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REVERSIBLE BLOCK OF AXONAL CONDUCTION BY CURARE AFTER TREATMENT WITH COBRA VENOM*†

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Abstract—After treatment of squid giant axon with cobra venom (CV), cetyltrimethylammonium chloride (CTA) or a combination of the two, *d*-tubocurarine (curare) caused reversible block of conduction at concentrations as low as 5×10^{-4} M. On control axons, $1{\cdot}4 \times 10^{-2}$ M curare had little effect on electrical activity. Other compounds rendered active by the treatment included chlorisondamine and protamine; the actions of physostigmine and di*iso*propylfluorophosphate were potentiated. Several quaternary ammonium compounds, among them prostigmine and acetylcholine, were not rendered active after pretreatment. Most tertiary amines affected the untreated squid axon action potential in almost the same concentrations that affect the synapses of the single electroplax. In contrast to lipid-insoluble quaternary ammonium compounds, which did not significantly alter the action potential of the untreated axon, three lipid-soluble quaternary compounds were as effective on the axon as on other conducting membranes. The results indicate that a strong lipid barrier surrounds the squid axon. A mechanism for the breakdown of this barrier by CV and CTA is discussed. Possible explanations are presented for the inability of this treatment to permit effects of some lipid insoluble quaternary compounds.

INTRODUCTION

NACHMANSOHN proposed in 1940 that the acetylcholine (ACh) system plays a central role in the conduction of impulses in nerve axons, and much experimental support for this theory has accumulated in the following two decades.[1] According to the theory, ACh released during activity changes the ionic permeability of the conducting membrane by its action on a receptor protein. The rapid inactivation of the ester by cholinesterase permits the receptor protein to return to its resting condition.

One of the major objections to the theory has been the failure of *d*-tubocurarine (curare), ACh, and other quaternary ammonium compounds to affect conduction in nerve axons, in contrast to their powerful actions on junctions. These findings have been explained by Nachmansohn on the basis of the existence of barriers, primarily lipoidal in nature, which prevent lipid-insoluble quaternary compounds from penetrating to the active membrane. Experimental evidence for the existence of barriers has been obtained by the demonstration that ACh labeled with ^{15}N and neostigmine

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applied to squid giant axons in high concentrations failed to penetrate to the axoplasm, whereas the lipid-soluble tertiary ammonium compounds physostigmine and trimethylamine were found in the axoplasm.[2, 3] Moreover, lipid-soluble nitrogen derivatives affect electrical activity of axons. Some of them, such as physostigmine and the tertiary analog of prostigmine, are known to have a relatively high affinity for cholinesterase, while others, such as procaine and tetracaine, have a high affinity for the receptor protein. The effectiveness of the local anesthetics procaine, tetracaine, and dibucaine in blocking the electrical activity of the conducting membrane of the electroplax[4] parallels their capacities for binding to the receptor protein, which has been isolated from electric tissue of electric eel.[5]

In recent years, however, evidence has been obtained that under certain conditions even quaternary ammonium derivatives may act directly on axonal conduction. For example, Dettbarn[6] demonstrated that curare rapidly and reversibly blocks electrical activity at Ranvier nodes of a single sciatic nerve fiber where, according to electron microscope studies,[7] the axonal membrane is covered only by a very thin and porous structure. Walsh and Deal[8] have reported that, after exposure of frog sciatic nerves to the detergent cetyltrimethylammonium bromide (CTAB), axonal conduction was reversibly blocked by curare, ACh, prostigmine and other quaternary ammonium derivatives. Armett and Ritchie[9] found a direct effect of ACh on *C*-fibers of the rabbit's desheathed vagus nerve.

The present experiments were designed to reduce the permeability barriers surrounding the axon in order to test whether lipid-insoluble compounds which are highly active at the synapse, but ineffective on intact axons, would become effective. A variety of enzyme and detergents were used for this purpose.

In addition, various lipid-soluble compounds were tested, and the strength of their action on axonal conduction was compared with that found previously on the synaptic junctions of the electroplax.[4, 12] Most of these compounds react with a protein, isolated by curare precipitation in solution from electric tissue.[5, 10–12] This protein shows an affinity to many compounds which parallels their effectiveness in blocking electrical activity of isolated single electroplax, and thus it has an important characteristic which would be expected of an ACh-receptor protein.[4, 5, 10–13]

The squid giant axon was used in these studies because it is a single fiber and is non-myelinated, i.e., it is surrounded by a relatively thin lipid membrane.[14, 15] This preparation would be expected to be more sensitive to the effects of enzymes and detergents than a nerve bundle. Effects observed would be less difficult to interpret than if a multi-fibered preparation were used.

MATERIALS AND METHODS

The majority, although not all, of the small nerve fibers adjacent to the giant axon of squid (*Loligo pealii*) were removed. Removal of all the small fibers was considered infeasible, since the axons survived longer when the time required for dissection was kept to a minimum. The giant axon was ligated at the stellate ganglion and about 5 cm posterior to the ganglion. It was then freed from the mantle and placed in a chamber having a capacity of about 20 ml. The entire procedure took about 1 hr.

The axon was attached by threads to stainless steel hooks which maintained it submerged under sea water in the nerve chamber. The nerve was placed in such a fashion that when the sea water was removed it rested on five external Ag–AgCl

electrodes: two stimulating, two recording, one ground. Every 5 or 10 min the action current was recorded by removing the solution and stimulating a few times at 1 pulse per sec, using pulses of 0·1–msec duration of controlled recorded intensity. A Grass Instrument Co. S4 stimulator and P6 d.c. preamplifier were used. In a number of experiments the action currents were photographed directly from the screen of a Tektronix 502 dual beam cathode ray oscilloscope. At the minimal voltage required to stimulate the giant axon the small nerve fibers did not respond; thus, the electrical activity recorded is that of the giant axon alone.

