$  s.e. of two to twenty values. Electrophysiological records are seen on the right, with calibration of 0.5 V and 5 ms for the single responses, and 0.5 V and 200 ms for the 20 stimuli bursts. Pre-treatment with DFP greatly prolonged the duration of e.p.p.s.

In the initial experiments, the tissue was given bursts of 20 impulses in 1 s, in the presence or absence of physostigmine (eserine,  $10^{-4}$  M). Surprisingly, the release was strongly inhibited, reaching only  $27 \pm 5$  % of that measured in the absence of eserine (means  $\pm$  s.e.; three experiments with different animals; in each of them three treated and three control samples). Eserine has been shown to considerably prolong the duration of the electric discharge but reduce the ability of the organ to sustain repetitive activity (Feldberg & Fessard, 1942). This was also observed in our experiments and it was at first thought that, in the presence of eserine, only the first impulse was really effective in releasing ACh.

Further experiments were done to check this by giving fewer impulses. It was also found more convenient to use a pre-treatment with DFP rather than eserine which could interfere with ACh metabolism by some direct manner due to structural analogy. Pre-treatment with DFP offers the advantage that the cholinesterase is inactive but no drug is present at the time of stimulation. Before the prisms were

mounted on the electrodes for stimulation, they were incubated for 1 h in the presence of  $10^{-3}$  M-DFP. The excess drug was washed out during the 90 min period preceding the stimulation. After this treatment, the activity of acetylcholinesterase was reduced to less than 1 %.

The e.p.p.s generated by the DFP-treated tissue in response to a single stimulus had approximately the same amplitude as those of the controls. In contrast, their duration was greatly increased. In response to the 1 s bursts, the individual e.p.p.s could no longer be distinguished from each other; in fact the tissue only produced a 'fused' potential of long duration (Fig. 2, records).

The amount of radiolabelled transmitter was also reduced by DFP pretreatment, even after a single impulse. For each stimulation pattern (one, five, ten or twenty impulses) the release was only 20 % of the control (Fig. 2, graphs). This was observed using [ $^3$ H]acetate or [ $^{14}$ C]choline as precursors and also with the combination of [ $^{14}$ C]acetate and [ $^3$ H]choline. As seen in Table 1 and further in the text, pre-treatment with DFP did not lead to large changes in the tissue ACh measured before stimulation.

It was the initial rate of release which was the most affected by cholinesterase inhibitors. When more prolonged stimulations were given, for example 90 s at 10 Hz, the radioactivity released by treated samples increased slowly but reached 40–50 %

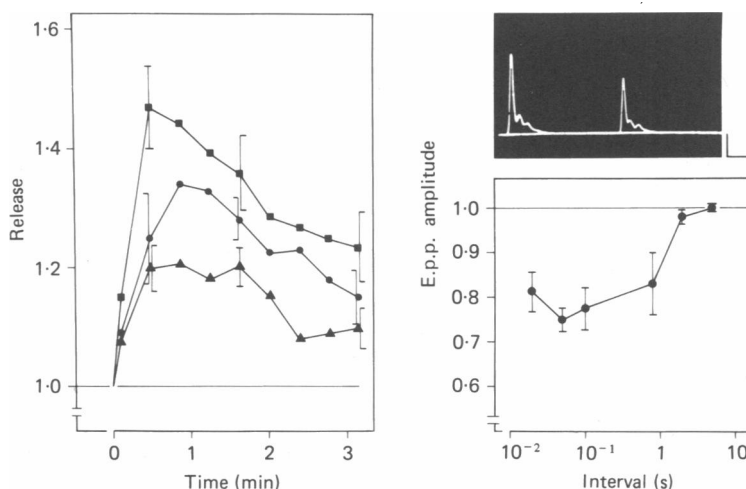


Fig. 3. ACh release and depression of synaptic transmission. A single stimulus (▲) or a pair of stimuli were applied at the time zero, after a long period of rest. The interval between the two stimuli was either 50 ms (●) or 5 s (■). Transmitter release was expressed as in Fig. 2 by the ratio of evoked release over the basal level of radioactivity. The curves are means  $\pm$  s.e. of five samples. At the 5 s interval, transmission had recovered from the depression. The second e.p.p. was identical to the first and the release by a pair of stimuli was not different than 2 times that of a single stimulus. At the 50 ms interval, the second e.p.p. was depressed and the release of radioactivity was significantly smaller than by the 5 s pair. The record shows two paired e.p.p.s with 50 ms interval (0.5 V and 10 ms). In the right hand graph, the amplitude ratio of the second to the first e.p.p. has been plotted as a function of the time interval between them.

of the control values at the end of the stimulation period. Finally, when the nerves were not excited by electrical stimulation but by high KCl concentrations (50 mM), only a slight reduction of release was caused by the presence of eserine or pre-treatment with DFP.

