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INHIBITION OF CHOLINE ACETYLTRANSFERASE ACTIVITY IN SQUID GIANT AXON*

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SUMMARY

Using a sensitive radiometric assay the choline acetyltransferase (acetyl-CoA: choline *O*-acetyltransferase, EC 2.3.1.6) activities of the squid fin nerve, giant axon, axoplasm, and axon envelope were determined.

Hexamethylene-1-[4(1-naphthylethenyl)pyridinium]-6-trimethyl ammonium bromide (Compound I) inhibited 94 and 88% of choline acetyltransferase in the intact giant axon at $5 \cdot 10^{-3}$ and $5 \cdot 10^{-4}$ M, respectively, when experiments were conducted in the dark. Exposure of solutions of the inhibitor to light caused inactivation ($5 \cdot 10^{-3}$ M gave only 33% inhibition). Compound I ($5 \cdot 10^{-3}$ M) depolarized the axon and blocked conduction equally well in the light and dark. Another choline acetyltransferase inhibitor 4-(1-naphthylethynyl)pyridine methiodide (Compound III) had similar effects as described for Compound I except that its potency as a choline acetyltransferase inhibitor was not inactivated by light. Pretreatment of the axon with 25 μg/ml of cottonmouth moccasin venom caused a 5-fold potentiation of the ability of Compounds I and III to block conduction, whereas the venom did not affect the choline acetyltransferase inhibition produced by these compounds. Compounds I and III ($1 \cdot 10^{-3}$ M) did not alter the maximal number of stimuli the squid axon could conduct when stimulated at 100 per second or when repetitively firing due to decreasing divalent cation content of media.

It is concluded that block of conduction and inhibition of choline acetyltransferase, by these compounds, are separate unrelated effects.

INTRODUCTION

Choline acetyltransferase (acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6),

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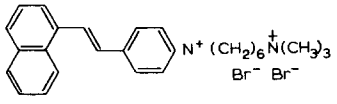
the enzyme catalyzing the synthesis of acetylcholine, is found in a great variety of conducting tissues[1,2]. In nerve endings, choline acetyltransferase appears to be present both in soluble and vesicular fractions[3–6]. This enzyme is necessary to maintain the level of acetylcholine, a known neurohumoral transmitter at certain junctional regions. Two markedly different explanations have been suggested for the presence of choline acetyltransferase in axons. Some investigators have suggested that choline acetyltransferase in the axon is simply in transit between the cell body where it is manufactured and the nerve ending where it is functional[7,8]. In contrast, according to the theory of axonal conduction as proposed by Nachmansohn and co-workers[9,10], choline acetyltransferase is essential for synthesis of acetylcholine all along the axon, and is not merely in transit from the cell body. The availability of a specific and potent inhibitor of choline acetyltransferase might allow us to decide between these two possibilities. If choline acetyltransferase is essential for axonal function then inhibition of the enzyme should eventually block conduction, whereas if the enzyme is merely in transit, inhibition would not be expected to affect physiological functions. Recently, Cavallito and co-workers[11–16] have described a group of styrylpyridine analogs, some of which are relatively potent and specific inhibitors of choline acetyltransferase. In this paper the effects of some of these inhibitors on choline acetyltransferase activity and axonal conduction of the squid giant axon are reported. The squid axon preparation was selected because the enzymatic activity in a single axon can be determined, thus eliminating the problems of interpretation associated with measurements of average activities in multifibred preparations[17]. In addition, in this preparation one can conveniently record electrical activity with intracellular as well as extracellular electrodes.

MATERIALS AND METHODS

Compound I

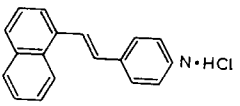
Hexamethylene-1-[4-(1-naphthylethenyl)pyridinium]-6-trimethyl ammonium bromide has been reported[11], but no physical properties given. This compound was synthesized by condensing 6-bromohexyltrimethylammonium bromide[18] with 4-picoline, according to the method of Gray *et al.*[19], to give hexamethylene-1-(4-methyl)-pyridinium-6-trimethylammonium bromide, m.p. 178°C. This was then condensed[19] with 1-naphthaldehyde to give the product as yellow-orange crystal, m.p. 169°C (decompn) (isopropanol). Analysis for $C_{26}H_{34}Br_2N_2$: calcd N, 5.24; found N, 5.04 (Analyses performed by Baron Consulting Co., Orange, Conn.).