Solutions of the various materials used—enzymes, detergents, neurotropic compounds—were prepared daily using filtered sea water buffered at pH 7·6 to 7·8 with 1 mM tris (hydroxymethyl) aminomethane (Tris). Sea water containing this concentration of Tris had no effect on the spike height over a period of several hours. After addition of compounds to the sea water, the pH of the solution was readjusted to 7·6–7·8, if necessary. The solutions were oxygenated during the course of the experiments.

ACh and dimethylaminoethyl acetate (DMAEA) were used in combination with $7{\cdot}3 \times 10^{-4}$ M physostigmine, a concentration which by itself did not affect axonal conduction.

Pretreatment procedures

A survey of various enzymes and detergents was first undertaken to determine the maximum concentration which could be applied for 30 min without detectably affecting the action potential. The enzymes and detergents in this concentration were then applied to fresh axons for 30 min, followed by fresh sea water for 10–15 min. The axon was then bathed in $1{\cdot}4 \times 10^{-3}$ M curare for 30 min, unless block of conduction was observed earlier. If an effect of curare was observed, reversibility of its action was tested in normal sea water. Pretreatment was considered ineffective if curare did not cause at least a 20 per cent decrease in spike height in 30 min.

In those experiments in which compounds other than curare were tested for potentiation, a modified procedure was used. The reasons for doing this are discussed in Results. The particular compounds were applied to axons, at the start of the experiments, for 30 min in combination with, per ml, 10 μg of *Naja naja* cobra venom (CV) plus 20 μg of cetyltrimethylammonium chloride (CTA). The axon was then kept in sea water for 10–15 min followed by a second application of the compound alone, in the absence of CV plus CTA. The compound remained in contact with the axon for 30 min, unless a pronounced effect was obtained sooner, in which case reversibility was checked.

Calculation of results

As an index of potency of those compounds which affect conduction in the squid axon, we used the "minimal active concentration" (MAC), i.e. that concentration which caused a 50 per cent decrease in the height of the spike in from 10 to 30 min. In those cases in which the number of experiments was few or in which there was considerable variation in the response, we have recorded the MAC as a range of concentrations. Other results are expressed as the mean per cent decrease in spike height $\pm$ standard deviation of the mean over a given period of time. If only one or two experiments were performed with a given compound the actual per cent decreases are recorded.

Compounds

Dibucaine (Nupercaine) and chlorisondamine (Ecolid) were kindly supplied by Ciba Pharmaceutical Products Inc. Prostigmine (neostigmine) was a gift of Dr. John Aeschlimann, Hoffman-La Roche, Nutley, New Jersey. Liquid dimethylaminoethyl acetate (DMAEA) and dimethylaminoethanol were obtained from Eastman Kodak Co. Three times crystallized DMAEA. HBr was prepared by Dr. S. Ginsburg of this Laboratory, who also prepared the lipid-soluble quaternary ammonium compounds: β-hydroxyethyldimethyldodecyl ammonium iodide (Norcholine 12), β-acetoxyethyldimethyldodecyl ammonium iodide (Noracetylcholine 12) and pyridine-2-aldoxime dodecyliodide (PAD). Methantheline (Banthine) and diphenhydramine (Benadryl) and protamine sulphate were commercially obtained.

Hydrolase mixture, an enzyme concentrate from *Aspergillus oryzae*, was obtained from C. F. Boehringer and Soehne, Mannheim, Germany. CTA, Span and Tween 20 were obtained from K and K Laboratories. The following enzymes were purchased from Mann Research Laboratory: purified lipase powder of microbiological origin, purified intestinal alkaline phosphatase, crystalline papain, trypsin and chymotrypsin and purified hyaluronidase.

TABLE 1. EFFECTIVENESS OF SEVERAL COMPOUNDS IN DECREASING THE ACTION POTENTIAL OF THE SQUID GIANT AXON

The measure of effectiveness recorded below is the minimal active molar concentration (MAC), which is that concentration required to decrease the height of the action potential 50 per cent in 10–30 min. For comparison, previously obtained MAC values for the action of these compounds on the neural response of the isolated single electroplax are added.[4, 12, 41] Number of experiments is indicated in brackets.

Compound	Squid axon MAC	Squid axon % reversibility	Electroplax MAC
Atropine	$1{\cdot}5$–$6{\cdot}0 \times 10^{-3}$ (3)	—	3×10^{-4}
Methantheline	$1{\cdot}0$–$2{\cdot}5 \times 10^{-3}$ (3)	0	5×10^{-4}
Physostigmine	$7{\cdot}3 \times 10^{-3}$ (6)	80	$7{\cdot}3 \times 10^{-4}$
Procaine	$3{\cdot}5 \times 10^{-3}$ (5)	100	$0{\cdot}5$–$1{\cdot}0 \times 10^{-3}$
Dibucaine	$2{\cdot}6 \times 10^{-5}$ (3)	0–50	$1{\cdot}3$–$2{\cdot}6 \times 10^{-5}$
Diphenhydramine	2–4 $\times 10^{-4}$ (3)	80	2×10^{-4}
Chlorpromazine	1×10^{-4} (6)	0	1×10^{-4}
Strychnine	5×10^{-4} (2)	90	—
Noracetylcholine 12	$\sim 5 \times 10^{-4}$ (1)	0	$1{\cdot}5 \times 10^{-5}$
Norcholine 12	$\sim 5 \times 10^{-4}$ (1)	60	$1{\cdot}5 \times 10^{-5}$
PAD	1–6×10^{-4} (2)	0	1–6×10^{-5}
Puffer fish poison	$\sim 0{\cdot}1$ μg/ml* (1)	100	$0{\cdot}025$ μg/ml

* Molecular weight unknown.

RESULTS

Control axons isolated in the manner described under Methods maintained their original spike heights ± 10 per cent for at least 4 hr, the longest period of any experiment. The mean action potential recorded with external electrodes and based on 170 axons was 18·7 mV, with a range from 10 to 40 mV.

