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THE PRESYNAPTIC EFFECTS OF QUATERNARY AMMONIUM COMPOUNDS ON THE ACETYLCHOLINE METABOLISM OF A SYMPATHETIC GANGLION

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Many quaternary ammonium compounds owe their interference with cholinergic transmission to a blocking action at the postjunctional receptor. Studies at the ganglionic synapse (Paton & Perry, 1953) and neuromuscular junction (Burns & Paton, 1951) have given greater definition to this well-known facet of the pharmacological activity of quaternary bases; their anticholinesterase action is also well documented (see review by Long, 1963). Yet in contrast little is known of their prejunctional effects, especially at the secretory nerve terminals.

With the introduction of the hemicholinium series by Long & Schueler in 1954 and subsequent investigations of the effects of the hemicholinium HC-3 (MacIntosh, Birks & Sastry, 1956; Birks & MacIntosh, 1961), it became clear that this quaternary base could interfere with the metabolism of acetylcholine in cholinergic nerve terminals. There seemed no *a priori* reason to suppose such effects to be confined only to hemicholinium. Indirect evidence followed, suggesting that triethylcholine also affected acetylcholine turnover in much the same way as did hemicholinium (Bowman & Rand, 1961).

The present paper describes the examination of various quaternary ammonium compounds, including the hemicholinium HC-3 and triethylcholine (triethyl-2-hydroxyethylammonium), upon presynaptic acetylcholine turnover, using the superior cervical ganglion of the cat. This ganglion, when isolated for perfusion, is particularly convenient for studying the functional acetylcholine metabolism of intact nerve endings because of its high density of cholinergic synapses (Brown & Feldberg, 1936b; MacIntosh, 1961).

Preliminary reports of part of this work have been made to the Canadian Federation of Biological Societies (Matthews, 1963) and to the British Pharmacological Society in January, 1965.

METHODS

Cats of medium size and either sex were anaesthetized first with ethyl chloride and ether and then with intravenous chloralose, 80 mg/kg. The right superior cervical ganglion was isolated for perfusion *in vivo* essentially by the method of Kibjakow (1933) as described by Feldberg & Gaddum

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(1934). The detailed procedures used in setting up and pressurizing the perfusion system were those described by Birks & MacIntosh (1961).

Perfusion was with plasma obtained from the fresh arterial blood of donor cats (anticoagulant; heparin sodium, 50 μg or 5 U/ml.); it contained by addition the anticholinesterase physostigmine sulphate, 3×10^{-5}M. Additionally one or more of the following substances was included in the plasma when a quaternary compound was to be tested: choline chloride; hemicholinium (HC-3) dibromide; hexamethonium chloride; tetraethylammonium chloride; triethylcholine chloride; troxonium iodide; and tubocurarine chloride. Concentrations given refer to the appropriate salt.

Perfusate samples were collected into tubes at accurately timed intervals and stored at 0° C until assayed for acetylcholine content 4 to 6 hr later. The perfusion pressure was sufficient to ensure a flow rate of 0.2 to 0.5 ml./min; in most experiments it ranged between 0.3 and 0.4 ml./min. Plasma was equilibrated at 20° C with 96.5% oxygen and 3.5% carbon dioxide (v/v) and was then rapidly filtered directly into the perfusion fluid reservoirs through a sintered-glass filter of medium porosity.

Stimulation of the distal stump of the cut preganglionic cervical sympathetic trunk was always begun 15 min after starting the perfusion and continued for 60 min. Rectangular pulses from an electronic stimulator assembly were delivered to the nerve through a stimulus isolation unit and bipolar platinum electrodes; the animal was earthed. A supramaximal voltage, pulse duration of 0.8 msec, and frequency of 20 shocks/sec were used. Maximal stimulation was ensured by moving the electrodes along the nerve towards the ganglion every 5 min. Additionally, the electrode assembly was such that paraffin oil (warmed to body temperature in the same way as the perfusion fluid) could be dripped on to the nerve between the electrode poles to minimize shorting.

A "drain-off" cannula, normally closed, was inserted into the distal stump of the external carotid artery to allow flushing out of the dead space of the arterial perfusion cannula and common carotid artery distal to the ganglion when rapid change-over of perfusion fluids was required. It was established in control experiments that a simple switching of reservoirs was without important effects on acetylcholine outputs.

At the end of 60 min stimulation the perfused ganglion was rapidly excised, placed in 10% trichloracetic acid solution at 0° C, and minced finely. The suspension was kept at 0° C for 105 min and extraction of acetylcholine for assay was then made by the method of Birks & MacIntosh (1961). The contralateral unperfused ganglion, to serve as a control, was excised and similarly treated immediately before perfusion was begun.

The content of acetylcholine in perfusate samples and ganglionic extracts was measured by comparison with standard acetylcholine chloride solutions, using the cat blood pressure preparation (MacIntosh & Perry, 1950). The standard acetylcholine solutions were prepared in the appropriate unperfused fluid remaining in the reservoirs at the end of a perfusion experiment and were stored with the samples of collected perfusate under identical conditions. Separate standard acetylcholine solutions prepared in a mixture (1:2) of 0.9% saline and distilled water were used for the assay of acetylcholine in ganglion extracts. The results of all acetylcholine assays are given in terms of nanograms (ng) of the chloride salt. Plasma containing added quaternary compounds did not interfere with the normal depressor response to injected acetylcholine, with the exception only of that containing the higher concentration of choline (2×10^{-2}M). In the latter instance a constant-volume injection technique was adopted to compensate for this effect. Stabilization of the cat blood pressure over many hours was achieved by means of a feedback drip of adrenaline through a needle thrust into the side-arm of the carotid arterial cannula, a method due to D. M. J. Quastel (personal communication).

