THE NUMBER OF TRANSMITTER
MOLECULES IN A QUANTUM: AN ESTIMATE FROM
IONTOPHORETIC APPLICATION OF ACETYLCHOLINE
AT THE NEUROMUSCULAR SYNAPSE

By STEPHEN W. KUFFLER and DOJU YOSHIKAMI

*From the Department of Neurobiology, Harvard Medical School,
Boston, Mass. 02115, U.S.A.*

SUMMARY

1. The sensitivity of the subsynaptic membrane of twitch muscles of the frog and snake to iontophoretically applied acetylcholine (ACh) was determined. Optimal placement of ACh micropipettes on to the postsynaptic membrane resulted in potentials that were similar, though not identical, to the miniature excitatory post-synaptic potentials (min e.p.s.p.s). A sensitive bio-assay was developed to measure the output of ACh from micropipettes; this allowed an estimate to be made of the upper limit of the number of ACh molecules in a quantum of transmitter that is released from the nerve to produce a min e.p.s.p.

2. The assay to calibrate the output of ACh from micropipettes used the end-plate of the snake muscle as an ACh concentration detector. The end-plate was situated within a few μm of an oil-water interface, and a 0·6 nl. droplet of Ringer solution containing a known concentration of ACh (1 μM or less) was formed in the oil phase. The droplet was brought to the interface and, upon touching it, discharged its contents into the Ringer phase immediately above the end-plate. This resulted in a membrane depolarization that was recorded with an intracellular microelectrode. By applying droplets containing various known ACh concentrations a standard curve was constructed. To measure the ACh output of micropipettes a 0·6 nl. droplet of Ringer solution was suspended in the oil. The ACh pipette tip was inserted into the droplet and several thousand pulses of ACh were then delivered. The ACh content of the test droplet was measured by comparing its effectiveness in depolarizing the end-plate with the standard curve. In this manner the number of ACh molecules released in a single pulse was determined as a function of charge passed through the pipette. The output of ACh was linear and an average of 30,000 molecules of ACh were released per pC.

3. The sensitivity of the subsynaptic membrane to iontophoretically applied ACh, using the linear slopes of dose–response curves, in preparations from frog and snake treated with anticholinesterases was usually about 5 mV/pC. It follows that 6000 molecules of ACh are sufficient to produce a depolarization of 1 mV in the subsynaptic membrane.

4. The mean min e.p.s.p.s of muscle fibres treated with anticholinesterase range from 1 to 3 mV. Since the ACh released from an iontophoretic pipette is less effective than the same amount released from the nerve, it is concluded that a quantum of transmitter consists of less than 10,000 molecules of ACh.

5. It is calculated that for each molecule of ACh released in a quantum there results a minimum net flow of 3000 univalent ions across the synaptic membrane.

<div align="center">INTRODUCTION</div>

The chemical synapse is an electrochemical transducer that converts the 'electrical' presynaptic nerve impulse into another electrical signal in the post-synaptic cell. The mechanisms in chemical synaptic transmission have been best analysed at the neuromuscular synapse in which acetylcholine (ACh) is released from nerve terminals in discrete multimolecular packets, or quanta, as was first demonstrated by Katz and his colleagues (Katz, 1969). Quantal release of ACh and of various other transmitters has also been shown in a variety of vertebrate and invertebrate synapses, and quantal synaptic transmission may be a common feature of chemical synapses. At the vertebrate neuromuscular synapse a single quantum produces a miniature excitatory post-synaptic potential (min e.p.s.p.) which is on the order of 1 mV, and normally several hundred quanta are simultaneously released in response to a nerve impulse in the presynaptic terminal (Katz, 1969; for recent reviews see Hubbard, 1973; Krnjević, 1974).

Our current inquiries explore issues that relate to both the secretion of ACh by the nerve terminals and to the post-synaptic responses that ACh produces. In particular, we have been concerned with events within the synaptic cleft after liberation of the transmitter. Thus, in the preceding paper (Hartzell, Kuffler & Yoshikami, 1975) we analysed the relative changes in ACh concentration in the synaptic cleft following release of ACh from the nerve. The present experiments were done to determine the actual numbers of ACh molecules that are secreted into the cleft.

Two approaches have been used for the determination of the amounts of transmitter liberated by individual nerve impulses. In the first, based on the work of Dale, Brown, Feldberg & Vogt (Dale, 1965), nerve bundles to muscles or ganglia were stimulated and the ACh that appeared in the

perfusate was assayed (see Discussion). The second approach, that we have used, became possible with the introduction of the method of ionto-phoretic application of ACh from micropipettes (Nastuk, 1953). For example, Krnjević & Miledi (1958) and Miledi (1962) applied ACh ionto-phoretically on to the neuromuscular junction so effectively that potentials with a time course almost as rapid as that of synaptic potentials were produced. On the assumption that it took similar numbers of ACh mole-cules for nerve terminals and micropipettes to produce the synaptic response, they calculated that several hundred thousand molecules of ACh were liberated in a quantum. Using the same method, similar values for the number of ACh molecules in a quantum were obtained by Dennis, Harris & Kuffler (1971) in parasympathetic nerve cells. Such determina-tions were only rough approximations, because they were based on an estimate of the fraction of the total current passed through the ionto-phoretic pipette that is carried by ACh ions. This transport number was assumed to be between 0·1 and 0·3.

The iontophoretic approach was re-investigated, since we thought that the use of such transport numbers for ACh was not appropriate when small and brief current pulses are passed through high-impedance pipettes. A quantitative assay was therefore developed to directly measure the relatively small numbers of ACh molecules that were delivered from ionto-phoretic pipettes. In addition, we took advantage of recent improvements in techniques and preparations that permit accurate placement of the tips of the pipettes on to the synaptic membrane under visual guidance (McMahan & Kuffler, 1971; McMahan, Peper & Spitzer, 1972; Peper & McMahan, 1972; Kuffler & Yoshikami, 1975). Our experiments show that less than 10,000 molecules of ACh are released in a quantum.

METHODS

The preparation of the externus obliquus abdominis muscle of the garter and ribbon snakes (*Thamnophis*), the cutaneus pectoris muscle of the frog (*Rana pipiens*), as well as the Ringer solutions, were described in a previous paper (Kuffler & Yoshi-kami, 1975).

