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THE EFFECT OF ACETYLCHOLINE ON TOUCH RECEPTORS IN FROG'S SKIN

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A wide variety of sensory receptors appears to be sensitized by the direct action of acetylcholine. In many respects their pharmacological properties appear similar to those of autonomic ganglia, in that the action of ACh is paralleled by nicotinic substances, and inhibited by those of the ganglion-blocking type. There is strong evidence, however, that ACh is not directly concerned in the initiation, by the natural stimulus, of impulses at sensory endings, since ganglion-blocking drugs inhibit the ACh response without interfering with the response to the natural stimulus (Douglas, 1952; Douglas & Gray, 1953; Diamond, 1955). This poses two problems: (1) the possible physiological significance of the ACh sensitivity of sensory endings; (2) the mode of action of ACh. No definite answer has been given to the first of these; the second remains controversial since the various experimental techniques applied to different preparations have given results which are apparently inconsistent with one another.

The experiments described in this paper show that ACh sensitizes touch receptors in frog's skin, and support the view that ACh is not normally concerned in the initiation of impulses. Its mode of action has been analysed by quantitative measurements made on isolated touch receptors functioning in the presence of ACh.

METHODS

English frogs were used throughout. Preparations for whole-nerve recording were made from the skin over the dorsal lymph-sac, together with its cutaneous nerves. The skin was mounted, inner side uppermost, on a platinum-covered (earthed) cork base, and the whole preparation was immersed in liquid paraffin. Action potentials were recorded from the cutaneous nerves through a pair of platinum electrodes.

For single-fibre recording, preparations were made from the skin over the gastrocnemius muscle. This was isolated, together with its nerve supply, and about 0·5 cm of single axon was dissected free at the junction of the cutaneous branch with the tibial nerve. The largest axons (9–12 μ) were selected as most probably subserving touch. The preparation was mounted on an air-gap insulator, a microscope being used to ensure that no node of Ranvier remained in the air-gap. The design of

the insulator was based on that of Tasaki (1953) and the construction was of Perspex, rigidly mounted on a heavy iron base which also supported the crystal stimulator used in most experiments (Fig. 1).

The skin was stimulated by a fine glass stylus attached to one corner of a Rochelle salt crystal mounted as described by Gray & Malcolm (1950). Both square and linearly increasing voltage pulses could be applied to the crystal, causing corresponding movements of the stylus. Oscillation of the crystal was damped by a drop of oil on the free corner; the appropriate viscosity was discovered by trial while observing the voltage output of a second crystal, similarly damped, driven by the first crystal. Under optimal conditions, a 'square-pulse' from the crystal had a rising time of 0·15–0·25 msec.

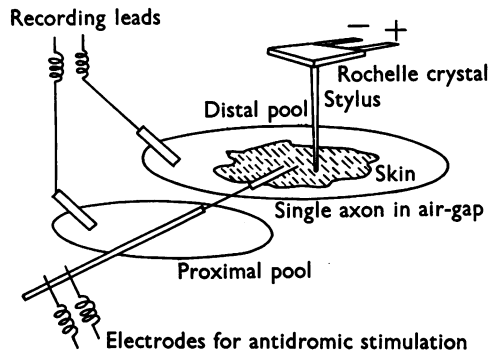

Fig. 1. A diagram showing the arrangement of the preparation and stimulator on the bridge-insulator.

The recording electrodes were silver/silver-chloride and led to a cathode-follower through low-capacity leads with cathode-coupled screening. Grid current was less than 10^{-12} A. The output of the cathode-follower fed an oscilloscope through appropriate amplifiers.

Before mounting the preparation, the skin was lightly scraped on the inside to remove excess mucus-like material, and soaked for 15–20 min in a large volume of Ringer's solution. Without this treatment ACh effects were erratic if present at all. The quantity of Ringer's solution added to the distal pool was known, since drugs were added in small quantities in concentrated form to the solution already present. The solution could not be removed without risk of some minor movement between skin and stylus, resulting in an apparent shift in threshold.