#### *Release by paired impulses*

The depression of transmission in experiments such as that of Fig. 1 was explained by a decline of the amount of ACh released in the successive nerve impulses. This could be more properly analysed by measuring the release due to paired impulses separated by various time intervals.

Fig. 3 shows that, under this condition, the second e.p.p. was smaller in size than the first one. The depression was maximum for an interval of 50 ms. Both the amplitude and area of the second e.p.p. were reduced to  $75 \pm 2.6$  and  $74 \pm 8\%$  respectively (mean  $\pm$  s.e.,  $n = 5$ ). Recovery from this depression was a rather slow process and was completed only after 5 s.

We have thus measured the liberation of labelled transmitter by two shocks separated by an interval of 5 s. As expected from the electrophysiological records, the release was two times larger than that measured after a single stimulus. On the other hand, when the two impulses were separated by only 50 ms, the release was significantly less than by the 5 s pair, and significantly more than by the single impulse (Fig. 3).

In the experiment, five tissue fragments were stimulated for each condition and the radioactivity was counted for nine successive time intervals after stimulation. This gave a total of  $3 \times 45 = 135$  measurements which all were used in the statistical analysis. Two methods were used. In the first, the  $F$  values of the multifactorial analysis demonstrated that the release by the 50 ms pair of stimuli was significantly larger than that by a single impulse ( $0.01 < P < 0.02$ ), and significantly smaller than that by the 5 s pair ( $0.001 < P < 0.002$ ). In the second method, for each point in time, the mean response to the single shock was taken as unity and the other values normalized in consequence. In this way the release by the 50 ms pair was found to be  $1.76 \pm 0.16$  times larger than that by the single stimulus ( $P < 0.001$ ). The release by the 5 s pair was larger than that by the 50 ms pair ( $P < 0.05$ ) and not significantly different from 2 times the release by a single stimulus ( $2.3 \pm 0.2$  times,  $P = 0.19$ ).

Thus a very satisfying accordance was found between the biophysical and the biochemical results. The depression affecting the second e.p.p. corresponded to a reduction in the amount of the radioactivity in the perfusate overflow.

#### *Release by trains of longer duration*

The electric organ of *Torpedo* is not able to sustain repetitive activity for a long time. The amplitude of the e.p.p. diminishes in a characteristic manner in continual stimulations at 5 or 10 Hz. Its decline is not monotone, but interrupted by one or several plateaux before reaching a very low level after approximately 1800 stimuli (Dunant *et al.* 1972, 1974).

The release of ACh was also measured in stimulations of longer duration to determine to what extent these phenomena were explained by a progressive failure



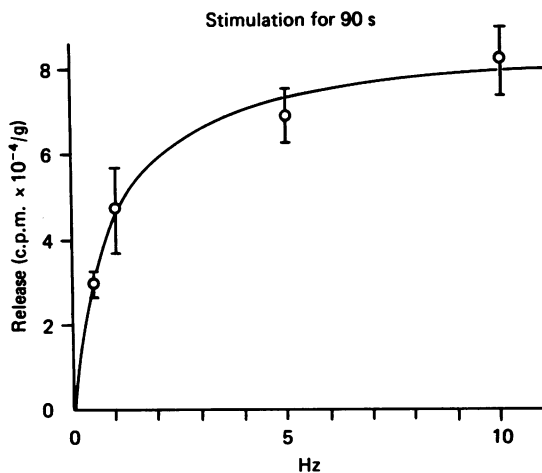


Fig. 4. Transmitter release by the electrogenic tissue, when stimulated at the frequencies of 0.5, 1, 5 and 10 Hz for 90 s. A nearly maximum rate of release was reached with the 10 Hz frequency. The ordinate refers to the total radioactivity released over the background during 3 min (the 90 s stimulation period plus 90 s post-activity). Means  $\pm$  s.e. of four prisms for each condition. Labelling by [ $^3\text{H}$ ]acetate.

of the release process. The prisms were stimulated for 90 s at the frequencies of 0.5, 1, 5 and 10 Hz. As in the experiments of 1 s bursts, more transmitter was released by the higher frequencies. However, the relationship between release and the number of stimuli showed that, in these longer stimulations, a nearly maximum rate of release was already reached with the 10 Hz frequency. Half of this rate corresponded to approximately 1 Hz. These results agreed well with the electrophysiological changes. Indeed the e.p.p. amplitude decreased more rapidly when the frequency was higher.