Effects on the electrical activity of the intact axon

The compounds tested which affect conduction in the squid axon are listed in Table 1; except for puffer fish poison of unknown structure, they are either tertiary

nitrogen derivatives or lipid-soluble quaternary nitrogen derivatives which have a dodecyl group replacing a methyl group on the nitrogen (Noracetylcholine 12, Norcholine 12, and PAD). These latter three compounds, in concentrations similar to those applied to the squid axon, have been found to block conduction in the lobster axon and in the electroplax.[4, 16] Di*iso*propylfluorophosphate (DFP) is readily reversible only at its MAC or lower. Higher concentrations or longer periods of application caused irreversible block of conduction as has previously been found.[17, 18]

In contrast to the other tertiary nitrogen compounds tested upon the squid axon, the following two required very high concentrations to produce even a relatively small effect: dimethylaminoethanol 2×10^{-1} M, 19 per cent decrease of spike height (two experiments); dimethylaminoethyl acetate $1{\cdot}5 \times 10^{-1}$ M, $21{\cdot}6 \pm 3{\cdot}1$ per cent (five experiments). By contrast, dimethylaminoethyl acetate has an MAC of 8×10^{-4} M upon the neurally evoked spike of the electroplax. The ineffectiveness of DMAEA upon the squid axon is perhaps surprising since tertiary nitrogen compounds are generally considered to be lipid soluble. Using the Hestrin technique,[19] we have found, however, that this compound is almost completely insoluble in olive oil.* Although olive oil is not a model for the nerve lipid, the experiment indicates lipid insolubility, which might explain lack of effect of DMAEA on the axon.

With a series of quaternary nitrogen compounds, no significant effect (greater than 10 per cent) on electrical activity of the axons was observed, when applied for 30 min in high concentrations. These compounds are listed with the highest molar concentrations used, and the number of experiments shown in brackets: acetylcholine 9×10^{-2} (6); carbamylcholine 5×10^{-2} (2); choline 6×10^{-2} (2); prostigmine 2×10^{-2} (4); succinylcholine 5×10^{-3} (2); decamethonium 5×10^{-2} (3); chlorisondamine 2×10^{-2} (3). Protamine sulfate was also tested and had no effect at 10 mg/ml. Weak effects, about 25 per cent decreases, were observed with two quaternary nitrogen compounds, the molar concentrations of which are listed as well as the mean percentage decreases in spike height $\pm$ standard error: benzoylcholine 9×10^{-2}, $24 \pm 3{\cdot}5$ (3); *d*-tubocurarine $1{\cdot}4 \times 10^{-2}$, $23 \pm 3{\cdot}3$ (4). In contrast to their ineffectiveness upon the squid axon, all of the above compounds, except choline and protamine sulfate, block the response to indirect stimulation of the electroplax in 10^{-4} to 10^{-6} M concentrations.[13]

Pretreatment experiments

The enzymes and detergents used were selected on the basis of reports in the literature which indicated that they might be effective in altering membrane structure. The effects of the detergent CTAB used by Walsh and Deal[8] were already mentioned. It is also known that cationic and non-ionic detergents affect the permeability of red cell membranes.[20] Lysozyme and trypsin cause changes in the membrane of sea urchin eggs,[21] while lipase accelerates the lytic action of alcohol on the red cell surface.[22] Cobra venom phospholipase A has marked effects on mitochondrial membranes and on various metabolic enzymes,[23, 24, 42] as well as apparently releasing ACh from its bound form in the brain.[25] Phospholipases block axonal conduction and have marked

* Solubility of DMAEA in olive oil was determined as follows: solid DMAEA was added to the oil, the mixture shaken for several hours and the precipitate removed by centrifugation. The oil was extracted with water, and, after concentration of the aqueous solution, the DMAEA was determined with the Hestrin technique.[19]

effects on the structure of the nerve axon.[26–29] Hyaluronidase decreases the time for block by procaine in intact frog sciatic nerves.[30]

Despite the reported structural changes caused by the above enzymes and detergents, curare in $1{\cdot}4 \times 10^{-3}$ M concentration in 30 min failed to affect electrical activity of squid giant axons after 30-min pretreatment of the axons with the following enzymes and detergents (concentrations in mg/ml and number of experiments in brackets): digitonin 0·02 (3), 0·1 (2); hyaluronidase 0·1 (7), 0·5 (1); hyaluronidase + trypsin + chymotrypsin 0·1 + 2·0 + 2·0 (2); hydrolase mixture 1·0 (2), 5·0 (1); lipase 0·5 (1), 3·0 (1); lysozyme 0·5 (1), 1·6 (1); papain 0·5 (1); alkaline phosphatase 0·1 (1), 0·33 (1), 1·0 (1); sodium desoxycholate 0·005 (1), 0·01 (1); Span 20, 0·5 (1), 5·0 (1); trypsin 0·5 (1), 2·5 (1); Tween 20, 0·5 (1), 5·0 (1). None of the enzymes and detergents listed affected axonal conduction by themselves, except hyaluronidase, digitonin and sodium desoxycholate, which in the highest concentrations listed caused, respectively, a 44, 27 and 20 per cent decrease in the spike height in 30 min.

On the other hand, after pretreatment with either CV or CTA or a combination of both, a marked effect of curare on electrical activity was obtained (Table 2). In a

TABLE 2. EFFECT OF CURARE ON SQUID AXON ACTION POTENTIAL FOLLOWING PRETREATMENT WITH COBRA VENOM (CV) AND/OR CETYLTRIMETHYLAMMONIUM CHLORIDE (CTA)

Pretreatment agents and curare were applied for 30 min, except in those experiments in which curare completely blocked conduction in less than 30 min. Reversibility was checked for 30 min. Number of experiments are given in brackets.