The Ringer's solution used contained: NaCl, 0·675%; KCl 0·015%; $CaCl_2$, 0·02%. ACh concentrations are given in terms of the iodide (mol. wt. 273·1) which was freshly dissolved for each experiment.

RESULTS

Initiation of impulses

The observations on the initiation of impulses by ACh were made on the skin from over the dorsal lymph sac of the frog, with the intact cutaneous nerves attached. The application of 10^{-4} g/ml. ACh was invariably followed by the appearance of a volley of impulses in the corresponding cutaneous nerve, followed not only by insensitivity to further applications of ACh but also by insensitivity to mechanical stimulation. Fig. 2 shows records of this phenomenon. A just supra-liminal mechanical stimulus was applied to the skin, and the resulting action potential was recorded. The stimulator was then switched

off, without interfering in any way with the skin or the position of the stylus. When the recording paper had been set moving at 1 in./sec, the skin round the tip of the stylus was flooded with 10^{-4} g/ml. ACh, producing the volley of impulses shown. Fig. 2 also shows that the preparation was rendered mechanically inexcitable after this treatment, but that this block was reversible, disappearing after 15–20 min washing with normal Ringer's solution. It may be objected that the washing had altered the relative positions of the skin and stylus, and a new tactile ending, previously unaffected by the ACh, was now being stimulated. The single axon preparation was therefore used to discover whether the block of normal function produced by excess ACh was genuinely reversible. In this method there is no paraffin to interfere with free

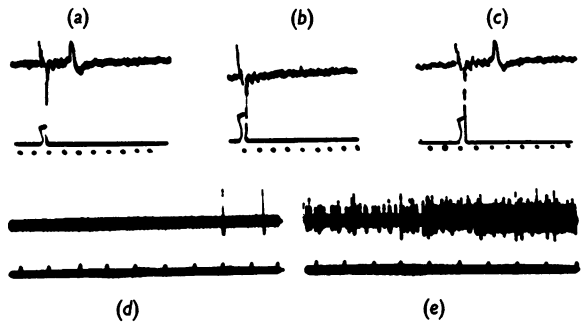

Fig. 2. Records from whole cutaneous nerve. (*a*)–(*c*) Upper trace, nerve recording; lower trace, electrical pulse to crystal; time-marks 1 msec. (*a*) Normal response in Ringer's solution; (*b*) mechanical insensitivity after 10^{-4} g/ml. ACh; (*c*) normal mechanical sensitivity after washing in Ringer's solution. (*d*)–(*e*) Upper trace, nerve recording; lower trace, time-marks 0·2 sec. (*d*) Control; (*e*) 5 sec after addition of 10^{-4} g/ml. ACh.

diffusion, and the 5–10 endings subserved by one axon (Marahashi, Mizuguchi & Tasaki, 1952) are certainly subjected to the same conditions. After this preparation had been rendered mechanically inexcitable by 10^{-4} g/ml. ACh, prolonged washing with Ringer's solution reversed the effect. The disadvantage of this preparation was that washing inevitably altered the relative positions of skin and stylus so that an apparent change in threshold was always observed. Nevertheless, the reversibility of the effect has been qualitatively, if not quantitatively, established.

Nicotine was found to have precisely the same actions as ACh; a concentration of 10^{-4} g/ml. excited a volley of impulses and then rendered the skin unresponsive to any further stimulation, chemical or mechanical. Tubocurarine in a concentration of 10^{-6} g/ml. did not affect the response of the skin to its natural mechanical stimulus, but prevented the response to applied ACh. The presence of 10^{-5} g/ml. atropine did not change any of the effects so far described, and had no demonstrable action of its own.

Threshold

The single-fibre preparation was always used when measurements of threshold were required. When a satisfactory preparation had been dissected out and set up on the bridge insulator, the approximate position of its receptive field was found by moving a light glass rod over the skin until action potentials were recorded. The stylus attached to the piezo-electric crystal could then be mounted over this point.