It is interesting to compare this relationship with the higher frequencies attained in the initial rate of release illustrated in Fig. 2.

#### *Stimulation via the nerve*

A few control experiments were performed to see if stimulation via the nerve gave the same results as the 'field' stimulation technique used in the present work (see Methods). Pieces of tissue containing two to six prisms were carefully dissected with their nerve intact. They were labelled with the precursors together with a number of other prisms dissected in the usual way. The latter were stimulated by the usual 'field' shocks and the former via their nerve supply. All received twenty impulses in 1 s.

No significant difference was found between the amounts of radioactivity released by the two groups of samples. The release factor, which is the ratio between the peak of evoked release over basal radioactivity, was  $1.40 \pm 0.09$  in 'field' stimulation and  $1.33 \pm 0.14$  in stimulation via the nerve (means  $\pm$  s.e.;  $n = 4$ ). The larger dispersion constantly found in experiments via the nerve was certainly due to the difficulty to dissect small fragments without damaging their nerve supply.

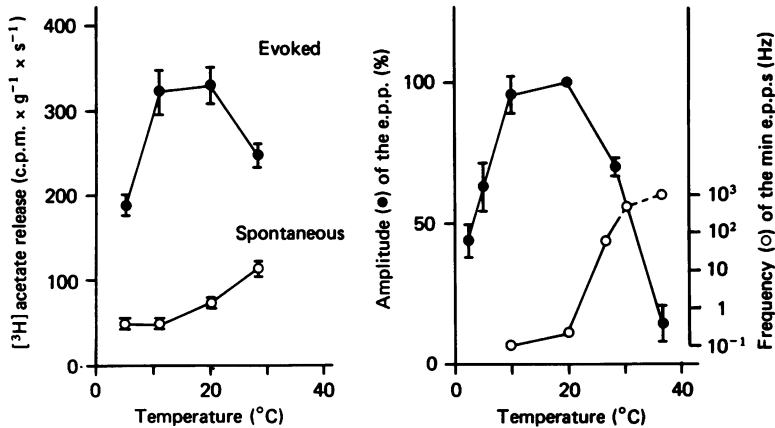


Fig. 5. Effects of temperature on ACh release and e.p.p. in electric organ. Spontaneous release (○) was the basal radioactivity counted in absence of stimulation, whereas evoked release (●) was the mean radioactivity in the four vials corresponding to the peak response to twenty impulses in 1 s. Labelling by [<sup>3</sup>H]acetate, approximately 15  $\mu$ Ci/ml. Means  $\pm$  s.e. of four samples. In the right-hand graph, the amplitude of the first e.p.p. (●) recorded in the same samples, has been expressed as per cent of the value measured at 20 °C. The apparent frequency of miniature e.p.p.s (○) was obtained from a different experiment. Values over 10<sup>2</sup> m.e.p.p.s per second are not indicative of a real frequency since the m.e.p.p.s were 'fusing' at over 25 °C and could no longer be counted individually.

#### *Effects of temperature*

Since the *Torpedo* is a poikilotherm animal, it was of interest to investigate the release of transmitter at different temperatures. In these experiments, the prisms were superfused by the normal saline at various temperatures which were determined in the tissue using a thermistor.

The spontaneous release of transmitter was estimated from the background level of radioactivity in the absence of any electrical stimulation. It remained low and constant at 5 and 12 °C but increased at temperature of 20 °C and higher. This can be compared with the apparent frequency of miniature e.p.p.s which remained very low until approximately 20 °C and increased sharply at higher temperatures (Fig. 5).

The evoked release of transmitter was analysed in response to a burst of twenty impulses in 1 s. The values shown in Fig. 5 were the averaged radioactivities counted in the four vials corresponding to the peak of release. The evoked release was impaired at 5 °C. It reached a maximum and apparently constant value between 10 and 20 °C. At higher temperatures the evoked release was reduced as the spontaneous release increased.

This pattern was in a rather good accordance with the changes in e.p.p. amplitude which was low at 2–5 °C, maximum and constant between 10 and 20 °C, and low again at higher temperatures. However, parameters of e.p.p., other than the amplitude have long been known to behave differently. Both the synaptic delay and the rising and falling times were very long at 3 °C and became simply shorter as the temperature was raised.