Compound II

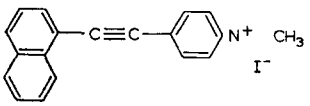
4-(1-naphthylethenyl)pyridine hydrochloride was prepared essentially by methods reported by Cavallito *et al.*[12]. It was found more efficient to isolate the product from the reaction mixture as the hydrochloride, m.p. 265°C (decompn). No melting point was previously reported[12]. Analysis for $C_{17}H_{14}ClN$: calcd C, 76.25; H, 5.27;

N, 5.23; found, C, 76.33; H, 5.31; N, 5.27 (Analyses performed by Baron Consulting Co., Orange, Conn.).



Compound III

4-(1-naphthylethynyl)pyridine methiodide was prepared according to reported method[12]. The intermediate dibromo adduct has a m.p. of 249–250°C; reported m.p. of 145–147°C (ref. 12) was a typographical error and should have been 245–247°C (C. J. Cavallito, personal communication). The methiodide had m.p. 251–255°C, reported m.p. 248–251°C.



Tissues

The giant axon from the hindmost stellar nerve of the squid (*Loligo pealii*) was used in these experiments except as noted. Those axons used for enzymatic measurements were carefully dissected free of all adhering small nerve fibres and as much connective tissue as possible. Both ends of the intact axon were tied off in order to avoid loss of axoplasm during enzymatic assay. After the enzymatic assay the weight of the string was determined and subtracted from the weight of the string *plus* axon. Separate measurements were sometimes made on the axoplasm extruded from the giant axon as previously described[20] and on the envelopes (giant axon *minus* axoplasm). The squid giant axons used for electrical recordings of activity were not freed of adhering small nerve fibres as the action and resting potentials can be monitored in the presence of these adhering fibres. We also used bundles of fin nerves which lie in close proximity to the giant axon, and axons from the legs of the spider crab.

Measurement of choline acetyltransferase activity

The enzyme assay conditions of Diamond and Kennedy[21] and Schrier and Schuster[22] were modified as follows. 3–10 mg of tissue were placed intact in or homogenized in 30 μl of filtered sea water (buffered to pH 7.8 with 0.001 M Tris) containing 0.01 M choline chloride and $6 \cdot 10^{-4}$ M physostigmine. This low concentration of buffer was used so as not to substantially alter the osmotic condition of the nerve tissue. In many experiments appropriate concentrations of Compounds I, II or III were also present in the sea water. Following a 10-min pre-incubation, 10 μl of $3.43 \cdot 10^{-4}$ M [1-^{14}C]acetyl-CoA (in distilled water) was added ($3 \cdot 10^5$ cpm). The radioactive substrate, having an activity of 58.1 mCi/mM, was obtained from New England Nuclear Corp. (lot No. 471–206). To this was immediately added 10 μl of $32.6 \cdot 10^{-4}$ unlabeled acetyl-CoA (Nutritional Biochemicals Corp.) dissolved in sea water containing twice the usual salt concentration. Acetyl-CoA at these concentrations forms a solution of pH 5.5 in distilled water or 7.6 under the conditions of

our experiments, *i.e.* 0.001 M Tris, in sea water, previously adjusted to pH 7.8. 10 μl of sea water were then used to wash down the mortar and pestles of the micro-homogenizers (500 μl capacity) in which the incubations were carried out. After 45–180 min incubation at 21–22°C, the homogenizer was placed in dry ice to stop the enzymatic reaction. The reaction mixture was then diluted to 1.2 ml with distilled water in order to reduce the salt concentration of the sea water, in preparation for the chromatographic assay.

A column chromatographic procedure was used to separate the labelled substrate, [1-^{14}C]acetyl-CoA, which is held by an anion exchanger from the labelled product, [1-^{14}C]acetyl choline, formed by the tissue choline acetyltransferase and not retarded on the exchanger. The 1.2-ml diluted incubate was directly applied to a microchromatographic column containing 100 μl of Dowex 1-X8 resin 100–200 mesh (Bio-Rad) in the acetate form. Three fractions collected directly in scintillation vials corresponded to the following additions to the column: 1, diluted incubation mixture (1.2 ml); 2, distilled water to wash out homogenizer and syringe (1 ml); 3, distilled water (1 ml); the radioactivity in these fractions represents synthesized acetylcholine (not held by anion exchanger). To correct for presumed impurity of the [^{14}C]acetyl-CoA, the entire procedure was repeated except for omission of tissue; the average cpm obtained were 1101 $\pm$ 116 ($N = 9$) which usually represented less than 10% of the counts obtained during an assay. Radioactivity was measured using 15 ml Bray's solution[23] in a Packard Tri-Carb liquid scintillation counter. The preparation of the micro-column has been described previously[24].