The threshold of the ending was found by determining the magnitude of the least mechanical square pulse of length 0·4 msec which would produce an action potential in the single axon; this magnitude was determined by reading the calibrated step-by-step potentiometer controlling the amplitude of the electrical pulse driving the piezo-electric crystal. After careful exclusion of all possible extraneous sources of mechanical interference or instability, the spontaneous variation in the threshold of the receptor was less than the discrimination of the measuring apparatus. In each preparation the threshold in

TABLE 1. Effect of ACh on threshold of frog's skin

Preparation no.	No. of determinations	Threshold stimulus in 10^{-6} g/ml. ACh (% of control)	Percentage reduction in threshold
1	6	92	8
2	6	89	11
3	6	91	9
4	12	88	12
5	12	92	8
6	12	88	12
		Mean reduction in threshold	10
		Standard deviation	1·9

Ringer's solution was determined either six or twelve times, according to what other measurements were to be made. When this number of control threshold determinations had been made, one drop of 10^{-4} g/ml. ACh solution was added to the distal pool, thus bathing the skin in 10^{-6} g/ml. ACh. After allowing 5–10 min for diffusion, the threshold was redetermined.

A comparison between the control threshold and that in the presence of ACh was prevented in many experiments by extraneous mechanical interference. In the six preparations listed in Table 1, however, complete determinations of the thresholds in Ringer's solution and in 10^{-6} g/ml. ACh were made. This represents, in all, 108 observations of threshold, 54 in both Ringer's solution and in ACh. In every instance the threshold in the presence of ACh was reduced below that of the control. The reduction ranged from 8–12% with a mean of 10% (S.D. 1·9).

Latency

Gray & Malcolm (1951) showed that in the touch receptor of frog's skin the latency between the application of the stimulus and the appearance of an

action potential varied inversely as the size of the stimulus. It was of interest in the present investigations to determine whether or not this relationship was affected in any way by the presence of ACh.

A two-channel oscilloscope was used in these latency experiments, one channel recording the electrical pulse to the piezo-electric crystal, the other the electrical response of the single axon. The latency could thus be determined by direct measurement from a photograph.

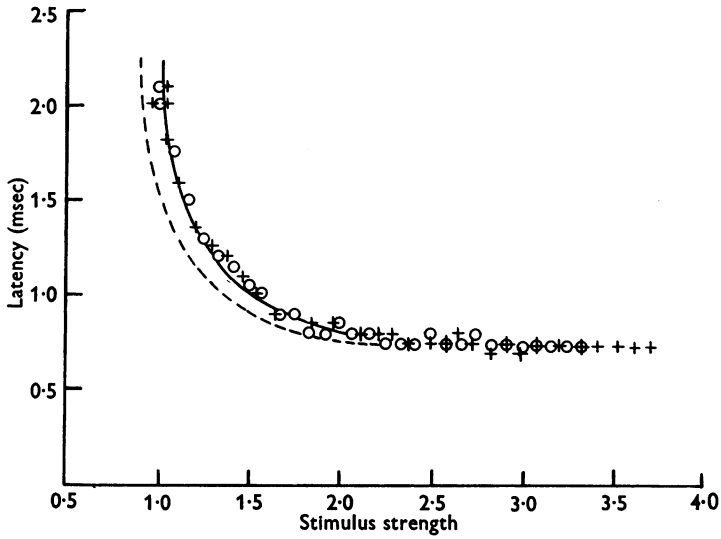

Fig. 3. Latency curves for the receptor in Ringer's solution (○) and in 10^{-6} g/ml. ACh (+). Ordinate: latency, msec. Abscissa: stimulus strength in multiples of the threshold. The dotted line indicates the position of the latency curve in ACh when stimulus strength is plotted in terms of the control (Ringer's) threshold.