Pretreatment	Conc. (μg/ml)	% Decrease by curare Mean ± s.e. $1{\cdot}4 \times 10^{-3}$ M	% Decrease by curare Mean ± s.e. $5{\cdot}6 \times 10^{-3}$ M	% Reversibility mean ± s.e.
None	—	3·7 ± 1·8 (7)	—	—
None	—	—	11·4 ± 2·9 (8)	—
CV	10	45·6 ± 9·2 (7)	—	39·5 ± 16·5
CV	10	—	72·8 ± 1·1 (3)	64·7 ± 17·6
CTA	30	56·2 ± 15·8 (4)	—	—
CTA	20	—	44 2 ± 14·2 (4)	86·7 ± 8·1
CV + CTA	10 + 20	52·4 ± 14·0 (5)	—	68·8 ± 14·2
CV + CTA	10 + 20	—	66·7 ± 7·3 (20)	81·9 ± 5·3

number of experiments, block of conduction was complete and readily reversible (Fig. 1). Block of conduction (Fig. 1; c, h) was complete despite greatly increased stimulus strength or altered polarity of stimulation. The stimulus artifact happens to be reversed in Fig. 1(c) because the polarity of this particular stimulus was reversed in an attempt to evoke an action potential. The concentrations of CV and CTA used did not detectably affect axonal conduction in 30 min and if the axons were returned to sea water they conducted normally for several hours. With higher concentrations of CTA, 150μg/ml, or CV, 25μg/ml, complete irreversible block of conduction was obtained in from 15 to 30 min. The electrical activity of frog sciatic nerve has been reported to be unaffected by exposure to 1:200 CV.[31]

In about 20 per cent of the experiments with CV plus CTA, recorded in Table 2, the pretreatment was ineffective in the period of observation (30 min). The potentiation of curare recorded in Table 2 would be even more pronounced if these experiments were omitted from the calculated results. In 25 per cent of the experiments $5{\cdot}6 \times 10^{-3}$ M curare caused complete block in from 2 to 10 min; reversibility in sea

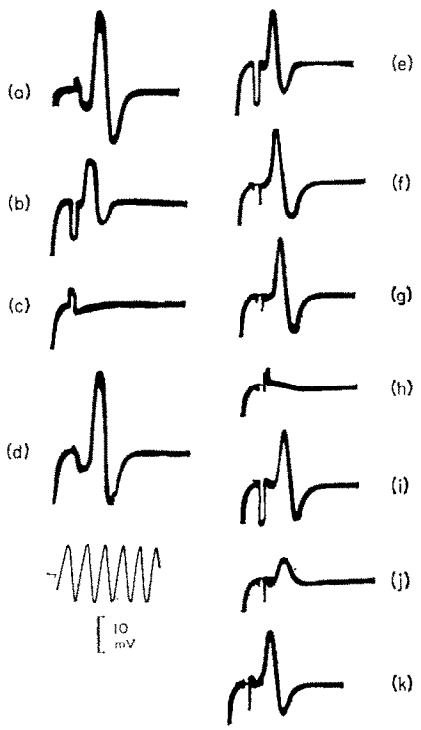

FIG. 1. Effect of curare on squid axon following pretreatment with (per ml) 10 μg of cobra venom (CV) plus 20 μg of cetyltrimethylammonium chloride (CTA). Photographs a to d from experiments on one axon, and e to k from another axon. (e) Control prior to CV plus CTA. (a, f) After 30-min pretreatment with CV plus CTA. Pretreatment had no effect on spike height. (g) After 10-min rinse with sea water. (b) 5 min and (c, h) 10 min after application of $1{\cdot}4 \times 10^{-3}$ M curare. Conduction was completely blocked despite greatly increased stimulus strength. (d, I) 10 min after return to sea water. (j) 15 min after application of $4{\cdot}2 \times 10^{-4}$ M curare. (k) 10 min after return to sea water. Time signal: 850 c/s.

water was good in most cases (Table 2). In the other experiments, curare was of intermediate effectiveness and was left in contact with the nerve for 30 min.

An axon rendered sensitive to the action of curare remained so over a long period of time. For example, curare was equally effective whether pretreatment was followed by placing in sea water for 10 min or 95 min (Fig. 2).

Following pretreatment, it was observed that repeated applications of curare, even when interspersed with 30-min periods in sea water, frequently resulted in a progressive enhancement of its effect. For example, in four experiments, 5×10^{-4} M curare in 30 min had no effect on the action potential. However, this same concentration, in two out of four experiments, caused complete block of the action potential of

pretreated nerves after they had responded reversibly to $5{\cdot}6 \times 10^{-3}$ M curare (Fig. 3.) This enhancement may be due to subminimal amounts of curare which remained within the nerve fibers even after they were returned to sea water. These results are in contradistinction to findings on untreated nerves, in which curare and other lipid insoluble compounds were as ineffective during a second application as during the first.

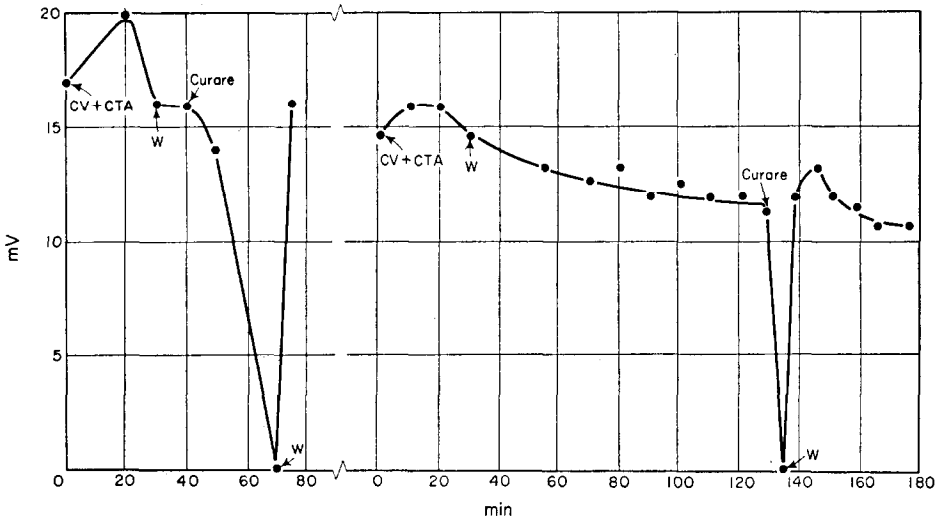

FIG. 2. Effect of $1{\cdot}4 \times 10^{-3}$ M curare on squid axon rinsed in normal sea water (W) for 10 min (left) and for 95 min (right) after the application, per ml, of 10 μg of cobra venom (CV) plus 20 μg of cetyltrimethylammonium chloride (CTA).