All results for ganglionic acetylcholine content and output determinations are expressed as percentages of the initial content (taken in each case as being equal to that of the contralateral unperfused ganglion: Birks & MacIntosh, 1961). This manoeuvre facilitates comparison of results from groups of ganglia differing widely in acetylcholine content and according to Quastel (1962) reduces the variance of the means from averaging replicate experiments by about one-half.

The statistical significance of difference between means was assessed by Student's *t*-test.

RESULTS

Resting sympathetic ganglia contain a preformed store of acetylcholine. Extraction of this store from eighty ganglia yielded a resting content (mean and standard error) per ganglion of 284 ± 7.7 ng of acetylcholine, slightly more than the 266 ± 5.8 ng ($n = 50$) found by Birks & MacIntosh (1961) but less than the 295 ± 60 ng (standard deviation) determined by Quastel (1962).

When ganglia were repetitively stimulated acetylcholine was liberated. The time-course of this output for a group of five control ganglia is illustrated by the upper line of Fig. 1, in which the output, expressed as a percentage of the initial content, is plotted on a log scale against time. The ganglia were perfused with plasma containing physostigmine (3×10^{-5}M) but no added quaternary salt. Initially the output declined, but after about 5 min it stabilized at a level which was well maintained. At the end of 60 min stimulation the ganglia had released, in acetylcholine, about four times their initial content. At extraction the final content showed an increase of 23% above the initial level.

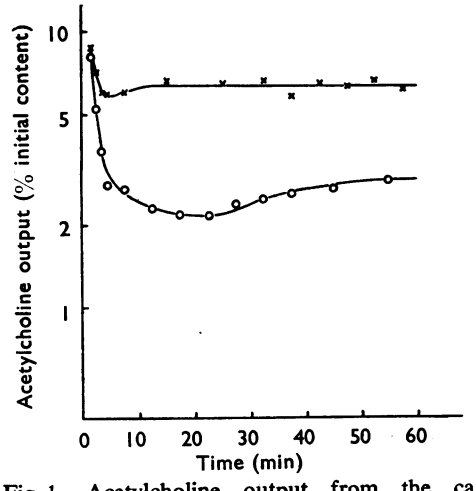

Fig. 1. Acetylcholine output from the cat superior cervical ganglion perfused with plasma containing physostigmine; ×, 3×10^{-5}M (mean of six experiments); O, 3×10^{-4}M (mean of three experiments). Ordinate; acetylcholine output expressed as a percentage of the initial ganglionic acetylcholine content on a log scale; that occurring in the first minute has been omitted for clarity. Abscissa: time (min). Preganglionic stimulation at 20 shocks/sec throughout.

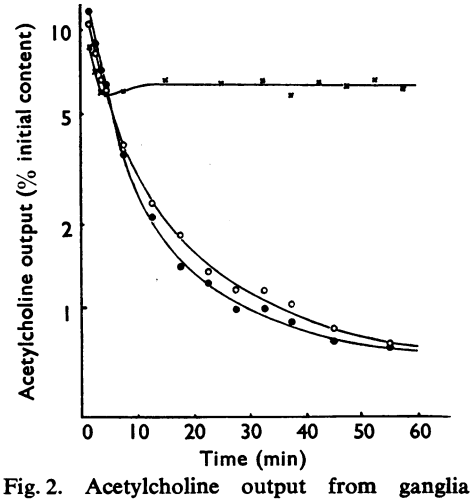

Fig. 2. Acetylcholine output from ganglia: effect of hemicholinium. Perfusion with plasma containing physostigmine, 3×10^{-5}M; ×, alone (mean of six experiments); O, with hemicholinium, 2×10^{-5}M (mean of four experiments); ●, 2×10^{-4}M (mean of four experiments). Other details as for Fig. 1.

When the plasma concentration of physostigmine was raised tenfold from the control concentration of 3×10^{-5}M to 3×10^{-4}M, acetylcholine outputs, initially unaffected, were later maintained below control levels (Fig. 1) while ganglion acetylcholine contents rose by 200%. Although the total output of acetylcholine was reduced significantly by 57% there was no corresponding reduction of synthesis (Table 1).