All micropipettes and micro-electrodes were pulled from Pyrex glass tubing (1·2 mm o.d. with a 0·3 mm wall thickness) on the same horizontal puller. Only the filament-heat setting was varied. Higher filament-heat produced pipettes with longer shanks and higher resistances.

The oil used in these experiments (Text-fig. 1*A*) was a high density (specific gravity about 1·9), low viscosity 'liquid Teflon' fluorocarbon (Fluorolube SF-5, Hooker Chemical Co., Niagara Falls, New York, U.S.A.).

Glass electrodes. Intracellular recording electrodes (Text-fig. 1, electrode *c*) con-taining 4 M potassium acetate had resistances of 20–40 MΩ, and were mounted on a Leitz micromanipulator. The micro-electrode (10 MΩ) for grounding the droplet (Text-fig. 1, electrode *d*) contained 3 M-NaCl and was mounted on a second Leitz

manipulator with a dual probe holder that also carried the transfer pipette (see below).

Iontophoretic ACh pipettes (Text-fig. 1, pipette f). These contained 1 M acetylcholine chloride and had resistances of 150–300 MΩ. They were the same as those used in previous studies (Kuffler & Yoshikami, 1975; Hartzell *et al.* 1975). The following modifications provided a conducting path for a braking current when the pipette tip was in the non-conducting oil phase. The external surface of the shank and tip was painted with electrically conducting colloidal silver except for the last 100 μm of the very tip. This last 100 μm was 'shellacked' with a thin coating of egg white or occasionally with saliva. When the coating had dried and the pipette was wetted with Ringer solution, the hydrophillic protein layer provided a thin 'wick' extending from the very tip of the pipette back to the silver coating. A fine silver wire lead (Text-fig. 1, wire *g*) was attached to the silver-painted surface. The ACh pipette was manipulated with a Beaudouin pneumatic micromanipulator (see Kuffler & Yoshikami, 1975).

Transfer pipette (Text-fig. 1, pipette e). The exterior surface of a short shank micropipette was coated with a thin layer of wax and its tip was broken off to provide an enlarged bevelled opening with about a 15 μm diameter (see Text-fig. 2). The wax made the outer surface of the pipette hydrophobic. The back of the pipette was attached to a flexible polyethylene tubing which led to a 2 ml. screw-driven syringe. The entire device was filled with oil, so that solutions in the tip of the pipette could be drawn in or extruded by adjusting the screw-drive of the syringe. The pipette was held in the dual-probe holder along with the 3 M-NaCl droplet-grounding micro-electrode (see above). Thus, both the transfer pipette and the grounding micro-electrode were moved together by the Leitz manipulator. In addition, the two probes could be moved independently by screw controls on the probe holder.

Dispensing capillaries (h, Text-fig. 1 B). These were short-shanked pipettes (total length, about 2 cm) with their tips ground to an inner diameter of about 200 μm. The other end of the dispensing capillary was fitted with a 1 cm long polyethylene tube extension and a small cap. The capillaries and extensions were filled usually with 0, 0·4, 0·6 and 0·8 μMACh in Ringer solution containing neostigmine 3 × 10^{-6} g/ml. These solutions were freshly made before each experiment. The polyethylene extension is hydrophobic and prevented capillary retention of the aqueous fluid contents when some of it was removed from the tip. The cap minimized evaporation from the extension. A radial array of four of these dispensing capillaries were mounted together on a simple manipulator with coarse controls.

Microscope optics. Zeiss Nomarski optics with a × 40 water immersion objective lens was used during iontophoretic application of ACh on to end-plates under visual guidance, as described previously (Kuffler & Yoshikami, 1975). When the preparation was covered with oil, this lens could also be immersed in it without appreciable loss of optical clarity. The lens has a working distance of 1·5 mm. During the ACh bio-assay part of the experiment, two dry objectives were used, a × 33 (Leitz UMK-50) and a × 10 (Leitz UM-2), with working distances of 6 and 15 mm. The microscope nose-piece turret was modified to allow smooth positioning of the objectives without disturbing the impaled preparation.

Procedures for the bio-assay

Sensitivity to iontophoretically applied ACh. The same recording and ACh ionto-phoretic methods were used as previously described (Kuffler & Yoshikami, 1975). They are briefly summarized here. Selected small areas of the muscle preparation were treated with collagenase. This allows the presynaptic terminals to be removed
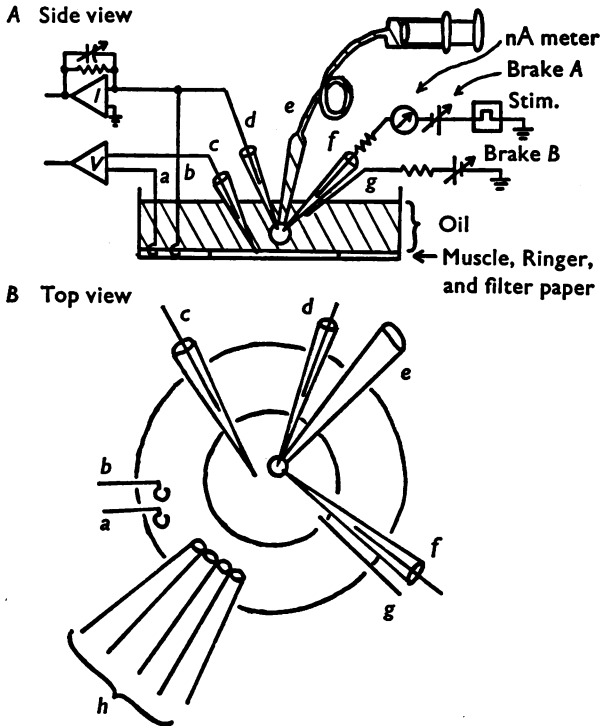
Text-fig. 1. *A*, sketch of a side view of the experimental arrangement for the bio-assay of ACh. The muscle is pinned down on to a thin layer of Sylgard and is surrounded by a single sheet of filter paper that is saturated with Ringer solution. Oil covers the entire preparation, leaving only a thin film of Ringer above the muscle (see also Text-fig. 2). *a*, Ag/AgCl reference electrode for the voltage amplifier (*V*) that monitors membrane potential. *b*, Ag/AgCl electrode that maintains the bath at virtual ground via a current to voltage converter (*I*). These two electrodes are in contact with the extracellular fluid space of the muscle via the filter paper. *c*, intracellular potassium acetate micro-electrode for membrane potential measurement. *d*, NaCl micro-electrode that grounds the droplet through the current-measuring device (*I*). *e*, transfer pipette for taking aqueous samples from dispensing capillaries (*h*, in Text-fig. 1*B*), extruding them as droplets and manipulating them in oil. *f*, ACh-filled micropipette is connected to a stimulus isolation unit through a current-limiting resistor (1000 MΩ) to a nano-ammeter and battery (brake *A*) that monitors and provides braking current. *g*, fine Ag wire in electrical contact with the tip of the ACh pipette (Methods) connected through a current-limiting resistor (1,500 MΩ) to a battery (brake *B*). It maintains the braking current when the pipette tip is in oil. *B*, top view of ensemble, including the array of four dispensing capillaries (*h*) not shown in *A*; one is filled with plain Ringer solution and the rest contain Ringer solutions with three different ACh concentrations. The tips of these capillaries are all immersed in the oil.