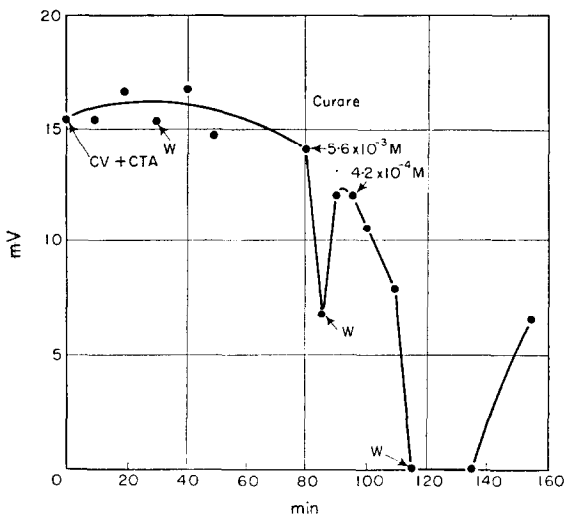

FIG. 3 Increased effect of curare on repeated application to squid axon pretreated with (per ml) 10 μg of CV plus 20 μg of CTA. Curare was first applied in $5{\cdot}6 \times 10^{-3}$ M concentration for 5 min, and returned to sea water (W) for 10 min. Subsequent application of $4{\cdot}2 \times 10^{-4}$ M curare blocked conduction. The lower concentration of curare, if applied immediately after pretreatment was ineffective.

TABLE 3. ACTION OF LIPID-INSOLUBLE QUATERNARY NITROGEN DERIVATIVES AND PROTAMINE SULFATE ON ELECTRICAL ACTIVITY OF SQUID GIANT AXON AFTER PRETREATMENT WITH COBRA VENOM (10 μG/ML) AND CETYLMETHYLAMMONIUM CHLORIDE (20 μG/ML)

The results are given as percentage decrease of electrical activity after 30 min exposure. The results are expressed as mean $\pm$ standard error where three or more experiments were performed. Otherwise the individual data are recorded.

Compound	Molar conc.	% Decrease
Acetylcholine	$2{\cdot}2 \times 10^{-2}$	$19{\cdot}5 \pm 5{\cdot}2$
(+ physostigmine)	$4{\cdot}5 \times 10^{-2}$	$21{\cdot}0 \pm 6{\cdot}2$
	$9{\cdot}0 \times 10^{-2}$	$37{\cdot}0 \pm 4{\cdot}4$
Benzoylcholine	$2{\cdot}3 \times 10^{-2}$	20
	$4{\cdot}6 \times 10^{-2}$	26
	$9{\cdot}3 \times 10^{-2}$	29, 48
Carbamylcholine	$2{\cdot}8 \times 10^{-2}$	0
	$5{\cdot}5 \times 10^{-2}$	0
Choline	$5{\cdot}7 \times 10^{-2}$	22, 19
Neostigmine	$1{\cdot}7 \times 10^{-2}$	3, 5
	$3{\cdot}3 \times 10^{-2}$	8
Decamethonium	$2{\cdot}4 \times 10^{-2}$	5
	$4{\cdot}8 \times 10^{-2}$	$9{\cdot}0 \pm 5{\cdot}0$
Chlorisondamine	$4{\cdot}6 \times 10^{-3}$	18, 19
	$1{\cdot}1 \times 10^{-2}$	100, 80*
	$2{\cdot}3 \times 10^{-2}$	73, 60*
Protamine sulfate	2·5 mg/ml	33
	5·0 mg/ml	$71{\cdot}6 \pm 10{\cdot}8$

* In 10–20 min.

TABLE 4. POTENTIATION OF THE ACTION OF TWO CHOLINESTERASE INHIBITORS ON THE SQUID AXON AFTER PRETREATMENT WITH COBRA VENOM (10 μG/ML) AND CETYLTRIMETHYLAMMONIUM CHLORIDE (20 μG/ML)

The effects are given as percentage decrease of electrical activity. The results are expressed as mean $\pm$ standard error where three or more experiments were performed. Otherwise the individual data are recorded.

Compound	Molar conc.	After pretreatment		No pretreatment
		% Decrease	Time	% Decrease, 30 min
DFP*	$5{\cdot}0 \times 10^{-4}$	38	30	—
	$1{\cdot}0 \times 10^{-3}$	83, 88	30	0, 0
	$5{\cdot}0 \times 10^{-3}$	75	6	$71{\cdot}2 \pm 16{\cdot}6$
Physostigmine	$7{\cdot}3 \times 10^{-4}$	$8{\cdot}3 \pm 2{\cdot}6$	30	—
	$1{\cdot}8 \times 10^{-3}$	$48{\cdot}2 \pm 9{\cdot}2$	10–30	$10{\cdot}3 \pm 1{\cdot}5$
	$7{\cdot}3 \times 10^{-3}$			$44{\cdot}3 \pm 3{\cdot}2$

* Because DFP is an irreversible inhibitor of cholinesterase, it was not applied in combination with CV plus CTA, but was applied only once, after CV and CTA.

In Tables 3 and 4 data are presented for effects of various other compounds after pretreatment with CV plus CTA. The compounds were initially applied to the axon for 30 min in combination with (per ml) 10 μg of CV plus 20 μg of CTA and, after placing the nerves for 10 min in sea water, the compounds alone were reapplied for an additional 30 min. This method was used since the compound was then in contact with the axon for a longer period of time and thus lesser degrees of potentiation could be observed. The data recorded in Tables 3 and 4 are taken from the results obtained with the second application of the compounds. Potentiation of the action of chlorisondamine, protamine sulfate, physostigmine, DFP and possibly acetylcholine, benzoylcholine and choline was observed. As with curare, block of conduction was readily reversible in all cases. As an illustration, the effects of chlorisondamine and protamine are shown in Fig. 4. Procaine, 7×10^{-4} M and $1{\cdot}7 \times 10^{-3}$ M (four experiments) was also tested after pretreatment with CV plus CTA, and was not potentiated.