TABLE 1

EFFECTS OF QUATERNARY AMMONIUM COMPOUNDS ON THE ACETYLCHOLINE METABOLISM OF THE CAT SUPERIOR CERVICAL GANGLION

The physostigmine experiments acted as controls for the other drugs. Final ganglion contents are expressed as percentages of initial values (=100). * Reduction statistically significant ($P<0.05$) ; † mean of two experiments ; ‡ mean of four experiments

Drug	Concentration ($M\times10^{-5}$)	No. of expts.	Final ganglion content (%)	Reduction (%) compared with controls of Output	Synthesis
Physostigmine	3	6	123	—	—
	30	3	300	57*	9
Hemicholinium (HC-3)	2	4	30	69*	88*
	20	4	29	71*	89*
Triethylcholine	20	3	40	70*	86*
	200	3	35	83*	99*
Tetraethylammonium	20	3	45	63*	78*
	200	3	37	78*	95*
Troxonium	2	3	46‡	53*	69*
	20	1	30†	68*	92*
Tubocurarine	10	5	144	16	10
Hexamethonium	20	5	134	0	0

A different effect was observed with the hemicholinium compound, HC-3. This caused a progressive decline in acetylcholine output (Fig. 2) but with a corresponding powerful inhibition of acetylcholine synthesis and a depletion of ganglionic acetylcholine content. Thus, as shown in Table 1, perfusion with hemicholinium, 2×10^{-5}M, depressed acetylcholine output by 69% and synthesis by 88% while acetylcholine stores at extraction were reduced 70% below the initial content. All these reductions were statistically significant.

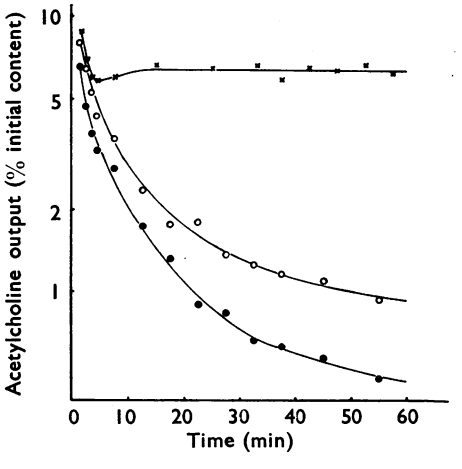

Fig. 3. Acetylcholine output from ganglia: effect of triethylcholine. Perfusion with plasma containing physostigmine, 3×10^{-5}M ; ×, alone (mean of six experiments) ; O, with triethylcholine, 2×10^{-4}M (mean of three experiments) ; ●, 2×10^{-3}M (mean of three experiments). Other details as for Fig. 1.

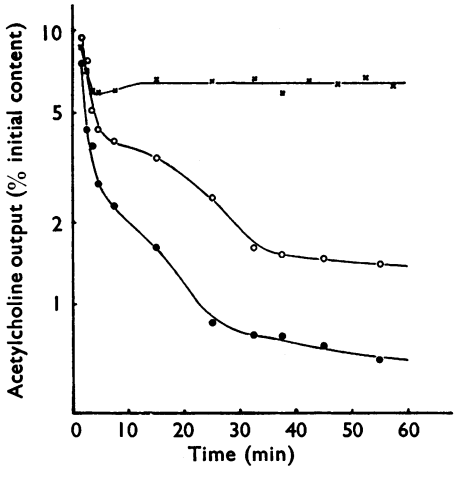

Fig. 4. Acetylcholine output from ganglia: effect of tetraethylammonium. Perfusion with plasma containing physostigmine, 3×10^{-5}M ; ×, alone (mean of six experiments) ; O, with tetraethylammonium, 2×10^{-4}M (mean of three experiments ; ●, 2×10^{-3}M (mean of three experiments). Other details as for Fig. 1.

An inhibition of acetylcholine synthesis associated with a declining output and depletion of ganglionic acetylcholine stores was also found when the plasma contained triethylcholine or tetraethylammonium (Figs. 3 and 4; Table 1). These compounds, which are structurally related, differ little in terms of their overall inhibitory potency upon acetylcholine metabolism but both have, on a molar basis, about one-tenth the inhibitory potency of hemicholinium, taking as the basis for comparison the percentage reduction of acetylcholine output and synthesis shown in Table 1. However, whereas a tenfold increase in the concentration of triethylcholine or tetraethylammonium (from 2×10^{-4}M to 2×10^{-3}M) led to a greater inhibition of synthesis, producing concomitantly a more rapid decline in output (Figs. 3 and 4) and an eventual greater depletion of ganglionic stores, increasing the hemicholinium concentration from 2×10^{-5}M to 2×10^{-4}M was without further effect. In the case of hemicholinium there was, statistically, no significant difference either between the output curves (Fig. 2), the reduction of synthesis, or the depletion of ganglionic contents at the two different concentrations.

The output curves during triethylcholine perfusion can be seen to conform closely to those obtained with hemicholinium (compare Figs. 2 and 3), the output declining exponentially. On the other hand the pattern of output seen in the presence of tetraethylammonium was different and seems worthy of special comment (Fig. 4). At both concentrations of tetraethylammonium tested the output curves showed a characteristic inflexion between 7 and 30 min after starting stimulation; this effect was not seen with any other compound tested.

The substance troxonium, introduced recently by Bhatnagar, Lam & McColl (1964), is of particular interest since it is the triethylcholine ester of trimethoxybenzoic acid. Like triethylcholine and hemicholinium it inhibited ganglionic acetylcholine turnover resulting in a diminished total output (Fig. 5; Table 1). Thus in the group of three ganglia perfused with a concentration of 2×10^{-5}M, the inhibitory effect of troxonium was about 80% that of the same concentration of hemicholinium, whilst in one experiment at 2×10^{-4}M it appeared about as effective as equal concentrations of hemicholinium or triethylcholine (Table 1). Experiments with high concentrations of troxonium were made difficult by the progressive desensitization to acetylcholine which appeared to develop in assay cats exposed to repeated doses of plasma containing this drug.