Venom treated axons were exposed for 30 min to 25 μg/ml of cottonmouth moccasin venom (*Agkistrodon piscivorus*) (20–22°C) prior to being carefully dissected free of adhering small nerve fibres for enzyme assay. Experiments in the dark were carried out in the presence of a red bulb of the type used in photographic dark rooms. The results are recorded as pmoles of acetylcholine synthesized per mg wet wt. per 90 min (mean $\pm$ S.E.).

Recording of electrical activity

The external recording of the action potential of the squid giant axon has been previously described[25]. Two procedures were adopted to determine the maximum number of stimuli which an axon could conduct. In one procedure, axons were incubated for 30 min either in normal sea water or in sea water containing a choline acetyltransferase inhibitor, then stimulated at a rate of 100 per sec while being kept moist with the incubation solution. The time required for block of the action potential was noted. In a second procedure, axons were incubated with appropriate solutions, after which spontaneous firings were induced by exposure to an artificial sea water solution containing one fourth of the usual concentration of calcium and magnesium[26]. The duration and total number of spontaneous or repetitive firings were recorded (the rate varying from 100–200 per sec).

Intracellular recording of resting and action potentials was carried out with the aid of a Bioelectric Instrumentation NF1 amplifier through a 10–15 MΩ glass micropipette filled with 3 M KCl. Extracellular stimulation was by means of a Grass SD5 stimulator with built-in stimulus isolation unit using a pair of No. 32 platinum wires spaced 5 mm apart. Only those preparations which showed an initial action potential of greater than 85 mV were used. Action and resting potentials were recorded

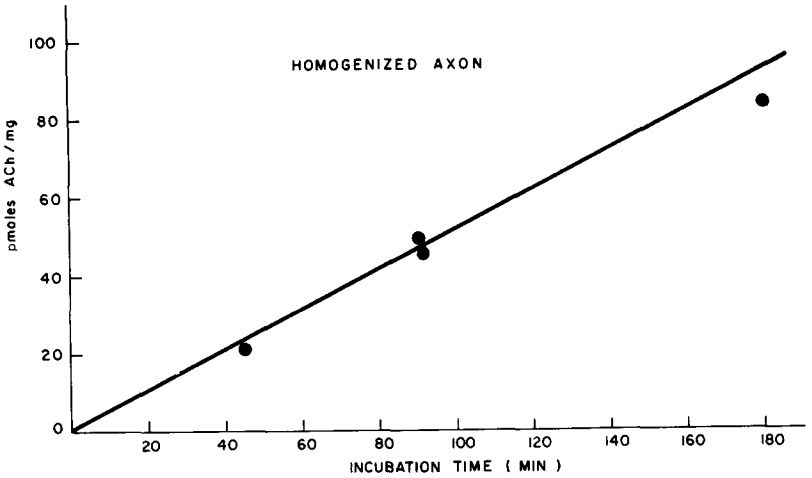


Fig. 1. Choline acetyltransferase activity in homogenized squid giant axons. One aliquot of homogenate was removed at 45 min, two aliquots at 90 min and one at 180 min. All were assayed for synthesis of acetylcholine (ACh) by the described procedure.

every 10 min and photographed with a Polaroid oscilloscope camera. All experiments were carried out between pH 7.5–8.0, at room temperature (20–22°C).

RESULTS

Enzymatic assays

The linearity of synthesis of acetylcholine, with respect to time of incubation, under the assay conditions described, are shown in Fig. 1; this data also illustrates the reproducibility of the analytical procedure used. A similar plot of enzymatic activity in the intact axon, with respect to time, is not possible because of the variation of activity in these preparations.