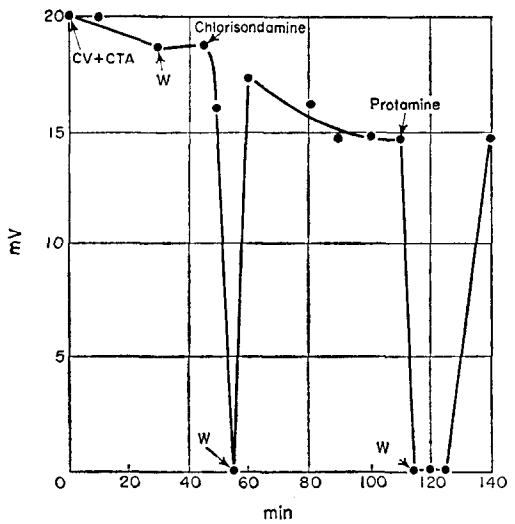

FIG. 4. Block of conduction of squid axon by chlorisondamine $1{\cdot}1 \times 10^{-2}$ M and protamine sulfate 5 mg/ml, after pretreatment with 10 μg of CV/ml plus 20 μg of CTA/ml.

Those axons which failed to respond to a given compound were rinsed with sea water and exposed to $5{\cdot}6 \times 10^{-3}$ M curare to ascertain whether failure to affect conduction might have been due to ineffectiveness of the pretreatment. Since curare still blocked electrical activity in about 90 per cent of the experiments in which this procedure was used, this does not seem to have been the main cause.

The effect of ACh at a given concentration was about three times greater on the treated than on the untreated axon. However, since the concentrations of ACh required were high and the effects relatively small, there is some question about the validity of this effect, and indeed it appeared that the effect of choline at comparable concentrations might also have been potentiated about three fold.

Although ACh, choline, benzoylcholine and decamethonium were relatively ineffective in reducing the spike height after pretreatment, they did produce, in the highest concentrations listed in Table 3, an unexpected effect which is difficult to explain. When the first application of these compounds, combined with CV plus CTA,

was replaced with sea water, complete block of electrical activity occurred; after 15 to 45 min in sea water the action potential returned to normal. This phenomenon was never observed with control axons, or on axons which were pretreated with CV plus CTA alone, washed and then bathed in the compounds. Once an axon had recovered, this block could not be produced a second time. It is possible that CV plus CTA, by reducing the barriers surrounding the conducting membrane, rendered it sensitive to the osmotic change which occurred when the solution containing 0·1 M quaternary compound in sea water was replaced by normal sea water.

Spontaneous firings

As previously indicated, liquid DMAEA had only a small effect on the height of the conducted impulse. However, at a concentration of $7{\cdot}5 \times 10^{-2}$ M or higher it caused spontaneous firings at a rate of from 50 to 100 per sec for about 30 min. Recrystallized DMAEA.HBr did not cause firings. When the two preparations were combined, firings still occurred indicating that it was not the bromide ion which suppressed firing. Using the Hestrin procedure,[34] the purity of the liquid DMAEA was about 96 per cent, as compared with recrystallized DMAEA.HBr, which was assumed to be 100 per cent pure.

Spontaneous firings were noted when the Ca^{2+} and Mg^{2+} concentrations in an artificial sea water[32] were reduced to 10–25 per cent of normal. Ammonia, 100 μg/ml, pH of 9·5, also produced firings, while sea water at this same pH had no marked effect on electrical activity. Once the firings had ceased the action potential could be evoked, but the firings could not be produced again even after prolonged washing. Pretreatment with CV and CTA prevented the spontaneous firings caused by DMAEA or low Ca^{2+} and Mg^{2+}. These results were not investigated further and no explanations are offered at this time.

DISCUSSION

Although much evidence has recently accumulated which demonstrates the essential role of the ACh-receptor protein in axonal conduction,[1] a number of questions require further investigation. In the present paper, two major problems have been studied which have some bearing on the nature of the structural barriers surrounding the axon and the presence of receptors in the axon.

The first problem concerns an attempt to obtain an action of lipid-insoluble quaternary ammonium ions, which are ordinarily inactive on the axon, but which react in solution with the isolated receptor protein and which interfere with electrical activity at synaptic junctions. The present approach, using enzymes and detergents to reduce permeability barriers, may in addition provide important clues about the chemical nature of such barriers.

The prototype of lipid-insoluble compounds, generally assumed to prevent the action of ACh by competition with the receptor protein, is curare. Until recently this action had been demonstrated only on junctions and formed one of the basic arguments for the idea of a special chemical mechanism in synaptic transmission. The assumption of a limitation of curare action to the junction became untenable by the two recent findings that curare blocks axonal conduction at Ranvier nodes[6] and in whole frog sciatic nerves after exposure to the detergent CTAB.[8] Since curare has a high affinity to the receptor protein in solution[10, 11] and is also a powerful blocking

agent at junctions, it appeared to be a most suitable compound for testing the effectiveness of agents in reducing barriers which may protect the receptor in the axon.

Of the fourteen enzymes and detergents tested, one detergent, CTA, and one enzyme mixture, CV, proved to be effective in reducing the barrier to the extent that curare rapidly and reversibly blocked electrical activity of the axon. The concentrations required were relatively low, 20 μg of CTA per ml and 10 μg of CV per ml, and the time of exposure relatively short, 30 min. Since exposure to these agents for from 50 to 60 min irreversibly blocked axonal conduction, it was important to carefully control the time of application to exclude a strong direct action of CV on the conducting membrane. After this pretreatment, curare blocked electrical activity in 80 per cent of the experiments. However, in the best experiments the concentrations of curare required are still about 10^{-3} M, a concentration which is from 100- to 1000-fold as high as at junctions. This difference is likely due to a relatively incomplete reduction of permeability barriers surrounding the axon, so that only a small fraction of the externally applied curare is able to penetrate and react with the receptor of the membrane. Another explanation may be the high ionic content of the sea water as discussed below. It may be noted that even lipid-soluble cholinesterase inhibitors must be applied to the squid giant axon in rather high concentrations to block electrical activity. Even when block occurs, only a small fraction of the outside concentration is found within the axoplasm.[2, 3] In 20 per cent of the pretreated axons, in which even the high concentration of curare was ineffective, the barrier may not have been sufficiently reduced. No electron microscope studies have as yet been made, although these obviously would be essential for a detailed interpretation of the data. Another desirable check would be the use of radioactive curare, in order to permit a determination of concentration in the axoplasm at the time when block of conduction occurs.