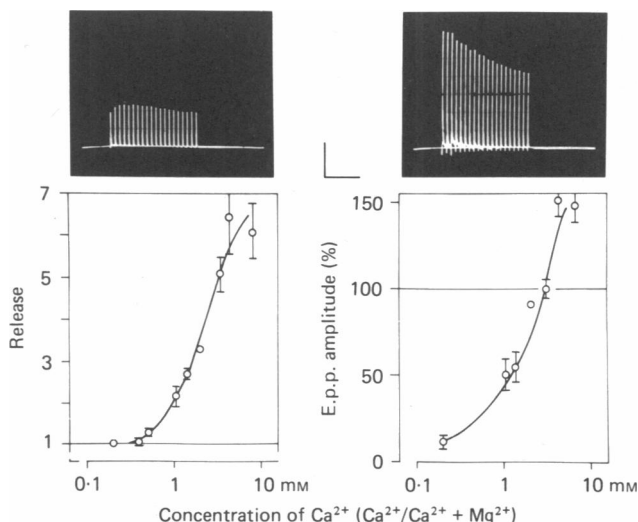


Fig. 6. Effects of Ca and Mg on ACh release and e.p.p. in the electric organ of *Torpedo*. Transmitter release is expressed as the ratio of the peak of evoked release over the basal radioactivity. Means  $\pm$  s.e. of three to ten values. Labelling by [<sup>3</sup>H]acetate. E.p.p. amplitude is the percent of the e.p.p. measured in the usual physiological solution (4.4 mM-CaCl<sub>2</sub> and 1.3 mM-MgCl<sub>2</sub>). Electrophysiological records show bursts of twenty impulses in 1 s. The one at the left was obtained in 1.1 mM-CaCl<sub>2</sub> and no MgCl<sub>2</sub>. The first electroplaque potential was of a small size but the subsequent responses exhibited no depression, rather some degree of facilitation. On the record of the right hand side (4.4 mM-CaCl<sub>2</sub>; no MgCl<sub>2</sub>) the first e.p.p. was larger and the following ones showed a progressive depression. Calibration: 1 V and 400 ms.

#### Calcium and magnesium

It has been known for a long time that the external Ca<sup>2+</sup> is required for the release of ACh (Harvey & MacIntosh, 1940; see Katz, 1969).

Fig. 6 shows that the release of ACh is dependent on Ca<sup>2+</sup> and inhibited by Mg<sup>2+</sup>. To allow comparison between different animals and different labelling conditions, the release function has been expressed as the ratio between the peak of the evoked release over the background level. The latter did not show any significant change as a function of Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations in these experiments.

The concentration-response curve of Ca<sup>2+</sup> for ACh release was found to be rather steep (Fig. 6). The Hill's coefficient was 3.2, when measured in the experiments in which no Mg<sup>2+</sup> was present. The Ca<sup>2+</sup> concentration corresponding to half of the maximum release was about 0.55 mM.

As expected, the amplitude of the e.p.p. showed a parallel Ca<sup>2+</sup> dependency. Interestingly, at high Ca<sup>2+</sup> concentrations the successive e.p.p.s of a twenty impulses burst underwent a marked progressive depression. In contrast, at low Ca<sup>2+</sup> concentrations, the first e.p.p. was of lower amplitude but the subsequent responses remained rather constant or showed some degree of facilitation (Fig. 6, records).

#### 4-aminopyridine

4-aminopyridine is expected to increase evoked transmitter release (Molgo, Lemeignan & Lechat, 1975) and we tested this effect in the electric organ. The prisms were labelled, washed and mounted as usual and then superfused with the

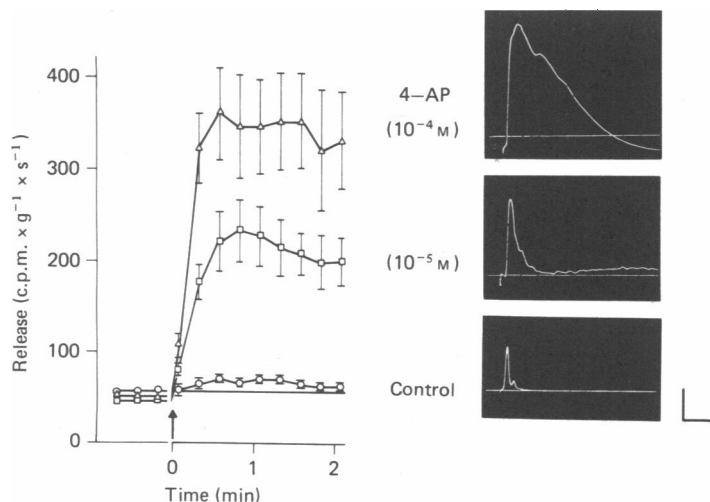


Fig. 7. Effects of 4-aminopyridine (4-AP) on ACh release and e.p.p. At the arrow, the prisms were stimulated by a single nerve impulse. 4-aminopyridine greatly enhanced evoked transmitter release but did not affect spontaneous release. Means  $\pm$  s.e. of four values. Labelling with [ $^3\text{H}$ ]acetate, 10  $\mu\text{Ci/ml}$ . The records show the dramatic enlargement of e.p.p. by 4-aminopyridine. Calibration: 1 V and 10 ms.

medium containing  $10^{-5}$  M or  $10^{-4}$  M-4-aminopyridine. A single stimulus was applied to each sample and ACh release was compared with the electrophysiological response.