The choline acetyltransferase activity was markedly different in the various tissues (Table I). The intact fin nerve is 4 times more active than the intact giant axon. The homogenized giant axon had enzymatic activity markedly lower than the intact, although special care was taken to prevent axoplasm loss prior to homogenization. In contrast, homogenization did not alter fin nerve activity. The linearity

TABLE I

LOCALIZATION OF CHOLINE ACETYLTRANSFERASE ACTIVITY

Activity is expressed in pmoles acetylcholine synthesized per mg wet wt per 90 min incubation.

Preparation	*No. of expts*	*Activity*
Intact fin nerve	3	860 ± 190
Intact giant axon	6	220 ± 40
Homogenized giant axon	7	40 ± 6
Intact axon *minus* axoplasm	4	83 ± 40
Axoplasm	2	1120 ± 350

of enzymic activity over the 180-min incubation period (Fig. 1) indicates that the enzyme is not especially labile. The addition of bovine serum albumin (2 mg/ml) also did not alter enzymic activity. The axoplasm appears to have most of the choline acetyltransferase activity of the giant axon, with the envelope only having small amounts. Several experiments were conducted in which the intact squid axon was incubated with the omission of either choline or the acetylcholinesterase inhibitor physostigmine; under these conditions only about 5% of the normal activity was observed. In view of recent publications[27,28] reported since the completion of these studies, the conditions of enzyme incubation may not be optimal and thus the values reported in Table I may reflect more accurately relative, rather than absolute, enzymic activities.

The choline acetyltransferase activities of control and inhibitor treated axons

TABLE II

INHIBITION OF CHOLINE ACETYLTRANSFERASE IN THE SQUID GIANT AXON IN THE PRESENCE AND ABSENCE OF LIGHT

The number of experiments is indicated in parentheses. Activity is expressed as mean ± S.E.

*Inhibitor**	*Molarity*	*pmoles acetylcholine per mg per 90 min*		*Inhibition (%)*
No inhibitor	—	220 ± 40	(6)	—
I (dark)	$5 \cdot 10^{-3}$	14 ± 11	(2)	94
	$5 \cdot 10^{-4}$	26 ± 13	(2)	88
(light)	$5 \cdot 10^{-3}$	150 ± 70	(3)	33
III (dark)	$5 \cdot 10^{-3}$	8 ± 8	(2)	96
	$5 \cdot 10^{-4}$	40 ± 30	(3)	82
	$5 \cdot 10^{-5}$	140	(1)	37
(light)	$5 \cdot 10^{-3}$	40 ± 30	(2)	82
	$5 \cdot 10^{-4}$	90 ± 30	(2)	59
	$5 \cdot 10^{-5}$	155	(1)	30

* See Materials and Methods for formulae of inhibitors.

are shown in Table II. Inhibitor II was very insoluble in sea water, however a saturated solution (less than $5 \cdot 10^{-4}$ M) caused approx. 35% inhibition in intact axons and 15% in homogenized axon (dark). It has been reported that compounds of the Types I and II are active inhibitors only as *trans* isomers whereas light induces a conversion into *cis* forms which are much weaker inhibitors[13–15]. We also found Compound I to be a much weaker inhibitor if experiments were carried out in a room well lit by both fluorescent bulbs and sunlight. In contrast, Compound III which cannot undergo an isomerization showed little if any decrease in potency on exposure to light (Table II). Because of the variability from one experiment to another, it was impossible to quantitate with confidence small changes in activity.

Some intact squid axons were exposed for 30 min to 25 or 100 μg/ml of cottonmouth moccasin venom prior to enzymatic assay. Their choline acetyltransferase activities (220 ± 70, $N = 3$) were identical to those observed in axons not pretreated with venom (Table I). In axons pretreated with 25 μg/ml venom, inhibitor I ($5 \cdot 10^{-4}$ M) caused 64% inhibition in the dark and no inhibition in the light, which is similar to that observed in axons not exposed to venom (Table II).

TABLE III

EFFECT OF CHOLINE ACETYLTRANSFERASE INHIBITORS ON ACTION POTENTIAL OF THE SQUID GIANT AXON

Number of experiments carried out is indicated in parenthesis. CM means that axons were pretreated for 30 min with 25 μg/ml cottonmouth moccasin venom. This exposure to venom had no effect on the action potential.