The control threshold was determined in the usual way, and then the size of the mechanical pulse was increased until the limit of the stimulator had been reached (i.e. approximately 4 × threshold). At each step of the potentiometer a photographic record was made, relating latency to that particular stimulus strength. When the complete relationship had been determined in Ringer's solution, ACh was added, making the distal pool concentration 10^{-6} g/ml., and the whole procedure was repeated.

To compare the curves of latency against stimulus strength for the receptor in Ringer's solution and in 10^{-6} g/ml. ACh, the change in threshold had to be allowed for. When this was done by plotting each curve in terms of its own threshold, the curves were identical. Three such curves were obtained from three preparations, of which Fig. 3 is typical. When all stimulus strengths were expressed in terms of the appropriate threshold, all points were found to

fall along one curve. The interrupted line in Fig. 3 represents the position of the latency curve in ACh if plotted in absolute units. The shift between this and the control curve is purely horizontal, representing the threshold change; there is no shift along the time axis. Hence, ACh does not affect the time-course of the processes underlying the initiation of an impulse.

Rate of recovery

After the initiation of an impulse, the threshold of a touch ending is for a brief period infinitely high (absolute refractory period) and then returns to normal. The time-course of this process constitutes a recovery curve. Experiments were done to discover whether it was altered by the presence of ACh.

Conditioning action potentials were initiated in the ending by stimuli of constant strength, and the threshold of the ending was determined after various intervals. The size of the test pulses was known from the calibration of the controlling potentiometer; the time intervals between the two action potentials were recorded photographically from the oscilloscope. This interval between the first (conditioning) action potential and the second (test) action potential was measured from the trace as the time interval *BD* (Fig. 4) and this value plotted against the threshold of the second action potential.

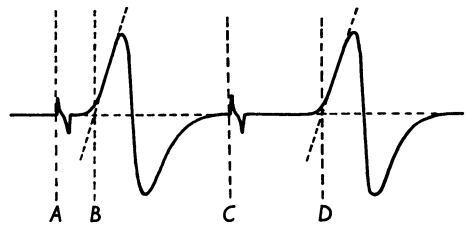

Fig. 4. Diagrammatic representation of the oscilloscope traces in determining rates of recovery. *A*, conditioning pulse artifact; *AB*, latency of supra-liminal conditioning action potential; *C*, test-pulse artifact; *CD*, threshold latency of test action potential.

When a complete recovery curve for an ending had been obtained in Ringer's solution, the whole procedure was repeated in the presence of 10^{-6} g/ml. ACh. For the two recovery curves to be comparable, allowance had to be made for the shift in threshold, as in the case of the latency curves, by expressing each in terms of its own threshold.

When this was done, the curves were found to be identical. Three such experiments were completed, of which the results shown in Fig. 5 are typical. If the ACh recovery curve is expressed in absolute units, as in the interrupted line in Fig. 5, there is a vertical shift corresponding to the change in threshold, but no shift along the time axis. Hence, the recovery of excitability after the initiation of an impulse is unaffected by ACh.