Further evidence for the reduction of the barriers is the reversible block of electrical activity by chlorisondamine, a well known di-quaternary ganglionic blocking agent. Here again the concentrations required to block in from 10 to 20 min are rather high (10^{-2} M); this suggests that an incomplete reduction of the barriers had occurred. In addition, the action of two lipid-soluble compounds, DFP and eserine, is increased about fivefold, as would be expected if the lipid barrier were reduced but not removed, since the active concentration of these two compounds is still much higher than the usual ones effective at junctions. In addition to being powerful cholinesterase inhibitors, DFP and physostigmine may also react with the receptor. On the other hand, the permeability barrier breakdown might be extensive since protamine sulfate, a highly positively charged protein of molecular weight about 6000, which reacts with the ACh receptor protein in solution and blocks synaptic transmission,[12] but is completely inactive on the axon without treatment, blocks electrical activity after treatment.

Some quaternary ammonium compounds which are very potent at the synapse were still relatively inactive after the same treatment. ACh, 0·1 M, decreased electrical activity by 37 per cent in 30 min, an effect which is much weaker than with curare. High concentrations of carbamylcholine, decamethonium and prostigmine were completely inactive even after treatment. Several factors must be considered which may be responsible for these findings. As indicated by the observations discussed above, the reduction of the permeability barrier is probably neither uniform nor

complete, and therefore probably only a very small fraction of the outside concentration will cross the barrier. In these circumstances, only those compounds which have a high affinity for receptors present may affect conduction. Indeed, *in vitro*, curare and chlorisondamine are bound more strongly to the receptor protein than those quaternary ammonium compounds which do not become active after treatment.[5, 10–12] Another factor may be differences in lipid solubility. However, the *in vitro* determination of solubility in nerve lipid is difficult, since a suitable model for this lipid is unavailable. Penetrability of some of these compounds into the pretreated axon will be studied directly through the use of radioactive labeling. Finally, the high ionic strength and high Ca^{2+} concentration of sea water might be contributing factors in weakening the action of some quaternary ammonium compounds. It has been observed that increased Ca^{2+} and Mg^{2+} concentrations may suppress the action of decamethonium and curare at junction[12, 33] and reduce binding to the receptor protein in solution.[12] The fact that ionic strength and Ca^{2+} concentration are considerably lower in mammalian Ringer's solution may partly account for the finding that ACh, even without any treatment, acts on *C*-fibers of rabbit vagus.[9] The same applies to the effects observed by Walsh and Deal with ACh and prostigmine on the frog sciatic fiber after treatment with CTAB.[8] On the other hand, the combination of some tertiary compounds with the receptor, both *in vivo* and *in vitro*, is actually enhanced by increasing the Ca^{2+} and Mg^{2+} concentrations in the physiological solution used.[12] This might explain why the tertiary nitrogen compounds acted as strongly in sea water on the squid axon as on the electroplax where the concentration of salts is much lower. Obviously, more experiments are required to elucidate these questions. Still more efficient experimental conditions and treatment may be found under which most or all of the compounds may become effective. The period during which squid axons were available, and these studies were carried out, was limited to two months. Thus only a broad survey of the problem was possible.

CV contains many enzymes, including phospholipase A, hyaluronidase and proteolytic enzymes.[34, 35] Hyaluronidase and several proteolytic enzymes failed to render curare active. Thus, it appears likely that the active principle of CV in our studies is phospholipase A. This will be checked in future experiments. CV and phospholipase A and C rapidly caused block of conduction and depolarization in lobster axons and with phospholipase C marked changes in the structure of the membrane were reported.[26–29] On the basis of studies with various enzymes, Tobias assumes that the lobster axonal membrane contains, among other components, lecithin, which he assumes to be essential for nerve function.[28, 29] A high percentage of the total phospholipid in the nervous system is lecithin.[36] It is present in the isolated receptor protein preparation, although the significance of this finding has not yet been elucidated.[12] Lecithin interacts with cationic and anionic detergents, but not with non-ionic detergents such as Span and Tween 20.[37–39] Of the enzymes and detergents used, only CV and CTA interact with lecithin, and these were the only agents which rendered curare active.

An important result of these studies is the evidence that curare affects axonal conduction once the barrier protecting the receptor is removed. The successful use of enzymes, in this case probably phospholipase A, for such an effect has opened a new approach for the analysis of the chemical nature of the barrier.

The second problem investigated and reported in this paper is the effectiveness of lipid-soluble compounds, known to react with the acetylcholine-receptor protein in solution,[5, 10–12] in blocking electrical activity of squid giant axons. The strength of this action is compared to that at synaptic junctions of the isolated single electroplax, in which barriers are minimal, and thus approximate affinity to receptors present may be determined. The similarity of the concentrations required in the two types of preparations is quite remarkable. One could not, however, expect exact agreement, even if the permeability barriers were completely absent, because of species differences and different experimental conditions.

Among the tertiary compounds, DMAEA was found to be inactive even in high concentrations; this may be due to its rather poor lipid solubility (see Results) and is in agreement with the observations that because of its poor penetrability it is unsuitable as a substrate for testing cholinesterase activity of the intact electroplax.[40] Moreover, it does not affect the response of the electroplax to direct stimulation after curare had blocked the synaptic junctions.[13]

In addition to tertiary amines, the effects of lipid-soluble quaternary ammonium ions were studied. Once again the concentrations required to affect axons and synaptic junctions were comparable.