Inhibitor	*Condition*	*Molarity*	*Exposure (min)*	*Decrease action potential (%)*
I	Dark	$5 \cdot 10^{-3}$	5	100 ± 0 (2)
		$2.5 \cdot 10^{-3}$	30	40 ± 17 (2)
		$1 \cdot 10^{-3}$	30	10 ± 4 (4)
	Light	$5 \cdot 10^{-3}$	5–15	97 ± 3 (4)
		$2.5 \cdot 10^{-3}$	30	45 ± 10 (2)
		$1 \cdot 10^{-3}$	30	0 ± 0 (4)
	Dark + CM	$1 \cdot 10^{-3}$	10	100 ± 0 (3)
		$2.5 \cdot 10^{-4}$	30	0 ± 0 (2)
III	Dark	$5 \cdot 10^{-3}$	20–30	60 ± 10 (2)
		$2.5 \cdot 10^{-3}$	30	0 ± 0 (4)
		$1 \cdot 10^{-3}$	30	0 ± 0 (5)
	Light	$5 \cdot 10^{-3}$	30	100 (1)
		$2.5 \cdot 10^{-3}$	30	20 ± 2 (4)
		$1 \cdot 10^{-3}$	30	0 ± 0 (2)
	Dark + CM	$1 \cdot 10^{-3}$	10	100 ± 0 (2)
		$2.5 \cdot 10^{-4}$	30	0 ± 0 (2)
	Light + CM	$5 \cdot 10^{-4}$	10–15	100 ± 0 (4)
		$2.5 \cdot 10^{-4}$	10–20	40 ± 20 (4)

In six experiments the effects of Inhibitors I and III in homogenized axons (in the dark) were determined. The per cent inhibitions were similar to those observed in intact axons, for example $5 \cdot 10^{-3}$ M Compound I caused 70% inhibition while $5 \cdot 10^{-3}$ and $5 \cdot 10^{-4}$ M Compound III caused 100 and 90% inhibition, respectively.

Effects on conduction

The effects of Compounds I and III on the action potential recorded with extracellular electrodes is shown in Table III. The effects of both compounds were identical in the light and dark, about $5 \cdot 10^{-3}$ M concentrations being required to block conduc-

TABLE IV

EFFECTS OF CHOLINE ACETYLTRANSFERASE INHIBITORS ON MAXIMUM NUMBER OF STIMULI CONDUCTED BY THE SQUID GIANT AXON

Number of stimuli presented as means ± S.E.

Inhibitor	*Concentration (M)*	*No. of expts*	*Max. number of stimuli* × 10^{-3}
Stimulating at 100/s			
No inhibitor	—	4	405 ± 83
I	$1 \cdot 10^{-3}$	3	490 ± 16
III	$1 \cdot 10^{-3}$	3	423 ± 14
Spontaneous firings in reduced calcium and magnesium sea water			
No inhibitor	—	2	324 ± 54
I	$1 \cdot 10^{-3}$	3	280 ± 10
III	$1 \cdot 10^{-3}$	3	350 ± 40

tion. This effect was usually at least 50% reversible in less than 30 min. Venom pretreatment rendered the axons sensitive to both compounds, concentrations as low as $5 \cdot 10^{-4}$ M blocked conduction. The action of Compound III was potentiated both in the light and in the dark by venom treatment. A saturated solution of Compound II caused about a 50% decrease in spike height in 30 min (two experiments). Compound III ($5 \cdot 10^{-3}$ M) applied to axons from legs of spider crab caused a 85–100% decrease in the action potential in 30 min (two experiments).

The effects of these choline acetyltransferase inhibitors on the maximum number of stimuli which an axon could conduct are shown in Table IV. There was no difference in the results between experiments performed in light or dark, therefore the results were pooled. Neither compound markedly altered the number of action potentials an axon could conduct. After conduction was blocked and a maximum number of stimuli recorded, stimulation was interrupted for a few minutes or the axons were returned to normal calcium and magnesium sea water. Both control and inhibitor treated axons could then conduct many more stimuli, although this was not quantitated.