4-aminopyridine dramatically increased the size of the e.p.p. (Fig. 7). The mean surface of the e.p.p. was enlarged by factors of 5.1 and 20.9 at  $10^{-5}$  M- and  $10^{-4}$  M-4-aminopyridine respectively.

This quasi explosive potentiation of transmission was really due to a pronounced increase in the evoked ACh release since a single impulse in the presence of 4-aminopyridine liberated more radioactivity than, for example, a hundred repetitive stimuli under normal conditions. The potentiation factors for ACh release were 18.1 and 27.2 at the  $10^{-5}$  and  $10^{-4}$  M concentrations respectively, i.e. much more than that expected from the e.p.p. enlargement.

The basal level of radioactivity was not increased by 4-aminopyridine. In other experiments the perfusate was collected for a longer time and the radioactivity decayed similarly to the experiment of Fig. 1. The huge potentiation of nerve evoked release only concerned isolated impulses or the first of several successive impulses. When, in the presence of  $10^{-4}$  M-4-aminopyridine, a second stimulus was given at 0.1–2 s interval, it did not give rise to any measurable e.p.p.

#### *Synthesis, storage and release of double-labelled ACh*

In typical double label experiments the prisms were incubated with [ $^3\text{H}$ ]choline and [ $^{14}\text{C}$ ]acetate at the same molar concentration (8.3 or 8.4  $\mu\text{M}$ ). Table 1 indicates the amount of the radioactive precursors which were incorporated into ACh at the end of incubation, and also the day after, at the time of stimulation. To provide a better basis for comparison, all quantities have been expressed as nmol/g wet tissue. Thus the radioactivities have been converted from d.p.m./g to nmol/g on the basis of the specific radioactivities of the precursors added to the incubation medium.

TABLE 1. Tissue ACh in a double label experiment (nmol/g wet tissue  $\pm$  s.e.;  $n = 3$ )

|  | Total<br>[ <sup>3</sup> H]choline | Total<br>[ <sup>14</sup> C]acetate | [ <sup>3</sup> H]ACh | [ <sup>14</sup> C]ACh | Total ACh<br>(bio-assay) |
|--|-----------------------------------|------------------------------------|----------------------|-----------------------|--------------------------|
| At the end of incubation   | 19.7 $\pm$ 0.8                    | 19.3 $\pm$ 1.0                     | 4.9 $\pm$ 0.7        | 4.5 $\pm$ 0.5         | 607 $\pm$ 3              |
| Washed overnight   | 10.7 $\pm$ 0.4                    | 15.0 $\pm$ 0.9                     | 5.5 $\pm$ 0.2        | 3.6 $\pm$ 0.2         | 613 $\pm$ 36             |
| Washed overnight,<br>60 min, in DFP (10 <sup>-3</sup> M),<br>90 min of subsequent<br>washing | 11.4 $\pm$ 1.1                    | 15.1 $\pm$ 0.4                     | 6.4 $\pm$ 0.5        | 5.05 $\pm$ 0.3        | 604 $\pm$ 55             |

At the end of incubation, equal amounts of [<sup>3</sup>H]choline and [<sup>14</sup>C]acetate were found in the tissue. From this total radioactivity, approximately 25 % was incorporated into ACh. The <sup>3</sup>H to <sup>14</sup>C ratio of the labelled ACh was not significantly different from 1. It could therefore be concluded that the incorporation of choline and acetate into ACh proceeded at the same rate.

Surprisingly, only a small amount of radioactivity was lost during the long washing time at 7 °C and the subsequent superfusion. The loss of total [<sup>3</sup>H]choline was significantly larger than that of acetate. From the remaining choline, more than half was recovered as ACh. The amount of [<sup>3</sup>H]ACh, labelled from the choline precursor, was maintained during the washing period.