There is some evidence that the impairment by triethylcholine and hemicholinium of acetylcholine turnover at intact nerve endings can be reversed. Hitherto such evidence has been largely if not entirely indirectly based. A direct attempt at reversal was therefore made, either by washout of the inhibitory drug or by increasing the exogenous choline concentration with the inhibitor still present. It had been found previously that addition of extra choline alone to the perfusing plasma does not alter the output of acetylcholine (Birks & MacIntosh, 1961). In two experiments with hemicholinium, 2×10^{-5}M, perfusion was changed, after 30 min stimulation, to plasma without hemicholinium. At 30 min later, with continued stimulation, the output had recovered almost to the control level (Fig. 6). The ganglia were obviously able to resynthesize acetylcholine during this hemicholinium-free perfusion, for at extraction acetylcholine stores were only 8% below the initial level. In two other experiments choline was added to the perfusion fluid at 30 min at a concentration of 2×10^{-4}M, ten times that of the hemicholinium which remained present throughout. The increased choline concentration,

although less effective than simple washout of hemicholinium, nevertheless did produce some recovery of output and permit a partial restoration of ganglionic acetylcholine stores (Fig. 6 ; Table 2).

TABLE 2

REVERSAL OF INHIBITION OF ACETYLCHOLINE METABOLISM IN SYMPATHETIC GANGLIA

Acetylcholine outputs and contents are means and are expressed as percentages of the initial content

Drug	Concentration (M × 10^{-5})	Reversal at 30 min by	No. of expts	Acetylcholine: Output, 30-60 min (%)	Acetylcholine: Content 60 min (%)
Hemicholinium (HC-3)	2	—	4	26	30
	2	Choline, 2×10^{-4}M	2	48	50
	2	Fresh plasma	2	101	92
Triethylcholine	20	—	3	32	40
	20	Choline, 2×10^{-4}M	2	78	89
	20	Fresh plasma	2	111	102
Tetraethylammonium	200	—	3	21	37
	200	Fresh plasma	1	107	88

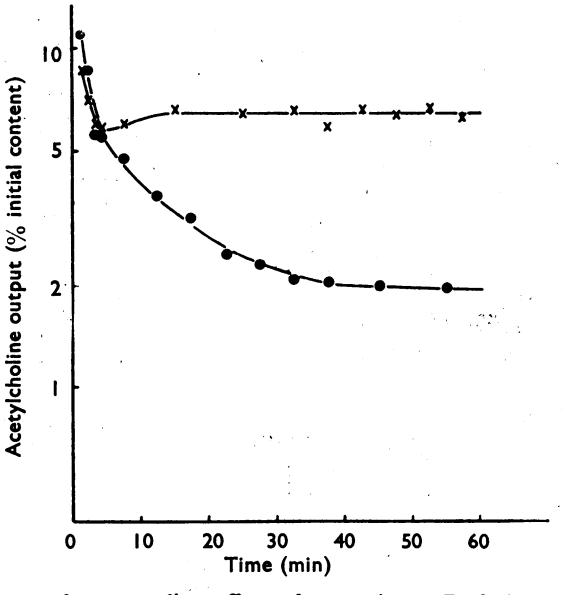

Fig. 5. Acetylcholine output from ganglia: effect of troxonium. Perfusion with plasma containing physostigmine, 3×10^{-5}M ; ×, alone (mean of six experiments) ; ●, with troxonium, 2×10^{-5}M (mean of three experiments). Other details as for Fig. 1.

With triethylcholine, 2×10^{-4}M, a marked recovery of output and restoration of acetylcholine content was evident upon switching to perfusion with plasma free from triethylcholine, as shown in Fig. 7. Addition to the perfusion fluid of choline at a concentration ten times that of triethylcholine also resulted in a good recovery of both output and acetylcholine stores (Table 2).

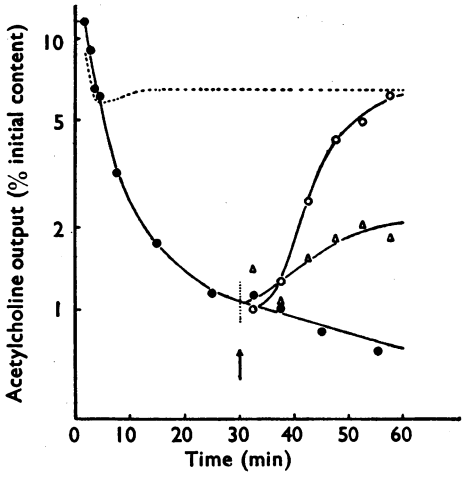

Fig. 6. Acetylcholine output from ganglia: reversal of hemicholinium action. Perfusion with plasma containing hemicholinium, 2×10^{-5}M: ● (mean of eight experiments before and of four experiments after the dotted vertical line at the arrow). At the arrow perfusion was changed to fresh plasma: ○ (mean of two experiments; or to plasma containing hemicholinium, 2×10^{-5}M, and choline, 2×10^{-4}M: △ (mean of two experiments). Physostigmine, 3×10^{-5}M, was present throughout all experiments. Control outputs (no added quaternary drug) are indicated by the dashed line. Other details as for Fig. 1.