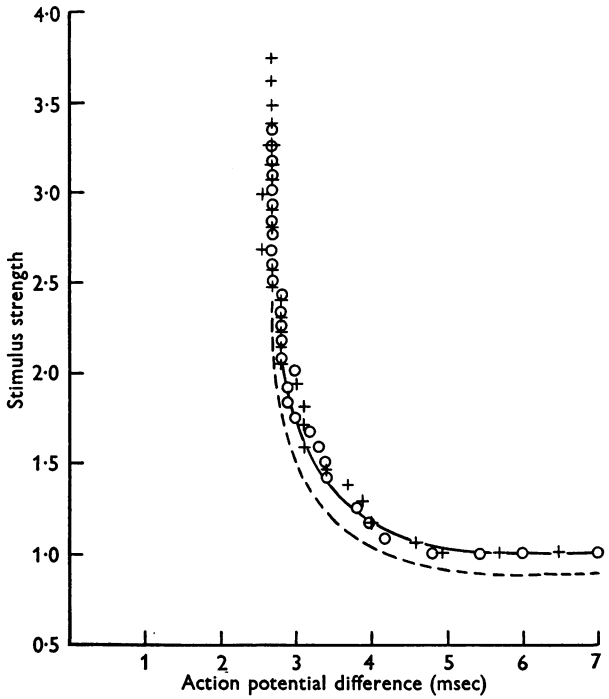

Fig. 5. Recovery curves for the receptor in Ringer's solution (○) and in 10^{-6} g/ml. ACh (+). Ordinate: stimulus strength in multiples of the threshold for that particular run. Abscissa: interval between action potentials, msec. The interrupted line indicates the position of the recovery curve in 10^{-6} g/ml. ACh when plotted in terms of the control (Ringer's) threshold.

Adaptation

In all the experiments described so far, 'square pulses' (see Methods) were used, and the minimum deformation of this type necessary to initiate an impulse has been termed the 'threshold of the ending'. If linearly-increasing pressures are applied to the ending, however, it is found that it withstands greater degrees of deformation before generating an action potential. There is a gradient below which no response can be evoked, regardless of the extent of the final deformation. This gradient is termed the 'critical slope', and is taken as a measure of the ending's ability to adapt to slowly rising pulses.

Experiments were carried out to see whether the critical slope was affected by ACh. A preparation was set up and its threshold to a square pulse of 1 msec duration determined six times as described above. To determine the critical slope, a triangular pulse with a rising time of about 60 msec was used; its amplitude was then gradually increased until an action potential appeared in the single axon (Fig. 6). Both this action potential and the shape of the

applied pulse were recorded oscillographically. The critical slope was determined six times, and the constancy of the square-pulse threshold was checked by a further six determinations. The determinations of the critical slope were expressed in terms of rheobases/sec (the rheobase being taken as approximately equal to the threshold to the square pulse of 1 msec duration (Gray & Malcolm, 1951)), and a mean critical slope was calculated.

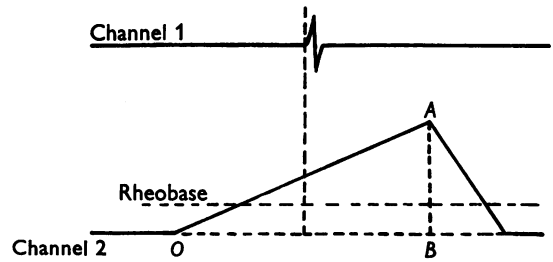

Fig. 6. The determination of the critical slope. The amplitude *BA* is increased until an action potential is recorded in channel 1. *AB/OB* is the critical slope, and is expressed in rheobases/sec.

TABLE 2. Effect of ACh on the reaction of skin to slowly-rising stimuli

No. of preparation	Mean critical slope in Ringer's solution (rheobases/sec)	Mean critical slope in 10^{-6} ACh (rheobases/sec)	Difference (rheobases/sec)	S.E. of difference
4	47	62	15	0·9
5	60	72	12	1·3
6	52	67	15	0·8

This whole procedure was then repeated in the presence of 10^{-6} g/ml. ACh, and a corresponding mean critical slope calculated, taking as rheobase in this case the threshold for the 1 msec square pulse with the preparation in ACh. Complete determinations, giving control mean critical slopes and mean critical slopes in ACh, were carried out in three preparations, and the results are tabulated in Table 2. Table 2 shows that in every preparation the presence of 10^{-6} g/ml. ACh brought about a highly significant increase in the mean critical slope, i.e. the ending adapted more rapidly.

Atropine

The best way of assessing any possible effect of atropine on these results would have been to add atropine to the distal pool after a complete experiment, and repeat all estimations on the same preparation. Unfortunately this was not possible, for ACh once added to the pool could not be removed without some movement between skin and stylus resulting in an apparent shift in threshold. The experiments done on preparations nos. 2, 3 and 6, therefore,

were carried out in the presence of 10^{-5} g/ml. atropine. This had no effect of its own, and did not affect the action of ACh qualitatively. By comparing preparations 2, 3 and 6 with preparations 1, 4 and 5 it is seen that atropine had no significant quantitative effect on the actions of ACh.