At the same time, the prisms retained 78 % of their total [<sup>14</sup>C]acetate. Despite this, the level of [<sup>14</sup>C]ACh was reduced, suggesting acetate transfer to other constituents of the tissue. Indeed appreciable amounts of [<sup>14</sup>C] radioactivity were found in regions other than that of ACh by chromatography using high voltage electrophoresis (unpublished data). Thus, at the time when the stimulation was to be applied, the <sup>3</sup>H to <sup>14</sup>C ratio was slightly but significantly higher than 1. The transmitter retained a little more radioactive choline than acetate. On the other hand, the amount of total unlabelled ACh had remained stable from the beginning of experiment, i.e. for about 24 h.

A few prisms of the same experiment were submitted at the end of the washing period to treatment with DFP (10<sup>-3</sup> M) for 1 h. The excess DFP was eliminated by a 90 min washing period. The total time of the experiment was identical to that of the previous samples. Table 1 shows that neither the radioactive nor the non-radioactive ACh were decreased by the DFP treatment. Consequently the reduction of transmitter release previously described for this condition can not be attributed to a reduction of the tissue content of transmitter.

<sup>3</sup>H and <sup>14</sup>C radioactivities were analysed in the superfusing medium as a function of time during stimulation (Fig. 8). A surprising but constant finding was that the resting release of acetate was higher than that of choline. This was repeatedly observed in several experiments on different animals and also confirmed by reversing the radioactivities of the precursors, namely by using [<sup>14</sup>C]choline and [<sup>3</sup>H]acetate.

In contrast, the evoked release of <sup>3</sup>H and <sup>14</sup>C radioactivities was a good reflection of the labelled ACh store. The evoked release of [<sup>3</sup>H]choline was slightly higher than that of [<sup>14</sup>C]acetate.

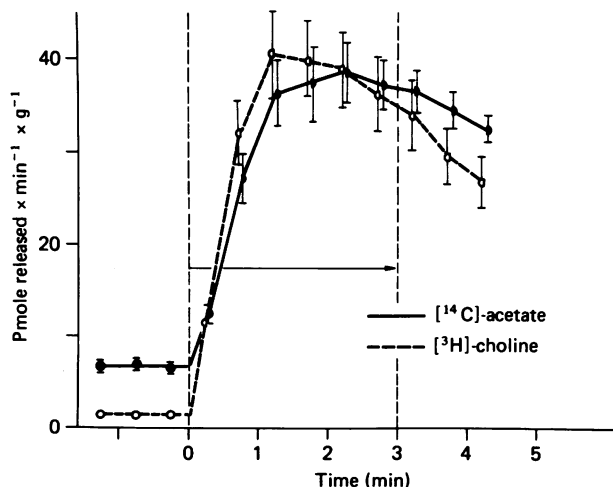


Fig. 8. Release of radioactive choline and acetate in a double labelling experiment. The tissue was incubated with [ $^{14}\text{C}$ ]acetate and [ $^3\text{H}$ ]choline at the same molar concentration. After the long washing period, the tissue ACh was labelled with a little more radioactive choline than acetate (see Table 1). The resting release of radioactivity, however, contained constantly more acetate than choline but, on stimulation, here at 10 Hz for 3 min (arrow), acetate and choline were released in a ratio corresponding to that of the store, in fact not far from 1. Thus most if not all the evoked overflow of radioactivity must come from the ACh released at the nerve endings by nerve stimulation. Further explanations in the text.

#### DISCUSSION

##### *ACh release as tested in the present work*

The evoked release of ACh has been measured in the electric organ of *Torpedo* by a somewhat unusual method.

It was the overflow of the breakdown products, acetate and choline, which was taken as an assay for the transmitter released by nerve impulses. This could be criticized since the amount of acetate or choline which escaped into the fluid is also expected to depend on other factors such as the rate of their re-uptake, unspecific binding, other metabolic routes etc. – nevertheless, we feel that this test can provide a good estimate of the released ACh, especially when the stimulation consists of single or a few impulses. The justification for this is as follows. (i) In the radioactivity which accumulated in the medium in response to stimulation, the choline to acetate ratio was the same as that of the ACh store; this ratio was in fact not far from 1. (ii) In a number of different stimulation patterns, the radioactivity recovered was that expected from analysis of electrophysiological events. (iii) The release of radioactivity and the e.p.p. were both affected in the same way and to the same extent by changes in external  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . (iv) Similarly, 4-aminopyridine, which is known to potentiate synaptic transmission by increasing evoked transmitter release, also increases the release as tested in the present experiments.