from some of the muscle fibres, leaving behind the exposed subsynaptic membrane, which appears as a groove in the case of the frog (Peper & McMahan, 1972) or as a crater in the case of the snake (Kuffler & Yoshikami, 1975). Any remaining acetyl-cholinesterase (AChE) was inactivated by treating the preparation either with methanesulfonylfluoride (MSF) or diisopropylfluorophosphate (DFP) (Hartzell et al. 1975) or by keeping the muscle in Ringer solution containing neostigmine, 3×10^{-6} g/ml.

The ACh pipette had a 3 nA negative braking current applied to it which is more than adequate to prevent leakage of ACh from the tip (Kuffler & Yoshikami, 1975). The ACh was delivered with a 1 msec positive current pulse, and the amplitude of the pulse was varied, often up to 10 nA (providing up to 10 pC). Fast intracellular potentials were invariably obtained when the ACh pipette tip was placed directly on to the exposed subsynaptic membrane.

Bio-assay of ACh. The assay for ACh utilized the end-plate of the snake fibres as an ACh detector. The muscle (usually not treated with collagenase) with its AChE inactivated was pinned out on to a 'Sylgard'-coated dish, as previously described (Kuffler & Yoshikami, 1975). A ring of Whatman no. 40 filter paper, soaked in Ringer, was laid around the muscle and made contact with it. All excess Ringer from the bath was removed, and a ½ cm thick layer of oil was placed over the entire muscle. Although the oil is denser than Ringer solution, it does not penetrate through the muscle layer, since surface tension maintains the oil-water interface just (less than 5μm) above the superficial muscle fibres. When connective tissue overlying the muscle surface was not trimmed away sufficiently, or when not enough of the excess Ringer had been removed, the oil-water interface was situated too far above the muscle surface for the preparation to be useful.
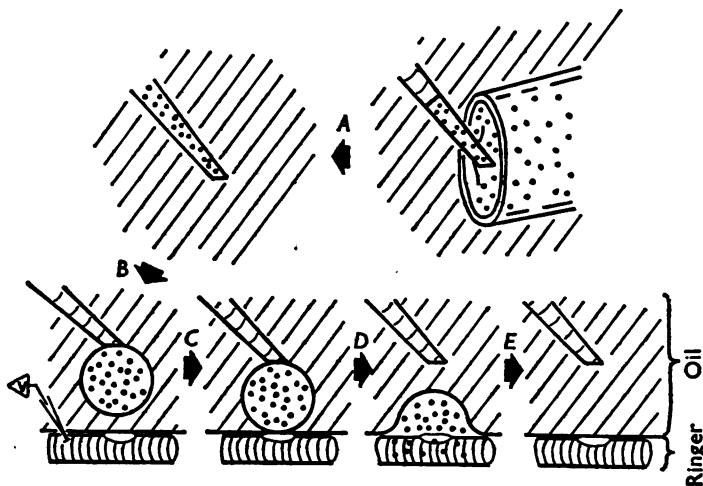
Text-fig. 2. Sequence of steps in the formation of a droplet, and its applica-tion to the end-plate. The oil phase is hatched and the ACh solution is stippled. The transfer pipette removes the aqueous fluid from a dispensing capillary (A) and is moved into position above the muscle (B) where it extrudes the droplet into the oil. The droplet is touched against the oil-Ringer interface (C) and discharges its contents into aqueous phase above the end-plate (D). The original state of the interface is restored (E), and the entire process can be repeated.

The various pipettes and electrodes mounted on their respective manipulators were then assembled above the preparation (Text-fig. 1). By viewing the muscle through the × 33 high-dry objective, one could clearly see the end-plates. An end-plate was selected on the upper surface of a superficial muscle fibre facing the oil-water interface. Intracellular potentials were usually recorded with an electrode about 150 μm away from the edge of the end-plate. Droplets of Ringer solution containing various ACh concentrations were accurately applied (see below) to the end-plate areas, and the resulting depolarizations were registered on a pen recorder (Brush 220) whose frequency response was adequate for the slow responses (see Text-fig. 5*A*).

The following procedure was used to apply solutions to the end-plate. The aqueous solution in the transfer pipette (see below) was extruded into the oil as a droplet. The droplet clung to the very tip of the pipette (Text-fig. 2, step *B*; Pl. 1). The wax on the outer surface of the pipette, being hydrophobic, prevented the droplet from creeping up the outer wall of the pipette. The droplet was then poised directly above the end-plate and slowly brought down on to the oil-water interface above the end-plate (Text-fig. 2, step *C*). Upon contact of the droplet with the interface, their boundaries fused (Text-fig. 2, step *D*), the droplet burst and gently discharged its contents on to the end-plate. During this process the droplet automatically detached itself from the transfer pipette. This entire procedure can be performed repeatedly with various solutions.