DISCUSSION

The results recorded above differ from those of Habgood (1950), who was unable to detect any effect of ACh applied to frog's skin. The effectiveness of applied ACh depends upon the method of preparing the skin; in all my experiments the skin was lightly scraped to remove excess mucoid material and then soaked in Ringer's solution for 15–20 min. When this was not done the effects of ACh were irregular. This scraping can be carried too far; Adrian (1931) has shown that the touch responses can be abolished in this manner, but Feng (1933) states that excitability returns after soaking in Ringer's solution.

There seems to be no doubt that the effects recorded were due to ACh acting upon the sensory nerve terminals. It had no effect on the nerve trunk, as was shown by placing the ACh in the proximal instead of the distal pool (Fig. 1), where it had direct access to the fibres since the nerve-sheath had been removed.

The only other structure likely to be affected by the ACh is smooth muscle, which is found in the skin in association with small vessels and mucus glands (Ecker & Wiedershein, 1896). The effects of ACh were in no way affected by the presence of 10^{-5} g/ml. of atropine, and it appears unlikely therefore that the observed effects could have been secondary to muscarinic effects on structures in the skin other than sensory nerve endings (Dale, 1914).

The effect of ACh on the cutaneous nerve endings is to increase their sensitivity to the natural stimulus of deformation of the skin. This reduction in threshold could be brought about if the specialized cutaneous terminals were depolarized by the ACh. In the two mechano-receptors where recording has been possible, the muscle spindle (Katz, 1950) and the Pacinian corpuscle (Gray & Sato, 1953), it has been shown that mechanical deformation gives rise to a local, graded depolarization from which, at a critical voltage, arises the propagated, all-or-nothing nerve action-potential. If the terminal portion of the frog's skin touch receptor is specialized in the same way, the actions of ACh can be understood by postulating that this specialized portion is also susceptible to depolarization by ACh. An alternative, though less likely, explanation is that the ACh lowered the critical voltage at which propagation takes place. Such evidence as is available, however, favours depolarization as being the explanation. The lowered threshold was accompanied by an increased rate of adaptation and, as Skoglund (1942) has pointed out, any process reducing the membrane potential increases accommodation in nerve. Similarly, Tasaki (1950) has shown that catelectrotonus of single nerve fibres results in an increased accommodation rate to exponentially increasing currents.

If similar processes are involved in the adaptation of the frog skin touch receptors, then the increased rate of adaptation which has been observed is consistent with the postulated lowered membrane potential.

The application to the skin of higher concentrations of ACh evokes a discharge of impulses and, eventually, a condition of block in which the skin is insensitive both to ACh and to natural stimuli. Identical effects are produced by nicotine, but those of tubocurarine are quite different. This substance renders the nerve endings insensitive to chemical stimulation by ACh, but leaves their response to deformation of the skin entirely unaltered. These facts suggest that the chemical and mechanical stimuli act on the ending independently, i.e. ACh does not normally act as a mediator in the initiation of an impulse by a mechanical impulse (Diamond, 1955). This confirms the original findings of Brown & Gray (1948) on the action of ACh on the mechano-receptors in the skin and mesentery of the cat. It must be assumed that the

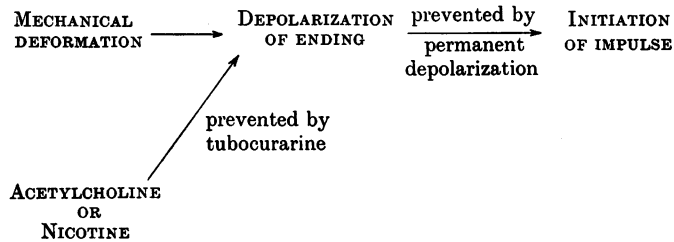

Fig. 7. Diagrammatic summary of suggested hypothesis.

initiation of impulses in sensory endings by ACh is a fortuitous consequence of its depolarizing action, and plays no part in the normal functioning of the ending.