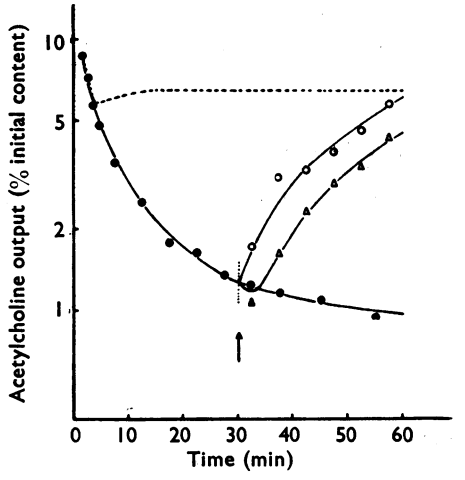

Fig. 7. Acetylcholine output from ganglia: reversal of triethylcholine action. Perfusion with plasma containing triethylcholine, 2×10^{-4}M: ● (mean of seven experiments before and of three experiments after the dotted vertical line at the arrow). At the arrow perfusion was changed to fresh plasma: ○ (mean of two experiments); or to plasma containing triethylcholine, 2×10^{-4}M, and choline, 2×10^{-3}M: △ (mean of two experiments). Physostigmine, 3×10^{-5}M, was present throughout all experiments. Control outputs (no added quaternary drug) are indicated by the dashed line. Other details as for Fig. 1.

One experiment with tetraethylammonium, 2×10^{-3}M, showed that its inhibitory action on acetylcholine turnover also could be effectively counteracted by changing to perfusion with fresh plasma. When the concentration of tetraethylammonium was 0.5×10^{-3}M or 5×10^{-3}M this reversal was still seen.

It is clear from the results given for each inhibitor in Table 2 that, whereas an increased choline concentration increases acetylcholine output twofold, simple washout of inhibitor raises the output to about four times the inhibited level.

In view of the finding that at least four quaternary ammonium compounds, one of them a ganglion-blocking agent well known for its postsynaptic action, are able to inhibit acetylcholine metabolism, other quaternary compounds used primarily for their postjunctional blocking action were also examined for any presynaptic activity. For example, although hexamethonium and tubocurarine are reputedly without important prejunctional effects (Paton & Zaimis, 1951; Brown & Feldberg, 1936a), in the case of tubocurarine recently this viewpoint has been questioned (Lilleheil & Naess, 1961; Standaert, 1964). However, although tubocurarine, 10^{-4}M, appeared to produce some small reduction in output (Fig. 8) this was not statistically significant (Table 1). Furthermore, not only did

hexamethonium, 2×10^{-4}M, not reduce output at all (Fig. 8) but with neither substance was there a significant reduction of synthesis nor did acetylcholine stores fall below control levels (Table 1). The concentrations of both tubocurarine and hexamethonium used in these experiments were adequate to block completely, or almost so, ganglionic transmission.

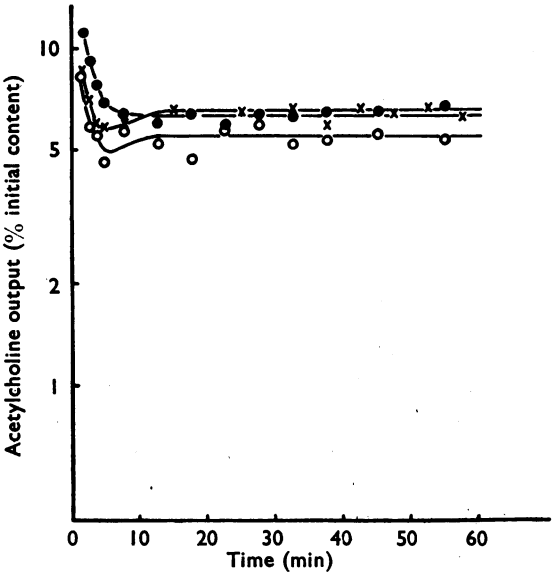

Fig. 8. Acetylcholine output from ganglia: effects of hexamethonium and tubocurarine. Perfusion with plasma containing physostigmine, 3×10^{-5}M; ×, alone (mean of six experiments); ●, with hexamethonium, 2×10^{-4}M (mean of five experiments); O, with tubocurarine, 10^{-4}M (mean of five experiments). Other details as for Fig. 1.

How quaternary compounds influence the immediate release of acetylcholine from preformed stores can be determined by looking at the initial rate of acetylcholine release for each group of experiments. Thus if the output of acetylcholine coming from the ganglion in the first 3 min of stimulation is measured, it is reasonably certain that this represents acetylcholine liberated only from previously manufactured stores uncontaminated by any material amount of transmitter newly synthesized in response to stimulation. Table 3 shows the total amount of acetylcholine released from ganglia in this first 3-min period; the effect of perfusion with various quaternary ammonium bases is shown. None of the compounds tested reduced the initial release of acetylcholine significantly. However, perfusion with hemicholinium, 2×10^{-5}M, or hexamethonium, 2×10^{-4}M, actually increased the initial output of acetylcholine; this increase was statistically significant.