Standardization of the assay. Aliquots of known ACh concentration were applied to the end-plate, as described above, to obtain a standard curve. Using the low magnification (× 10) objective to get a wide field of view, the transfer pipette was brought to the opening of one of the four dispensing capillaries and an aliquot was drawn into the pipette (see Text-fig. 2, upper right). A reticule in the microscope ocular was used to measure how far the shank of the pipette was filled. The magnification was then increased by the × 33 objective. When the contents of the transfer pipette were extruded as a droplet (Pl. 7 and Text-fig. 2, step *B*), its diameter was carefully measured with the reticule in order to determine its volume. After a few trials, one knew how much to fill the transfer pipette in order to produce a droplet with a diameter of 105 μm. This size droplet contains 0·6 nl., and was used throughout our experiments. It is about twice the diameter of the end-plate.

Calibration of the output from ACh pipettes. The transfer pipette removed 0·6 ml. Ringer solution from one of the dispensing capillaries and extruded it into a droplet (cf. Text-fig. 2). At this point the NaCl grounding electrode (*d*, Text-fig. 1) was inserted into the droplet (see Pl. 1).

The tip of the ACh pipette was normally left in the bath Ringer fluid immediately above the muscle with a 3 nA brake applied through brake *A* in Text-fig. 1*A*. When the ACh electrode was lifted into the oil phase, it lost electrical contact with the bath ground, and the braking current was then shunted through the silver wire lead (*g*, Text-fig. 1). Additional braking voltage was necessary to deliver enough braking current through the series resistance introduced by the protein 'wick' between the tip of the pipette and the silver wire lead. This voltage was provided by brake *B* in series with a 1500 MΩ current-limiting resistor. The 3 nA braking current of the pipette was continuously monitored with a nano-ammeter (Text-fig. 1*A*). The ACh pipette was then inserted into the droplet for the delivery of ACh pulses. It was essential that there was no interruption at any time in the braking current while the pipette was in oil. The braking current prevented leakage of ACh into the fine fluid film surrounding the tip of the pipette when it was in oil. Current pulses used to drive ACh out of the pipette and into the droplet were recorded via the low resistance NaCl ground electrode (*d*, Text-fig. 1*A*).

Several thousand pulses of ACh had to be applied into the droplet to produce an ACh concentration high enough to be reliably measured by the end-plate. The ACh pipette was pulsed at a rate of 40–50/sec. A separate experiment was performed to show that these pulse rates did not affect the output of ACh from the pipette. In this experiment no anticholinesterase was used, so that the responses that were produced with the ACh pipette abutting the post-synaptic membrane were very fast and completely over in less than 20 msec. The shapes and sizes of the post-synaptic potentials were essentially identical when the ACh pulse rate was varied between 0·5 and 50/sec.

<div align="center">RESULTS</div>

The experiments were designed to answer the following two questions: first, how much is the post-synaptic membrane depolarized per unit of charge that is pulsed through an iontophoretic ACh pipette, and secondly, how is this unit of charge translated into number of ACh molecules?

<div align="center">

The sensitivity of the post-synaptic membrane to
iontophoretically applied ACh

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Comparison of pipette-applied ACh responses with quantal potentials

The time course of miniature synaptic potentials in parasympathetic neurones and at neuromuscular junctions can be reproduced to within a fraction of a msec by the iontophoretic release of ACh from micropipettes (Dennis *et al.* 1971; Kuffler & Yoshikami, 1975). The 'artificial' synaptic potentials are slightly slower than those due to quantal release by nerve terminals. While such differences may seem small, they are probably significant in terms of a precise comparison of the number of molecules of ACh required to produce the two potentials.

Text-fig. 3 shows an example in which a miniature synaptic potential was imitated by pipette release of ACh on to a snake muscle fibre that was lightly treated with collagenase. To release ACh a 1 msec pulse was passed through the pipette that had a tip opening of about 100 nm and was placed at the edge of a synaptic bouton (Kuffler & Yoshikami, 1975). A min e.p.s.p. occurred spontaneously on one of the two superimposed traces and permits a ready comparison to be made of the responses. The rise time (10–90 %) of the pipette-evoked potential is 1·1 msec as compared with 0·75 msec for the miniature potential. This shows that the pipette does not exactly simulate nerve release of ACh (Discussion). ACh current pulses of 0·1–0·5 msec duration produced better imitations of quantal potentials; however, accurate measurements of the ACh-pulse currents were more difficult.

Post-synaptic sensitivity to ACh in mV/pC

The ACh pipette can be positioned on the post-synaptic membrane so that nerve release of ACh can be closely mimicked. Under such conditions,

it was previously shown (Kuffler & Yoshikami, 1975) that when the output of the ACh pipette is varied, one obtains sensitivity values of about 5 mV/pC from the linear slopes of the resulting dose–response curves. This value can be reproducibly obtained from both frog and snake muscles that are treated with anticholinesterases (see Kuffler & Yoshikami, 1975). The experiment of Text-fig. 4 is an example of a determination of membrane sensitivity. The subsynaptic membrane of a collagenase-treated snake
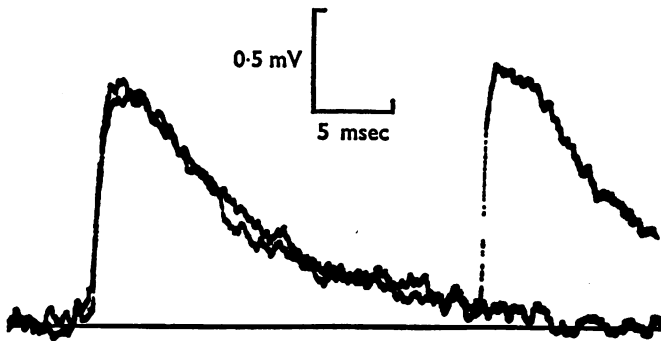
Text-fig. 3. Comparison of pipette-evoked ACh potential with min e.p.s.p. in a snake muscle fibre. One msec pulses were passed through an ACh pipette whose tip was at the edge of a synaptic bouton. Two iontophoreti- cally produced responses (left) are superimposed; during one of them a spontaneous min e.p.s.p. occurred (right). Rise time (10–90 %) of the response to the pipette-applied ACh is about 1·1 msec, and of the min e.p.s.p. about 0·75 msec. The preparation had been lightly treated with collagenase. This treatment removes some, but not all of the AChE. This accounts for the slightly slower than normal time course of both potentials, although no anticholinesterase was used.

muscle fibre was exposed by removing the motor axon together with its terminal synaptic boutons. An ACh pipette was positioned on to the synaptic membrane lining an empty crater that had previously been the seat of a bouton. By applying 1 msec current pulses of varying magnitude, a dose–response curve was obtained. Its linear portion had a slope of 6·5 mV/pC. The inset of Text-fig. 4 shows several samples of the potentials whose peak amplitudes were used in constructing the dose–response curve. The value in this example is among the highest obtained. In one set of experiments in which seven pipettes were tested on one snake preparation the mean post-synaptic sensitivity was 4 ± 1 mV/pC (mean $\pm$ 90 % con fidence limits).