It is then possible to formulate a consistent scheme for the effects of ACh on sensory receptors (Fig. 7). This scheme is consistent with the reported effects of ACh on a wide variety of receptors. Stimulation by ACh, followed by blocking of the natural stimulus by higher concentrations, has been reported in cold receptors in human skin (Bing & Skouby, 1950); thermal receptors in mammalian tongue (Dodt, Skouby & Zotterman, 1953); and for taste and touch receptors in frog's tongue (Landgren, Liljestrand & Zotterman, 1953). In mammalian skin and mesentery mechano-receptors (Brown & Gray, 1948) and in carotid sinus baroceptors (Diamond, 1955) it appears impossible to block the natural stimulus with an excess of ACh, although excess ACh prevents further excitation by the drug itself. It may be that in receptors of this type ACh block is non-depolarizing (cf. tubocurarine) rather than depolarizing (Thesleff, 1955), and it can be seen from Fig. 7 that this would not be expected to block the natural stimulus.

Many other explanations of ACh's action at sensory endings have been proposed. They have been based, in the main, on results from preparations containing many sensory units, and are not consistent with the findings in frog's skin. (1) It has been suggested that ACh acts by increasing the number of active sensory units. This is clearly inapplicable to the present findings, based as they are on the responses of an isolated unit. (2) An accelerated rate of recovery has been put forward to explain the action of ACh. In my experiments, however, it has been shown that the rate of recovery after an impulse was unaffected by ACh. (3) A diminished accommodation rate has been proposed as a possible mode of action. My experiments show that the reverse of this in fact takes place. (4) Skouby (1951) has suggested that the action of ACh at sensory endings may be due to 'improved transmission in the nerve trunk'. This implies an increased propagation velocity with consequent increased rate of arrival of impulses at the central nervous system; this might well explain the increase in subjective pain which Skouby measured, but cannot account for the objective findings in frog's skin. On the other hand, Skouby's results can be interpreted equally well by supposing that ACh reduced the threshold of the pain receptors with a consequent increase in the rate of firing (Adrian, 1928). (5) Bing & Skouby (1950), who described the sensitization of cold receptors in human skin by ACh, rejected the possibility that this was due to an excitatory action, since no concentration of ACh would initiate a cold sensation in the absence of a natural stimulus. The ACh they injected, however, was just as likely to initiate impulses from warmth as from cold receptors (Dodt *et al.* 1953) and, since their measurements were subjective, under these conditions it is reasonable to expect no change in thermal sensation.

SUMMARY

1. Acetylcholine has been shown to excite impulses in the cutaneous nerves of frogs when applied to the skin in a concentration of 10^{-4} g/ml. After the discharge of impulses had ceased the skin was found to be insensitive to mechanical stimuli. This block was reversible, disappearing after 15 min washing in Ringer's solution.

2. The presence of 10^{-6} g/ml. acetylcholine reduced the threshold to mechanical stimuli by a mean value of 10% (six experiments, range 8–12%).

3. Acetylcholine had no effect on the latency of impulse initiation or on the time-course of recovery of excitability after the passage of an impulse.

4. The critical slope for mechanical excitation (expressed in rheobases/sec) was significantly increased by acetylcholine (10^{-6} g/ml.).

5. Atropine did not affect the results. Tubocurarine prevented the actions of acetylcholine, but had no effect on the response to mechanical stimulation.

6. The site and mode of action of acetylcholine are discussed, and it seems probable that acetylcholine depolarizes the sensory receptor.

I am indebted to Mr P. Bell and Mr R. Featherstone for help with the apparatus. My thanks are due to Professor G. L. Brown for advice and encouragement throughout. To Dr J. A. B. Gray I am especially grateful for his guidance, both theoretical and technical, and for his constructive discussion of the manuscript.

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