Since, anatomically, the superior cervical and the nodose ganglia are closely adjacent both are necessarily perfused together. In some experiments the nodose ganglia were excised at the same time as the corresponding superior cervical ganglia and assayed for acetylcholine content. The nodose ganglion of the vagus has been noted previously to contain small quantities of acetylcholine (MacIntosh, 1941). In the present experi-

ments the mean acetylcholine content (mean and standard error) of nodose ganglia was, on the unperfused side, 28 ± 4.0 ng ($n=16$), 11% that of the content of corresponding superior cervical ganglia, 254 ± 18.2 ng ($n=16$).

TABLE 3
EFFECT OF QUATERNARY AMMONIUM COMPOUNDS ON THE INITIAL RELEASE OF ACETYLCHOLINE FROM THE SUPERIOR CERVICAL GANGLION OF THE CAT
The physostigmine experiments† acted as controls for the other drugs. Outputs are means and standard errors. * Difference statistically significant ($P<0.05$)

Drug	Concentration ($M \times 10^{-5}$)	No. of expts	Acetylcholine output in first 3 min as percentage of initial content.
Physostigmine	3	6	21·2±2·6†
	30	3	20·6±0·2
Hemicholinium (HC-3)	2	8	27·9±1·4*
	20	4	30·2±2·6
Triethylcholine	20	7	22·7±1·9
	200	3	16·8±2·0
Tetraethylammonium	20	3	25·3±2·4
	200	4	23·5±5·8
Troxonium	2	4	25·5±1·5
	20	2	32·4±2·4
Tubocurarine	10	5	21·6±1·2
Hexamethonium	20	5	29·7±1·7*

On perfusion with plasma containing physostigmine the unstimulated nodose ganglia gained acetylcholine (Table 4), even in the presence of drugs which inhibited acetylcholine turnover in stimulated perfused superior cervical ganglia; in a total of thirteen such experiments, there was a mean increase of 89%. This increase in acetylcholine content was significantly reduced ($P<0.01$) to only 11% in three other experiments with the high hemicholinium concentration (2×10^{-4}M).

TABLE 4
ACETYLCHOLINE CONTENT OF PERFUSED NODOSE GANGLIA
Acetylcholine contents are means, and are relative to the content of the contralateral unperfused nodose ganglion (=100)

Drug	Concentration ($M \times 10^{-5}$)	No. of expts	Acetylcholine content (%)
Physostigmine	3	1	177
Troxonium	2	3	179
	20	2	159
Hexamethonium	20	3	183
Hemicholinium	2	4	193
	20	3	111

DISCUSSION

The plasma-perfused ganglion can efficiently synthesize and release acetylcholine. When stimulated repetitively it manufactures and releases in 1 hr acetylcholine equivalent to about four times its initial content.

It is probable that the decline in output at the start even of control experiments represents predominantly acetylcholine liberated rapidly from the smaller more readily releasable fraction of acetylcholine stores. This fraction comprises, according to Birks & MacIntosh (1961), only about one-fifth to one-quarter of the total depot acetylcholine available for release. Hence its depletion would occur comparatively quickly, leading to an early decline in output. Subsequently the release of acetylcholine from the larger less readily mobilized fraction contributed increasingly to the total output; this is also progressively supplemented by newly synthesized acetylcholine, if no inhibitor is present.

A stable level of output is reached when acetylcholine derived from these two sources becomes sufficient to balance that released. The nadir of the output curve at 5 min, preceding the stable level, may coincide with the point at which fresh acetylcholine just begins to supplement liberation from preformed stores. Indeed, there is some evidence that synthesis of new acetylcholine is delayed and requires some 4 or 5 min stimulation to become fully effective (Quastel, 1962). As a corollary to this, after stimulation for a few minutes the ganglionic content should be less than the initial content, for acetylcholine would be released from its depots, partially depleting them. At this point the depots would be replenished little, if at all, by synthesis. Results from experiments with the blood-bathed ganglion stimulated *in vivo* in the absence of an anticholinesterase support this contention: after 5 min stimulation ganglionic acetylcholine contents were decreased by 13.4%, but after 60 min they were only 0.8% below the content of contralateral unstimulated ganglia (MacIntosh, 1963).

With a tenfold increase in physostigmine concentration acetylcholine outputs were decreased, but net synthesis was not affected, suggesting that in high concentration physostigmine inhibits a process involved in the maintenance of the release rather than the synthesis of acetylcholine. At concentrations up to 7.26×10^{-5}M physostigmine is without effect upon the conducted action potential in mammalian nonmyelinated nerve fibres (Armett & Ritchie, 1961). On the other hand, Wright (1956) has reported that high concentrations of physostigmine, from 5×10^{-3}M to 8×10^{-2}M, progressively but reversibly block high frequency conduction in nerves, apparently like local anaesthetics by affecting the normal permeability changes. It seems likely therefore that in the present experiments using the raised physostigmine concentration of 3×10^{-4}M some degree of intermittent transmission failure contributed to the decline in acetylcholine output. Synthesis was certainly not impaired, for ganglionic acetylcholine stores rose markedly. The precise location of this additional acetylcholine is uncertain. Whether it is cytoplasmic or particle-bound is not clear but it is evident that the ganglion is able to retain this grossly increased amount of acetylcholine intracellularly without significant leakage into the perfusate.