The remaining task in determining the efficacy of ACh at the membrane is to find the number of ACh molecules that a pC of charge carries out through the tip of the micropipette.
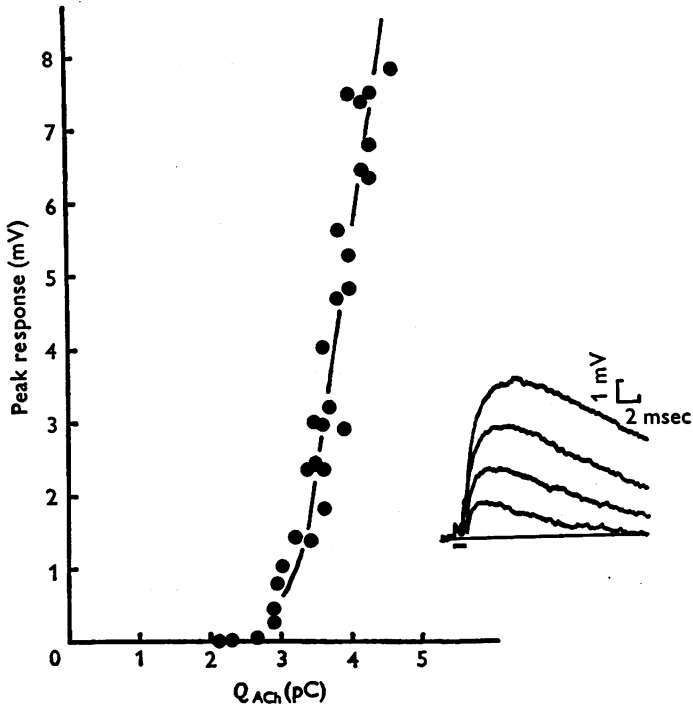
Text-fig. 4. Sensitivity of the post-synaptic membrane to iontophoretically applied ACh. The ACh pipette tip was placed on to a subsynaptic membrane area that had been exposed by removing the presynaptic terminal after treatment with collagenase. Acetylcholinesterase was inhibited with DFP. Dose–response curve (mV vs. pC) obtained by plotting the peak responses produced by varying 1 msec pulses of ACh. The linear part of the dose–response curve has a slope of 6·5 mV/pC. Inset, sample records of potentials from which the dose–response curve was constructed. The 1 msec pulse of current (bar) was varied in amplitude to pass 3·2, 3·6, 3·8 and 4·3 pC through the pipette.

Determination of the ACh output from pipettes

Our principal aim was to quantitatively determine the output of ACh from pipettes under conditions that were identical with those under which the post-synaptic sensitivities were determined. This required a highly sensitive assay.

Assay with radioactivity labelled ACh

We initially began our ACh output-determination experiments by using pipettes filled with 1 M tritiated ACh of the highest specific radioactivity that was commercially available (290 mCi/m-mole, labelled in the acetate moiety). The pipettes performed as usual in physiological tests; however

in order to obtain a sufficient output of ACh that could be reliably de-
tected in a scintillation counter, more than 40,000 pulses had to be collected
per sample. Under such conditions there was no easy way to know that
the pipette output had not changed during the protracted period of
pulsing (about 15 min per sample). Nevertheless, the results indicated
that for a pulse in the range of 10 pC, no more than 50,000–100,000 mole-
cules of ACh were released per pC of charge passed through the pipette.
These findings made us seek a more sensitive method, and at the same
time led us to a realization that what was desired was a system that
detected ACh concentrations rather than the absolute amounts of ACh.
This is because the concentration of ACh can, in principle, be greatly
increased merely by reducing the volume containing the ACh to be
measured. We therefore turned to a bio-assay procedure.

The bio-assay

The bio-assay which used the end-plate as an ACh-concentration de-
tector compared the depolarizing effect of ACh from two sources. One
source consisted of solutions of various known standard concentrations of
ACh. The other source consisted of solutions into which ACh had been
pulsed from the pipette and therefore contained unknown concentra-
tions. Samples from the two sources were tested alternately on the same
end-plate.