The effect of Compound I on the resting and action potentials was also recorded with intracellular electrodes. The results of two experiments in the dark (squares and

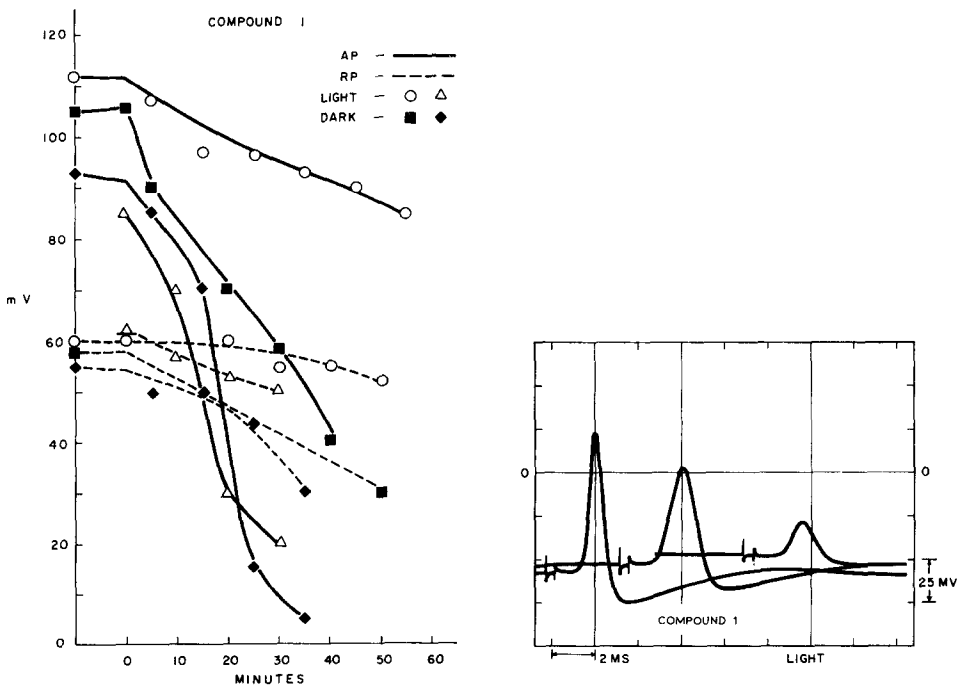


Fig. 2. The effects of Compound I ($5 \cdot 10^{-3}$ M) on resting potential (RP) and action potential (AP) of the squid giant axon.

Fig. 3. Effect of inhibitor on action potential and resting potential of squid giant axon. Three tracings from left to right at 5, 20 and 35 min after application of $5 \cdot 10^{-3}$ M Compound I to the axon. The distance from baseline to zero reference line indicates the magnitude of the resting potential. Note vertical and horizontal calibrations of 25 mV and 2 msec, respectively.

diamonds) and two in the light (circles and triangles) are shown in Fig. 2. In three of the experiments the effects were comparable, while in one (circles) the inhibitor had only a small effect. The actual tracings of the triangle experiment of Fig. 2 are shown in Fig. 3. Because of the minimal effects shown by Compound I in one of the light experiments, two additional experiments (by Dr. Alan Freeman) were performed, one in the light and one in the dark. With Compound I ($5 \cdot 10^{-3}$ M) a rapid (less than 10 min) and reversible depolarization (17 mV) in the light as well as in the dark (13 mV), accompanied by block of conduction, was observed.

DISCUSSION

The radiometric assay described for choline acetyltransferase activity was found to be suitable for detecting the micro amounts of enzymatic activity in the homogenized nerve preparations. There was, however, considerable variability in the results obtained from sample to sample using intact fin nerves or finely dissected axons (see standard errors of Table I); this may be partly explained by (1) small amounts of fin nerve fibres contaminating the axon preparation, if present, would induce a considerable error since they have on a weight basis about 4 times the choline acetyltransferase activity of the giant axon; (2) the difficulty in obtaining reproducible wet weights of the small amounts of axonal tissue; and (3) the variable presence of a natural occurring or introduced inhibitor.

A lower choline acetyltransferase activity in the squid axon was found in these experiments than was previously reported[29] with a less sensitive technique in which CoA is continuously reacetylated during the incubation. The data obtained confirms earlier observations of lower enzymatic activity found with synthetic acetyl-CoA as substrate compared with that found using an acetyl-CoA regenerating system[30–32], although the explanation remains unclear.

The axoplasm was found to contain most of the choline acetyltransferase activity of the axon; in contrast the axon envelope had only a small fraction of the enzymic activity. This finding would be compatible with observations that choline acetyltransferase is synthesized in the cell body and transported down the axoplasm[33]. It is also possible that the enzyme is synthesized in the axoplasm, as the axoplasm of the squid giant axon can synthesize protein[34,35]. Local axonal synthesis of acetylcholinesterase has been demonstrated[36–38]. In the axon of the Mauthner cell both axoplasm flow and local protein synthesis have been found[39].