Puffer fish poison acts by an unknown mechanism, probably not involving combination with the receptor,[41] while DFP exerts its irreversible effect through its anticholinesterase action, while its reversible effects may be due to combination with the receptor.[1]

The results presented provide support for the contention of Nachmansohn that the ACh-receptor protein, generally accepted as being present at the synapses, also exists in the axon.[1] Still to be determined is the chemical nature of this receptor through its *in vitro* isolation. The essentiallity of this component in nerve conduction is demonstrated by the fact that compounds which block synaptic transmission have a similar effect on the axon. Permeability and anatomical factors exist which must be overcome in order to reveal these similarities.

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REFERENCES

1. D. NACHMANSOHN, *Chemical and Molecular Basis of Nerve Activity*. Academic Press, New York (1959).
2. M. A. ROTHENBERG, D. B. SPRINSON and D. NACHMANSOHN, *J. Neurophysiol.* **11**, 111 (1948).
3. T. H. BULLOCK, D. NACHMANSOHN and M. A. ROTHENBERG, *J. Neurophysiol.* **9**, 9 (1946).
4. P. ROSENBERG and H. HIGMAN, *Biochim. et Biophys. Acta* **45**, 348 (1960).
5. S. EHRENPREIS and M. G. KELLOCK, *Biochem. Biophys. Research Comm.* **2**, 311 (1960).
6. W. D. DETTBARN, *Nature, Lond.* **186**, 891 (1960).
7. J. D. ROBERTSON, The molecular biology of cell membranes. In *Molecular Biology* (Edited by D. NACHMANSOHN). Academic Press, New York (1960).
8. R. R. WALSH and S. E. DEAL, *Amer. J. Physiol.* **197**, 547 (1959).
9. C. J. ARMETT and J. M. RITCHIE, *J. Physiol.* **152**, 141 (1960).
10. S. EHRENPREIS, *Science* **129**, 1613 (1959).
11. S. EHRENPREIS, *Biochim. et Biophys. Acta* **44**, 561 (1960).
12. S. EHRENPREIS, and S. EHRENPREIS and E. BARTELS. Unpublished observations.

13. P. ROSENBERG, H. HIGMAN and D. NACHMANSOHN, *Biochim. et Biophys. Acta* **44**, 151 (1960).
14. F. O. SCHMITT and N. GESCHWIND, *Progr. Biophys. Biophys. Chem.* **8**, 166 (1957).
15. R. VILLEGAS and G. M. VILLEGAS, *J. Gen. Physiol.* **43**, 73 (1960).
16. E. SCHOFFENIELS, I. B. WILSON and D. NACHMANSOHN, *Biochim. et Biophys. Acta* **27**, 629 (1958).
17. T. H. BULLOCK, H. GRUNDFEST, D. NACHMANSOHN and M. A. ROTHENBERG, *J. Neurophysiol.* **10**, 11 (1947).
18. T. H. BULLOCK, H. GRUNDFEST, D. NACHMANSOHN and M. A. ROTHENBERG, *J. Neurophysiol.* **10**, 63, (1947).
19. S. HESTRIN, *J. Biol. Chem.* **180**, 249 (1949).
20. B. A. PETHICA and J. H. SCHULMAN, *Biochem. J.* **53**, 177 (1953).
21. B. HAGSTROM and B. HAGSTROM, *Exp. Cell Res.* **6**, 532 (1954).
22. R. BALLANTINE and A. K. PARPART, *J. Cell. Comp. Physiol.* **16**, 49 (1940).
23. B. M. BRAGANCA and J. H. QUASTEL, *Biochem. J.* **53**, 88 (1953).
24. A. P. NYGAARD, M. W. DIANZANI and G. F. BAHR, *Exp. Cell Res.* **6**, 453 (1954).
25. B. M. BRAGANCA and J. H. QUASTEL, *Nature, Lond.* **169**, 695 (1952).
26. J. M. TOBIAS, *J. Cell. Comp. Physiol.* **46**, 183 (1955).
27. P. G. NELSON, *J. Cell. Comp. Physiol.* **52**, 127 (1958).
28. J. M. TOBIAS, *J. Cell. Comp. Physiol.* **52**, 89 (1958).
29. J. M. TOBIAS, *J. Gen. Physiol.* **43**, 57 (1960).
30. P. NORDQVIST, *Acta Pharm. Tox., Kbh.* **8**, 195 (1952).
31. N. K. SARKAR and S. R. MAITRE, *Amer. J. Physiol.* **163**, 209 (1950).
32. C. L. PROSSER, D. W. BISHOP, F. A. BROWN, JR., T. L. JAHN and V. J. WULFF, *Comparative Animal Physiology*. W. B. Saunders, Philadelphia (1952).
33. D. H. JENKINSON, *J. Physiol.* **152**, 309 (1960).
34. E. A. ZELLER, *Advanc. Enzymol.* **8**, 459 (1948).
35. E. E. BUCKLEY and N. PORGES (Editors), *Venoms*. American Association for the Advancement of Science. Washington (1956).
36. G. BRANTE, *Acta Physiol. Scand.* **18**, Suppl. 63, 1 (1949).
37. Z. BAKER, R. W. HARRISON and B. F. MILLER, *J. Exp. Med.* **74**, 621 (1941).
38. G. F. REDDISH, *Antiseptics, Disinfectants, Fungicides and Chemical and Physical Sterilization* (2nd Ed.) p. 51. Lea and Febiger, Philadelphia (1957).
39. C. A. LAWRENCE, *Surface Active Quaternary Ammonium Germicides* p. 126. Academic Press, New York (1950).
40. W. L. SCHLEYER, *Biochim. et Biophys. Acta* **16**, 396 (1955).
41. W. D. DETTBARN, H. HIGHAM, P. ROSENBERG and D. NACHMANSOHN, *Science* **132**, 300 (1960).
42. E. PETRUSHKA, J. H. QUASTEL and P. G. SCHOLEFIELD *Can. J. Biochem. Physiol.* **37**, 975, 989 (1959).