Standardization of the assay

Text-fig. 5 *A* provides an example in which droplets with a diameter of
105 μm (0·6 nl. in volume) were applied to the end-plate as sketched in
the inset (see also Text-fig. 2). Three concentrations, 4, 6 and 8 × 10⁻⁷ M,
ACh were used, and the peak depolarizations were plotted. Samples of the
responses are shown to the right of the curve. They rose to a peak in about
1 sec and reflect the rate of diffusion over the end-plate area. The small,
rapid deflexions on the base line and on the slow potentials are caused by
spontaneous min e.p.s.p.s whose peak heights are attenuated by the slow
response time of the pen recorder. Changing the diameter of the droplet
beyond 100 μm made only a small difference to the response heights,
indicating that the end-plate (usual diameter about 50 μm) was functioning
essentially as an ACh concentration detector. When droplets of Ringer
solution containing no ACh were applied, no responses were obtained.
This served as a control and indicated that the droplet by itself caused no
perturbation of the recording system. The concentration in the droplets
was converted into numbers of molecules of ACh and is indicated on the
lower portion of the graph. Thus, a 0·6 nl. droplet of 4 × 10⁻⁷ M ACh
contained 144 million molecules and produced a response of about 1 mV.
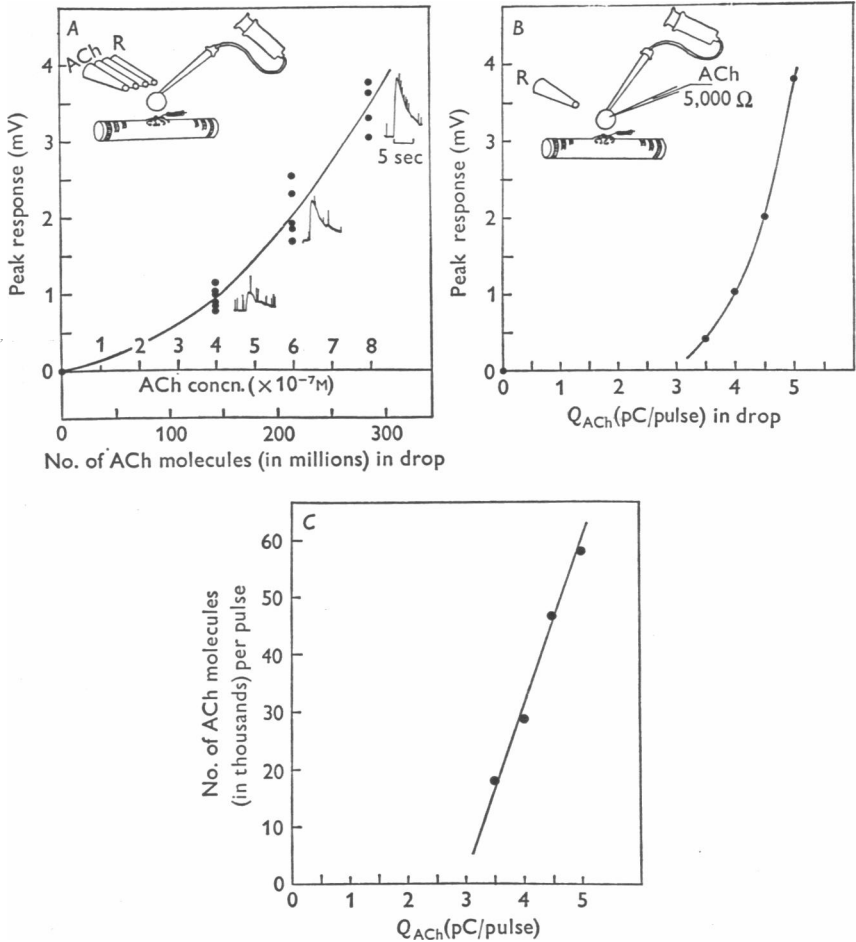
Text-fig. 5. Determination of the output of ACh from an iontophoretic pipette. This entire representative series was obtained from the same end-plate. *A*, standard curve for the assay. Three concentrations of ACh (0·2, 0·4, and 0·8 μM) were applied to the end-plate in droplets of fixed volume (0·6 nl.). No response was obtained when the droplet contained Ringer (*R*) solution only, and this serves as a control blank. The number of ACh molecules in each droplet was determined from the known ACh concentration and droplet volume. Some scatter was produced from the variation in response to identical concentrations. Note the rapid miniature potentials on the relatively slow ACh responses. *B*, pulsing of ACh into Ringer droplets. Each point represents the response to a 0·6 nl. droplet into which 5000 pulses of ACh had been released. Note zero pC pulse gives no response. *C*, the responses in *B* were converted directly into numbers of ACh molecules using the standard curve in *A* and are replotted to obtain the number of molecules per pulse as a function of pC of charge passed through the pipette. The slope indicates an essentially linear increase in output of ACh from the pipette with increasing dose; it provides a value of 28,000 molecules of ACh/pC.

Output from iontophoretic pipettes: the number of ACh molecules per pC

To determine the amount of ACh ejected from a pipette, positive current pulses of 1·0 msec duration and varying magnitude were passed through it while its tip protruded into a 0·6 nl. droplet of Ringer solution (Pl. 1). The droplet containing the unknown amount of ACh was then applied to an end-plate, and the resulting peak membrane depolarization was measured.

The tests illustrated in Text-fig. 5*B* were made on the same end-plate as the assay in Text-fig. 5*A*. Each point represents the quantity of ACh that was delivered into a droplet by 5000 pulses given at a rate of 40/sec over a period of 125 sec.

The quantity of ACh in each pulse did not change during this time, as tested in other experiments by direct iontophoretic application of the ACh to the subsynaptic membrane (see Methods). The charge passed through the ACh pipette was varied in steps of 0·5 pC, from 3·5–5·0 pC. In addition, a control was performed by inserting the pipette into a droplet for over 2 min without giving any pulses (zero pC point in Text-fig. 5*B*). As expected, no ACh output was measured provided the braking current was maintained at all times, even when the tip was in oil (see Methods). Output of ACh was detected, however, when the pulses were sufficiently large. For example, a drop of Ringer solution containing the ACh from 5000 pulses of 4 pC produced a potential change of close to 1 mV, similar to the response caused by the droplet containing 4×10^{-7} M ACh (or 144 million molecules of ACh) in Text-fig. 5*A*. When such a conversion is made for all the points in Text-fig. 5*B*, one obtains the number of ACh molecules delivered as a function of charge passed through the pipette, plotted in Text-fig. 5*C*. The output of ACh over the range of several pCs is linear with a slope of 28,000 molecules/pC. Thus, 28,000 molecules of ACh are discharged from the pipette per pC.

As seen from Text-fig. 5*B* and *C*, 3 pC pulses would have discharged no measurable amounts of ACh, because that much was needed to 'overcome' the braking current (cf. Text-fig. 4).

Variability of assays

The standard curves, such as shown in the sample experiment of Text-fig. 5*A*, were quite consistent; however, calibration curves for individual ACh pipettes were more variable. Fairly often, during the several hours required to complete an entire series, the fibre deteriorated, the properties of the pipettes changed, or the braking current of the pipette could no longer be maintained when the tip was in oil. When any one of these all too frequent discouraging events occurred, the experiment had to be

terminated before completing the series and the data were discarded. In experiments that were acceptable we calibrated eight different pipettes from as many different batches. Since the assay was time consuming, each pipette was calibrated only once with the expectation of obtaining a more representative sample. The measured ACh outputs from the eight pipettes ranged from 10,000 to 50,000 molecules/pC. The mean output was 30,000 ± 18,000 molecules/pC (mean ± 90 % confidence limits). This considerable scatter is greater than what might be expected from the sensitivity measurements that we have obtained (see above). We cannot easily explain this discrepancy until further improvements are made in the technique. Nevertheless, the average value of 30,000 molecules/pC is, at present, the best estimate we can make.