Shelley (1956) found physostigmine to have a significant inhibitory effect on acetylcholine synthesis in guinea-pig brain slices, but only in very high concentrations, above 2.7×10^{-3}M. Choline antagonized this effect. She concluded therefore that physostigmine competes with choline for the active centres of choline acetylase. When, however, the structural and functional integrity of neuronal tissue is preserved, as in the perfused ganglion, other factors may govern the precise nature of the inhibitory effect. Thus the existence of membrane barriers together with the operation of a highly efficient choline uptake mechanism during stimulation might tend to minimize the type of inhibition proposed by Shelley. Furthermore, a progressive interruption of nerve impulse traffic (see above) might intervene to reduce acetylcholine release by diminishing the rate of excitation-secretion coupling before any appreciable effects appeared on the synthesizing mechanism.

The accumulation by the unstimulated nodose ganglion of extra acetylcholine in the presence of an anticholinesterase suggests that it may form " surplus " acetylcholine in a similar way to the resting superior cervical ganglion. The 89% increase in acetylcholine content for the nodose ganglion certainly compares quite closely with the 93%

increase found by Birks & MacIntosh (1961) for the resting superior cervical ganglion. Only the high hemicholinium concentration interfered appreciably with the ability of the nodose ganglion to accumulate acetylcholine. It is not clear, however, whether the acetylcholine found in the nodose ganglion, generally regarded as a nonsynaptic structure, is of neuronal or extraneuronal origin.

The ability of hemicholinium to impair sympathetic ganglionic acetylcholine metabolism by inhibition of synthesis was confirmed. Moreover, the net inhibitory effect on acetylcholine turnover of triethylcholine, tetraethylammonium and troxonium, though less than that of hemicholinium, was otherwise almost indistinguishable from it. This suggests that all four substances may act presynaptically by the same mechanism. There is, for example, evidence that hemicholinium affects synthesis indirectly, preventing the transport of choline inward to the nerve terminals. Other quaternary compounds might act similarly. An alternative explanation exists: they might usurp the storage or binding sites for acetylcholine (MacIntosh, 1961). It is improbable that they are themselves acetylated or that they directly inhibit choline acetyltransferase (Gardiner, 1961 ; Hemsworth & Morris, 1964). Thus an effect may occur either at the level of the neuronal membrane, interfering with the choline carrier mechanism, or alternatively, or additionally, at an intracellular locus. In an endeavour to resolve these possibilities, the uptake and release of [^{14}C]-tetraethylammonium were studied in plasma perfused ganglia (Matthews, unpublished). These experiments were however not conclusive because any turnover of tetraethylammonium at ganglionic nerve endings was obscured by a considerable uptake of the labelled material by other adjacent tissues.

The results obtained in reversal experiments, too, unfortunately provide little discriminatory information about the precise site of action of hemicholinium or triethylcholine. Despite their almost identical net inhibitory effect (Table 1) the rate of recovery of output differed with these two substances. Recovery from inhibition by triethylcholine was more rapid both with fresh plasma and increased exogenous choline than the corresponding reversal of hemicholinium inhibition. Since a 10:1 molar ratio of choline to inhibitor was more effective in reversing an inhibition due to triethylcholine than one due to hemicholinium it seems that triethylcholine is more readily displaced from its site of action than hemicholinium. The capacity of hemicholinium to bind so effectively either at neuronal membranes or indeed any other site in the synthetic pathway or storage process must depend upon some factor other than a straightforward similarity to the choline substrate, for in terms of molecular size triethylcholine appears the closer analogue. Possession of a terminal bisquaternary configuration, whether interposed by an aromatic nucleus or not, alone cannot satisfactorily account for a potent inhibitory action, for the bisquaternary drugs tubocurarine and hexamethonium were without such effect. The antipodal arrangement and distance of separation of the choline moieties of the hemicholinium molecule must therefore remain the most important, probably critical, determinants of its high presynaptic activity in the cholinergic pathway.

The inflexion in the output curve seen during perfusion with tetraethylammonium might be explained by assuming that tetraethylammonium either directly increases acetylcholine release at this point or acts indirectly by accelerating synthesis or mobilization of newly formed acetylcholine. An effective increase in the mobilization of acetylcholine from the less readily releasable depot or of fresh acetylcholine would seem more likely

since any influence of the tetraethylammonium on the release mechanism, as such, should be apparent much earlier. Although the total synthesis of acetylcholine is obviously strongly inhibited during 1 hr of stimulation, it remains a possibility that tetraethylammonium induced a transient increase in the synthesis rate initially, sufficient to be reflected subsequently in the output curve.