Since the axoplasm represents about 80% of the wet weight of the intact axon it was somewhat unexpected to find the axoplasm having about 5 times the choline acetyltransferase activity of the intact axon. The intact axon, however, has strong permeability barriers which prevent the penetration of lipid insoluble compounds such as choline, acetylcholine, *etc.*[9,10,20,40]. It is possible, therefore, that in the intact preparation only that proportion of the enzyme which the externally applied choline can reach is being assayed. It is, however, more difficult to explain the consistent observation that the homogenized squid axon has only about 20% of the choline acetyltransferase activity of the intact preparation. This is apparently not due to instability of the enzyme in homogenized tissue, since the rate of enzymatic activity was linear over a period of 3 h. It is possible that an inhibitor of choline acetyltransferase might exist in the envelope and be freed during homogenization thereby

inhibiting the axoplasmic enzyme. To explore this possibility, 6 mg of homogenized axon with known activity was added to 4 mg of intact axon (activity previously measured). The choline acetyltransferase activity of the combination was that which would have been expected from a simple addition of the two known activities. This may indicate that there is no choline acetyltransferase inhibitor (at least in excess) in the homogenized tissue or that the inhibitor cannot reach the enzyme in the intact tissue. A more likely explanation for the decrease of choline acetyltransferase activity on homogenization might be (1) dilution of necessary cofactors; (2) the effect of sea water metals, *etc.*; (3) the loss of organized structure. While our incubation media contained all the co-factors known to be required in choline acetyltransferase assay, the necessity of additional factors for optimal activity in the squid axon cannot be ruled out. It is well known in enzyme chemistry that unless all necessary co-factors are provided in the incubation media, tissue slices will have higher activities than homogenates due to dilution of co-factor requirements. Insolubilization of enzymes even in artificial and relatively simple systems may decrease K_m values 50–100 times (ref. 41,42). It appears reasonable to assume, therefore, that in an organized particle of a subcellular structure enzymes may act at maximum speeds at lower concentration of substrates than they do in solution.

Using a rat brain supernatant as source of choline acetyltransferase, Cavallito and co-workers[11,12] reported the I_{50} for Compounds I and III as $9 \cdot 10^{-7}$ and $1 \cdot 10^{-6}$ M, respectively. On the intact squid axon, where strong permeability barriers would be expected to interfere with the penetration of these quaternary ammonium compounds[9,10,20,40], the I_{50} was much higher, about 10^{-4} M (Table II).

The squid giant axon can conduct hundreds of thousands of impulses even though about 80% of choline acetyltransferase is inhibited (Compound III, $1 \cdot 10^{-3}$ M, Tables II and IV). This is not a critical test for the acetylcholine theory of axonal conduction, as the squid giant axon has about 3 μg of acetylcholine per g (ref. 29); this "storage" form of acetylcholine would presumably be available even if 100% of the choline acetyltransferase were inhibited.

Compounds I and III at a concentration of $5 \cdot 10^{-3}$ M block conduction and inhibit choline acetyltransferase more than 90% (in the dark). However, the effects in the enzyme do not appear to be related to the block of conduction. Compound I is a much more potent inhibitor of the enzyme in the dark, whereas it blocks conduction equally well in the light or dark. Pretreatment of the squid giant axon with 25 μg/ml cottonmouth moccasin venom decreases the minimal concentration of Compounds I and III required to block conduction about 5-fold; however, this pretreatment has no effect on choline acetyltransferase inhibition by Compound I. Cottonmouth moccasin venom has been shown to decrease permeability barriers in the squid axon increasing thereby the penetration and potency of several quaternary nitrogen containing compounds[20,25,40,43–46]. It is therefore not surprising to find a similar effect with Compounds I and III which on the basis of structure we would expect to penetrate poorly. It appears that Compounds I and III cause a direct depolarization and block of conduction which is not related to inhibition of choline acetyltransferase. In a recent study testing many styrylpyridine choline acetyltransferase inhibitors on various neuromuscular preparations it was also concluded that their pharmacological effects cannot be attributed only to choline acetyltransferase inhibition[47]. Our results emphasize that in all studies using choline acetyltransferase inhibitors and intact

structures one must critically analyze if the effects observed are due to enzyme inhibition or due to other unrelated effects.

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