The number of molecules that produce the quantal e.p.s.p.

In the snake and frog the sensitivity of the post-synaptic membrane to iontophoretically applied ACh is about 5 mV/pC. This value, taken in conjunction with the average value of 30,000 molecules/pC for the ACh output from the pipettes, indicates that 6000 molecules of ACh are needed to produce a 1 mV potential. The justification for using the linear slope of the dose–response curve rests on our previous findings; that is, during the peak of its action the ACh released from a quantum does not saturate post-synaptic receptors, nor does it fall on to the potentiating portion of the curve, but fits on to the linear phase (Hartzell *et al.* 1975). When the AChE was inhibited, the mean min e.p.s.p.s in our preparations ranged between 1 and 3 mV with an average slightly above 1·5 mV. The average effect of a quantum, therefore, could be reproduced by approximately 10,000 molecules of iontophoretically applied ACh. For several reaons we conclude that the actual number of ACh molecules in a quantum is smaller than this (see Discussion).

DISCUSSION

The most attractive feature of the new assay is that it provides a direct determination of the number of molecules of ACh that are released from micropipettes. In addition, the bio-assay itself has some interesting features. The standard curve for the end-plate sensitivity to ACh (Text-fig. 5A) is non-linear. For example, doubling the dose of ACh from 0·4 to 0·8 μM increases the response from 1 to 3·5 mV. From this we see that the end-plate response is proportional to about $[ACh]^{1\cdot8}$. This observation confirms the findings in the preceding report (Hartzell *et al.* 1975). The high sensitivity of the assay allows one to detect at least 10^{-16} moles (sixty million molecules) of ACh, and in principle, by using small volumes

this assay could be used in other types of experiments where minute amounts of ACh have to be assayed.

The iontophoretic method provides an upper estimate. An over-estimate of the number of ACh molecules in a quantum is expected for several reasons. First of all, the responses to applied ACh are slower than the nerve-evoked potentials (Text-fig. 3). The major reason for this is that the pipette is almost certainly further away from the post-synaptic membrane than is the nerve terminal. Therefore, a larger quantity of ACh will be required from the pipette than from the terminal to produce a potential of the same peak height. Another possible reason for an over-estimate lies in the determination of the output of ACh from pipettes. We have shown that no leakage of ACh into the assay droplet occurred when no pulses were given (zero point in Text-fig. 5*B*). During a series of pulses, however, we do not know whether a small leakage occurs in the interval between pulses.

The fraction of the iontophoretic current that is carried by ACh. From the number of ACh molecules that are delivered per pC of total charge passed through the pipette, one can calculate that only about 1/600 of the total current is carried by ACh during the 1 msec pulse. This fraction is small for a number of reasons. For example, the ACh concentration in the tip of the pipette is expected to be considerably diluted by the braking current. Furthermore, in view of the high tip impedance of the pipettes some of the current must be capacitative. Thus, the fraction of the current carried by ACh probably varies, depending on, among other things, the type of pipette, its tip diameter, the amount of braking current, and the duration and the amplitude of the pulse. For this reason it was essential that the same pipettes and pulsing conditions be used both in the direct iontophoretic application of ACh on to the subsynaptic membrane and in the assay to determine the ACh output from the pipettes.

The amplification factor achieved by the gating action of ACh. By measuring the synaptic current produced by a quantum of transmitter, one can obtain the net number of charges passed across the membrane. Normally, with AChE fully active, the miniature excitatory post-synaptic current in snake fibres rises to a peak of about 3·5–4·0 nA in less than 0·5 msec and declines exponentially with a time constant (1/e) of about 1·5 msec (Hartzell *et al.* 1975). Thus, a net charge of about 5 pC is passed through the membrane during a min e.p.s.p. This is equivalent to thirty million univalent ions. Assuming 10,000 molecules/quantum we may conclude that the conductance change produced by one ACh molecule results in a net flux of more than 3000 univalent ions through the membrane. This ratio emphasizes the large amplification achieved by the valve or gating action of the ACh molecules in synaptic transmission. The effectiveness of a

quantum is increased about threefold when the activity of AChE is inhibited (Hartzell *et al.* 1975).

Other estimates of the number of ACh molecules per quantum. It may be useful to compare the present upper estimate of the number of molecules per quantum with data from experiments in which the release of ACh was measured in the diaphragm of the rat after stimulation of the phrenic nerve. For example, in relatively recent studies Potter (1970) used radioactively labelled ACh and found that about 4×10^6 molecules were released per end-plate for each impulse. The number of quanta per impulse released per end-plate in Potter's experiments is not known, but if one could assume that it was between 200 and 300 as suggested by Hubbard & Wilson (1973), one obtains an estimate between 12,000 and 22,000 ACh molecules for each quantum.

A likely minimum number of molecules per quantum was derived by Katz & Miledi (1972) from their analysis of ACh noise (see also Anderson & Stevens, 1973). They estimate that about 1,000 ionic conductance channels are opened up in the post-synaptic membrane by the action of a quantum of ACh at the frog neuromuscular junction. This indicates that at least 1,000 ACh molecules have to reach the subsynaptic membrane per quantum.

Quantal size and receptor density. Since we have shown that a quantum of transmitter does not saturate the post-synaptic receptors (Hartzell *et al.* 1975), it is of interest to compare our upper estimate of 10,000 molecules with the density of ACh receptors in the post-synaptic membrane. Studies with radioactively labelled α-bungarotoxin indicate that the end-plates of twitch muscles from rat and mouse have 7000–30,000 toxin-binding sites/μm^2 (Barnard, Wieckowsky & Chiu, 1971; Fambrough & Hartzell, 1972; Fertuck & Salpeter, 1974). Thus, assuming that each toxin-binding site represents a site on the receptor for binding ACh as well, it appears that 1 μm^2 of post-synaptic membrane can accommodate all of the molecules of ACh that are released in a quantum.