Thus the radioactivity of acetate and choline which increased over the background in response to nerve stimulation most probably came from the released transmitter. It must be emphasized, however, that only a small percentage of the transmitter

released at the nerve endings was recovered in the medium. The major part remained in the tissue, being mixed with the very large pool of endogenous transmitter and recycled into new ACh during and after synaptic activity (see Dunant *et al.* 1974, 1977).

Under the resting conditions, however, the radioactivity recovered in the medium had a choline to acetate ratio constantly smaller than that of the tissue ACh. This intriguing phenomenon suggested that substances other than ACh could have contributed to the release of radioactive acetate in the absence of stimulation. Additional experiments are in progress to elucidate this.

A few comments should also be made on the methods of stimulating the *Torpedo* electrogenic tissue. Care has always been taken to abundantly superfuse the prisms with the saline medium during stimulation. This allows the tissue to dissipate most of its electrical energy outside, which normally occurs for a fish which usually discharges in the well conducting sea water. When the electric organ is stimulated in air, the prisms are forced to work against high external resistance, thus producing high voltage but no external current. This drives a large leakage current back through the tissue whose proper resistance is rather low. Stimulation in this condition should be avoided since it causes artifactual lesions of the nerve endings (Dunant *et al.* 1976).

#### *The effect of anticholinesterases*

Since the pioneer works of Dale, Feldberg and co-workers, the release of ACh has been almost always measured after complete inhibition of the tissue cholinesterases. It was consequently very surprising to find that eserine or pre-treatment with DFP strongly reduced the evoked release, even by a single nerve impulse. The effect was mainly on the 'initial' release, as tested after a long period of rest. Anticholinesterases affected to a smaller extent the release by prolonged stimulations or increased KCl concentration.

It could be argued that, in the present experiments, the anticholinesterase effect was due to some special change in the radioactive pools of ACh. This criticism is not founded, since it was checked in the experiment of Table 1; neither the radioactive ACh nor the endogenous ACh were reduced in the tissue after DFP treatment. Moreover the release of endogenous transmitter was also impaired by anticholinesterase as found, but not noticed, in previous experiments in which the transmitter released in absence of inhibitor was measured by re-acetylation of choline (Dunant *et al.* 1972; Table 3).

A similar depression of ACh release by anticholinesterase drugs has been reported by Szerb & Somogyi (1973), working with mammalian brain slices. The effect was antagonized by atropine. The situation seemed less clear in mammalian sympathetic ganglia. Indeed, Perry (1953) found that the amount of ACh liberated by a given stimulation in the presence of eserine was greater than the amount of choline liberated by a similar stimulation in absence of eserine (see also MacIntosh & Collier, 1976). However, recent experiments by Sacchi *et al.* (1978) showed that eserine caused a drastic depression of ACh release in the isolated rat ganglion.

The decreased release of radioactivity by cholinesterase inhibitors could be attributed to some rapid re-uptake of the unhydrolysed ACh, resulting in a smaller

proportion of it escaping into the perfusate. This would be surprising since the uptake of intact ACh is usually considered as a much less effective process than that of choline or acetate (see MacIntosh & Collier, 1976).

Another explanation for this effect of anticholinesterase drugs may be found in the retro-inhibition of transmission by ATP, recently proposed by Meunier, Israël & Lesbats, (1975) and Israël, Lesbats, Manaranche, Marsal, Mastour-Frachon & Meunier (1977). The ACh released at rest, being no longer hydrolysed, would act on post-synaptic receptors, depolarizing the electroplaques and causing the release of ATP. This post-synaptic ATP, in turn, would act on the nerve endings by reducing further release of ACh. In the work of Szerb & Somogyi (1973), atropine did restore the efficiency of the release process; this speaks in favour of the above explanation, as did the recent experiments of Miledi *et al.* (1978) who showed that the release of ACh in the DFP-treated rat diaphragm was increased after treatment with  $\alpha$ -bungarotoxin. We tried similar experiments with the electric organ of *Torpedo*. However preliminary results showed that neither  $\alpha$ -bungarotoxin (kindly provided by Dr B. Fulpius, Geneva) nor tubocurarine could antagonize the reduction of release caused by anticholinesterase drugs.

#### *Ca<sup>2+</sup>, temperature and 4-aminopyridine*

Both the e.p.p. and evoked ACh release exhibited identical Ca<sup>2+</sup> dependency and identical inhibition by Mg<sup>2+</sup>. This provided an excellent control that the radioactivity counted in our experiments was faithfully related to the ACh released at the synapses by nerve impulses and not to some unspecific effect of the stimulating shocks. In addition, the dose-response curve for the Ca<sup>2+</sup> dependency of ACh release had a rather steep slope, confirming the conclusions of many electrophysiological experiments, namely that co-operation by 3-4 Ca<sup>2+</sup> ions seems to be required to trigger ACh release (see Ginsborg & Jenkinson, 1976, for discussion). Finally, the Ca<sup>2+</sup> and Mg<sup>2+</sup> experiments could provide useful information since the intraterminal compartmentation of ACh in this tissue has been found to also depend on the external bivalent cations (Babel-Guérin, 1974; Israël *et al.* 1974).