Tetraethylammonium is known to increase acetylcholine release under certain conditions (Douglas & Lywood, 1961 ; Matthews, 1961 ; Collier & Exley, 1963). It did not do so at the start of the present experiments. However, this might be because at a stimulation rate of 20 shocks/sec acetylcholine is liberated from its preformed depots at close to the maximum possible output per volley and only later might the output be increased indirectly as the result of an effect on the formation or mobilization of fresh acetylcholine. It becomes difficult then to account for the increased initial release seen with hemicholinium and hexamethonium perfusion unless this is ascribed to a different action. Perhaps some kind of base-exchange mechanism operates between these compounds, which are both bisquaternary compounds, and acetylcholine. A similar mechanism has been put forward to explain the sharp but temporary increase in the output of choline observed from stimulated ganglia perfused with hemicholinium (Birks & MacIntosh, 1961). Alternatively they might prevent uptake by other tissues of acetylcholine released from presynaptic terminals. However, a greater proficiency in the preparation and stimulation of the preganglionic nerve might in part account for these results ; they were certainly obtained from experiments performed in the later part of the experimental series. More experiments and a greater fractionation of acetylcholine output in the first 5 min of stimulation would be required to determine the precise nature and relative importance of the initial effects of these two quaternaries.

The fact that all the quaternaries examined were without significant *inhibitory* effect upon acetylcholine release initially is an interesting finding both in view of the postulation by Koelle (1961) of an autocatalytic release of acetylcholine and because of the suggestion (Lilleheil & Naess, 1961 ; Standaert, 1964) of a presynaptic action of tubocurarine. From the present results it appears that if presynaptic receptors for cholinergic transmitter action do exist then these must differ quite considerably from those at the postsynaptic site, particularly in their susceptibility to blocking agents. Especially, for example, were hexamethonium and tubocurarine without important presynaptic effects. This evidence confirms, for prolonged stimulation under conditions more optimal for acetylcholine metabolism, the lack of presynaptic activity of these quaternaries, described previously only for relatively short periods of stimulation and in experiments where turnover of acetylcholine must have been suboptimal (Brown & Feldberg, 1936a ; Paton & Zaimis, 1951). The results with tubocurarine agree with those obtained at the neuromuscular junction by Cheymol, Bourillet & Ogura (1962), who measured acetylcholine output from the rat diaphragm. Bhatnagar & MacIntosh (1966) found tubocurarine and hexamethonium to have a small but measurable inhibitory effect on acetylcholine synthesis by minced brain. Negligible inhibitory activity was observed in the present experiments. This is most probably due to the superior functional ability of the intact ganglion to turnover acetylcholine compared with the less optimal metabolism in nervous tissue whose structural integrity has been destroyed with consequent disruption of important limiting cell membrane barriers.

The results obtained in the present study show clearly that it can no longer be tacitly assumed that any quaternary ammonium base is devoid of presynaptic action unless subjected to direct test. Especially is this so when structurally the quaternary molecule resembles choline. It has been pointed out already, on the basis of these experiments (MacIntosh, 1963), that there seems no reason why the ability of various quaternary ammonium compounds to interfere with presynaptic acetylcholine turnover should parallel their ability to interfere with acetylcholine action postsynaptically, any more than there is to expect their blocking and anticholinesterase activities to be in constant ratio.

Only an intensive study of molecular configuration related to inhibitory activity on acetylcholine metabolism can be expected to define the structural requirements for a presynaptic inhibitor at cholinergic junctions. Nevertheless, the evidence so far obtained is suggestive. For example, an examination of gallamine for presynaptic effects might prove of considerable interest. This substance is notable for its postjunctional blocking activity. Yet structurally it can be considered essentially as three molecules of triethylcholine distributed about a benzene nucleus. Furthermore, results of recent experiments add to the probability that gallamine increases acetylcholine release (Jones & Laity, 1965). Thus it may resemble tetraethylammonium (see above) and triethylcholine (Roberts, 1962). But with high intensity nerve traffic it might well interfere with the synthetic pathway for acetylcholine, again as do triethylcholine and tetraethylammonium at the ganglion, and triethylcholine (Bowman & Hemsworth, 1965) and probably also tetraethylammonium (Matthews, unpublished) at the neuromuscular junction.

In conclusion it may be noted that a recent investigation has disclosed the presence of a specific choline uptake mechanism in the axon of the squid (Hodgkin & Martin, 1965), a process most probably associated with the neuronal membrane. This " carrier " system was inhibited, apparently competitively, by hemicholinium>triethylcholine> tetraethylammonium, as well as by acetylcholine and tetramethylammonium. Of these compounds, hemicholinium was again the most potent inhibitor. Tubocurarine did not inhibit choline transport in this system (K. Martin, personal communication).

SUMMARY

1. The superior cervical ganglion of the cat was perfused with plasma containing physostigmine and stimulated repetitively: ganglionic acetylcholine turnover was studied in the presence or absence of added quaternary ammonium compounds.

2. With prolonged stimulation in the presence of either hemicholinium, triethylcholine, tetraethylammonium or troxonium (2×10^{-5}M, or greater), acetylcholine outputs fell progressively below control levels, ganglionic acetylcholine stores were depleted and synthesis of acetylcholine was inhibited. These effects were reversed by perfusion with fresh plasma or by increasing the exogenous choline concentration.

3. In contrast, tubocurarine (10^{-4}M) and hexamethonium (2×10^{-4}M) affected neither the maintained output nor the synthesis of acetylcholine significantly.

4. Raising the physostigmine concentration tenfold, to 3×10^{-4}M, significantly inhibited the total output but not the synthesis of acetylcholine.

5. None of the compounds tested significantly reduced the initial release of acetylcholine ; only hemicholinium and hexamethonium increased it.

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