Quantal size and the concentration of ACh in the synaptic cleft. In our previous report (Hartzell *et al.* 1975) we estimated that a quantum of transmitter covers a post-synaptic area less than 2 μm^2. If the actual area involved were, say, 1 μm^2, then the volume of a 50 nm-wide synaptic cleft over this area would be 5×10^{-17} l. If, at the peak of the quantal synaptic current, 10,000 ACh molecules were distributed uniformly in this volume, the ACh concentration would be about 3×10^{-4} M. Although this is a rather a high concentration, ACh receptors at the post-synaptic membrane would not be saturated, since, as discussed above, there are probably more receptors in 1 μm^2 of subsynaptic membrane than ACh molecules in a quantum.

Quantal size and synaptic vesicles. A parallel question relates to the storage of ACh in the presynaptic vesicles. If 10,000 molecules were fitted into a vesicle with an inner diameter of, say, 50 nm, the ACh concentration would come to 260 mM; and for a vesicle of 40 nm diameter it would be 505 mM. If the contents of a 50 nm diameter vesicle were iso-osmotic with snake Ringer (about 320 m-osmole), it could accommodate about 6000 ACh molecules and 6,000 counterions. In this context one should note that Sheridan, Whittaker & Israel (1966) found that the synaptic vesicles of the electric organ of *Torpedo* contained an ACh concentration that is iso-osmotic with plasma.

From the present data we conclude that a maximum of 10,000 molecules is released into the synaptic cleft during a quantal event. Further refinements in the comparison of iontophoretic and nerve-released ACh responses, as well as in the ACh assay system, may improve the estimate.

REFERENCES

ANDERSON, C. R. & STEVENS, C. F. (1973). Voltage-clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol.* **235**, 655–691.

BARNARD, E. A., WIECKOWSKI, J. & CHIN, T. H. (1971). Cholinergic receptor molecules and cholinesterase molecules at mouse skeletal muscle junctions. *Nature, Lond.* **234**, 207–209.

DALE, H. H. (1965). *Adventures in Physiology.* London: William Clowes & Sons.

DENNIS, M. J., HARRIS, A. J. & KUFFLER, S. W. (1971). Synaptic transmission and its duplication by focally applied acetylcholine in parasympathetic neurones in the heart of the frog. *Proc. R. Soc.* B. **177**, 509–539.

FAMBROUGH, D. M. & HARTZELL, H. C. (1972). Acetylcholine receptors: number and distribution at neuromuscular junctions in rat diaphragm. *Science, N.Y.* **176**, 189–191.

FERTUCK, H. C. & SALPETER, M. M. (1974). Localization of acetylcholine receptor by ^{125}I-labelled α-bungarotoxin binding at mouse motor end-plates. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1376–1378.

HARTZELL, H. C., KUFFLER, S. W. & YOSHIKAMI, D. (1975). Post-synaptic potentiation: interaction between quanta of acetylcholine at the skeletal neuromuscular synapse. *J. Physiol.* **251**, 427–463.

HUBBARD, J. I. (1973). Microphysiology of vertebrate neuromuscular transmission. *Physiol. Rev.* **53**, 674–723.

HUBBARD, J. I. & WILSON, D. F. (1973). Neuromuscular transmission in a mammalian preparation in the absence of blocking drugs and the effect of D-tubocurarine. *J. Physiol.* **228**, 307–325.

KATZ, B. (1969). *The Release of Neural Transmitter Substances.* Liverpool: Liverpool University Press.

KATZ, B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol.* **224**, 665–699.

KUFFLER, S. W. & YOSHIKAMI, D. (1975). The distribution of acetylcholine sensitivity at the post-synaptic membrane of vertebrate skeletal twitch muscles: ionto-phoretic mapping in the micron range. *J. Physiol.* **244**, 703–730.

KRNJEVIĆ, K. (1974). Chemical nature of synaptic transmission in vertebrates. *Physiol. Rev.* **54**, 419–540.

KRNJEVIĆ, K. & MILEDI, R. (1958). Acetylcholine in mammalian neuromuscular transmission. *Nature, Lond.* **182**, 805–806.

McMAHAN, U. J. & KUFFLER, S. W. (1971). Visual identification of synaptic boutons on living ganglion cells and of varicosities in postganglionic axons in the heart of the frog. *Proc. R. Soc.* B. **177**, 485–508.

McMAHAN, U. J., SPITZER, N. C. & PEPER, K. (1972). Visual identification of nerve terminals in living isolated skeletal muscle. *Proc. R. Soc.* B **181**, 421–430.

MILEDI, R. (1962). Induction of receptors. In *Ciba Foundation Symposium on Enzymes and Drug Action*, ed. MONGAR, J. L. & DE REUCK, A. V. S., pp. 220–235. London: J. and A. Churchill.

NASTUK, W. L. (1953). Membrane potential changes at a single muscle end-plate produced by transitory application of acetylcholine with an electrically controlled microjet. *Fedn Proc.* **12**, 102.

PEPER, K. & McMAHAN, U. J. (1972). Distribution of acetylcholine receptors in the vicinity of nerve terminals on skeletal muscle of the frog. *Proc. R. Soc.* B. **181**, 431–440.

POTTER, L. T. (1970). Synthesis, storage and release of [^{14}C]acetylcholine in isolated rat diaphragm muscles. *J. Physiol.* **206**, 145–166.

SHERIDAN, M. N., WHITTAKER, V. P. & ISRAEL, M. (1966). The subcellular fractionation of the electric organ of *Torpedo*. *Z. Zellforsch. mikrosk.* **74**, 291–307.

EXPLANATION OF PLATE

Micrograph of a droplet of Ringer into which ACh is being pulsed. The 105 μm sphere is suspended in oil from the transfer pipette (right) from which it had been extruded. The fine tip of the ACh pipette (lower left) and the blunter NaCl grounding micro-electrode (lower right) protrude into the droplet. They pull slightly on the droplet, and therefore the transfer pipette appears a little off-centre. This micrograph was taken with bright field instead of Nomarski optics for greater focal depth. Blurred specks are dust particles in the optics.