The evoked release was optimal between 10 and 22 °C, i.e. in the temperature range which is physiological for *Torpedoes*. By comparison, ACh release in mammalian diaphragm is maximal near 37 °C, with a marked decrease at lower temperatures (Straughan, 1960).

4-aminopyridine has a strong anti-curare effect due to increased quantum content of the e.p.p. (Molgo *et al.* 1975). 4-aminopyridine most probably acts, like tetraethylammonium, by blocking potassium efflux, hence prolonging axon terminal spike and increasing Ca influx. This explains why 4-aminopyridine greatly potentiated the evoked, but not the spontaneous ACh release.

#### *Depression of transmission*

At most cholinergic synapses successive nerve impulses produce e.p.p.s of decreasing amplitude. It is the same phenomenon as the 'inhibition' first described by Wedensky (1891) which already concluded that it was due to functional alteration of nerve endings rather than of muscle fibres. This was later confirmed by physiologists who showed that depression was in fact a reduction of the quantum content



of the e.p.p. (Del Castillo & Katz, 1954) and that the sensitivity of the muscle membrane was unchanged (Otsuka, Endo & Nonomura, 1962).

Depression is also evident when, after a long period of rest, one gives a pair of stimuli separated by an appropriated interval. The second produces an e.p.p. which is smaller than the first. It was demonstrated by a direct method in this work that the depression was really due to a decrease in the amount of ACh released by the second impulse. When the interval was prolonged to several seconds, the two stimuli generated e.p.p.s of equal size and also released equal amounts of transmitter.

In prolonged stimulation of ganglionic and neuromuscular synapses, depression only affects transmission during the first stages of stimulation. Then a steady state is reached and may remain stable for a long time. Direct measurements have similarly demonstrated that transmitter output was greater in the initial phases of stimulation (Brown & Feldberg, 1936; see MacIntosh & Collier, 1976).

In its physiological discharge, the *Torpedo* produces short bursts of five to ten impulses at approximately 100 Hz. Therefore, the fish can use high frequencies for very short times due to its very high 'initial efficiency' of the release process. In repetitive activity, depression is prominent and the maximal rate of release declines as the stimulation is continued. However, the depression observed in repetitive stimulation of the *Torpedo* electric organ was only partly explained by presynaptic failure. The electromotive force, due to ionic gradient through the electroplaque membranes, also decreased by about 50 % after 1 min stimulation at 5 or 10 Hz. This also contributed to the reduction of e.p.p. amplitude (Dunant, 1976; Dunant *et al.* 1977).

The degree of depression was somewhat related to the amount of transmitter released in the first impulse. Therefore, the depression was pronounced in high  $\text{Ca}^{2+}$  whereas little depression or even facilitation occurred in low  $\text{Ca}^{2+}$ . This was in good accordance with the observation made on neuromuscular and other synapses (see Ginsborg & Jenkinson, 1976). An exaggeration of this was obtained with 4-aminopyridine, where the first e.p.p. was enormously potentiated whereas the following impulses did not produce any response.

It has been tempting, but disappointing, to correlate the neuromuscular depression with some decrease of the intraterminal ACh store. In the electric organ, a marked decrease was indeed observed in the early phase of a continual stimulation, but later the store increased again and oscillated near the initial level whereas the e.p.p. did not show a parallel increase (Dunant *et al.* 1974, 1977). In other synapses, it has been found that the ACh store was as large or sometimes larger when the e.p.p. was reduced by prolonged stimulations (see MacIntosh & Collier, 1976). To explain this, it has been proposed by Perry (1953) and by Birks & MacIntosh (1961) that a small compartment of 'immediately available transmitter' is situated in series between the process of release and the main depot of transmitter. It is this compartment which would be depleted during the initial depression of transmission; its refilling would be rate-limiting at the later stages of activity. An evident alternative hypothesis is that the efficiency of the release process itself would be affected in the phenomenon of depression. After having been activated by  $\text{Ca}^{2+}$ , the release process operating at the presynaptic membrane would not be able to release another packet of transmitter before the initial conditions are restored, perhaps by complete clearance of  $\text{Ca}^{2+}$ .

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