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ACETYLCHOLINE SYNTHESIS FROM RECAPTURED CHOLINE BY A SYMPATHETIC GANGLION

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SUMMARY

1. The recapture and re-use of choline formed by the hydrolysis of released acetylcholine (ACh) was studied in the superior cervical ganglion of the cat using radioactive tracer techniques. The ganglion's ACh store was labelled by perfusion, during preganglionic nerve stimulation, with Krebs solution containing [^{3}H]choline.

2. Preganglionic stimulation (5 Hz for 20 min) of ganglia containing [^{3}H]ACh released similar amounts of radioactivity when perfusion was with neostigmine–choline–Krebs or with hemicholinium–Krebs. This indicated that neostigmine does not increase transmitter release.

3. The amount of radioactivity collected from stimulated ganglia during perfusion with choline–Krebs was 39% of the amount of radioactivity collected during perfusion with medium containing neostigmine or hemicholinium. This difference in release was almost (85%) accounted for at the end of the experiment by extra radioactive ACh in the ganglia perfused with choline–Krebs. It is concluded that during preganglionic nerve stimulation approximately 50–60% of endogenously produced choline is recaptured for ACh synthesis; thus, during activity preganglionic nerve terminals appear selectively to accumulate choline.

4. However, chronically decentralized ganglia accumulated as much choline as did acutely decentralized ganglia, and this was interpreted as indicating that at rest preganglionic nerve terminals do not selectively accumulate choline.

5. Increased exogenous choline concentration increased the amount of radioactivity collected during nerve stimulation in the absence, but not the presence, of an anticholinesterase agent. The spontaneous efflux of radioactivity was little affected by changes in external choline levels. It is concluded that exogenous choline and choline made available from released transmitter compete for uptake into nerve terminals.

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INTRODUCTION

It has been suggested that cholinergic synapses re-use choline that is formed by the hydrolysis of released transmitter (Perry, 1953; Collier & MacIntosh, 1969; Potter, 1970; Bennett & McLachlan, 1972; Hanin, Massarelli & Costa, 1972). In part this hypothesis was supported by the observation that during preganglionic nerve stimulation the amount of acetylcholine (ACh) collected from a cat's superior cervical ganglion in the presence of eserine is about twice the amount of choline collected in the absence of eserine (Collier & MacIntosh, 1969). Before this observation can be unequivocally accepted as indicating that about 50% of choline that is formed from ACh hydrolysis is recaptured by the ganglion, an effect of the anticholinesterase agent upon ACh release must be ruled out. It has been suggested that anticholinesterase agents might increase ACh release (Riker, Roberts, Standaert & Fujimori, 1957; Takeshige & Volle, 1962, 1963; Carlyle, 1963; Standaert & Riker, 1967), or that the presence of anticholinesterase agents might result in a decreased ACh release (Szerb & Somogyi, 1973). One aim of the present experiments was to determine the effect of neostigmine on transmitter release in the cat superior cervical ganglion, so that a more definitive conclusion can be made about choline recapture.

A second aim of the experiments described in this paper was to identify the fate of the choline that was recaptured from hydrolysed ACh by the superior cervical ganglion, because this was not done in the earlier experiments of Perry (1953) and of Collier & MacIntosh (1969). Cholinergic and non-cholinergic neurones take up choline (Diamond & Milfay, 1972), and much of the ganglion's endogenous choline is located outside cholinergic nerve endings (Friesen, Ling & Nagai, 1967). If recaptured choline selectively entered cholinergic nerve terminals, it might be used mainly for ACh synthesis, but if recaptured choline entered non-cholinergic structures, it would be converted to other metabolites such as phosphorylcholine or phosphatidylcholine (Ansell & Spanner, 1968; Collier & Lang, 1969; Abdel-Latif & Smith, 1972).

METHODS

The methods used were similar to those described earlier (Collier & Lang, 1969; Collier & MacIntosh, 1969; Collier & Katz, 1971; Katz, Salehmoghaddam & Collier, 1973).

Ganglion perfusion. Cats (1·8–3·0 kg) were used; anaesthesia was induced by ethyl chloride followed by ether and was maintained by chloralose (I.V., 80 mg/kg). The superior cervical ganglion was perfused by the method of Kibjakow (1933) as modified by Feldberg & Gaddum (1934) and described by Collier & Lang (1969). In some experiments, the right ganglion was perfused and the left ganglion was removed before the start of perfusion and used as the control for ACh content. In other

experiments, both ganglia were perfused. Perfusion was with Krebs solution (mM: NaCl 120, KCl 4·6, $CaCl_2$ 2·4, KH_2PO_4 1·2, $MgSO_4$. $7H_2O$ 1·2, glucose, 9·9, $NaHCO_3$ 25) which was equilibrated with 5% CO_2 in O_2 throughout the experiment to maintain its pH at 7·4 at 37° C. The perfusion medium, unless it is stated otherwise, contained choline chloride (10^{-5} M), either unlabelled (British Drug Houses) or (Me^3H)-labelled (100 mc/m-mole, New England Nuclear); Krebs solution containing choline (10^{-5} M) is referred to as 'choline–Krebs solution'. In the experiments that measured choline uptake by chronically decentralized ganglia, the choline concentration in the perfusion medium was varied and its specific activity was changed so that the medium always contained the same amount of radioactivity (1 μc/ml.). Where indicated, neostigmine bromide (3×10^{-5} M, Sigma) or hemicholinium No. 3 dibromide (5×10^{-5} M, generously provided by Dr V. B. Haarstad, Tulane University) was added to the perfusion medium.

Nerve stimulation. In all experiments, the preganglionic nerve was cut low in the neck. When necessary, the nerve was stimulated with supramaximal rectangular pulses (5 V, 0·3 msec) and during stimulation the electrode was moved a few millimetres proximally along the nerve every 5 min.

Ganglion extracts. Acid soluble material was extracted from the ganglion by the procedure described by Birks & MacIntosh (1961). The ganglion was removed quickly, minced finely in 2·0 ml. 10% (w/v) ice-cold trichloro-acetic acid (TCA) solution, and allowed to stand at 0° C for 90 min. The TCA extract was recovered, TCA was removed by shaking the extract with water-saturated ether, and the aqueous solution (pH 4–6) was aerated briefly to remove residual ether. Separate aliquots of this aqueous extract were used for the bio-assay of ACh and for separating labelled ACh from other labelled material. In some experiments, the residue that remained after the ganglion had been extracted with TCA was allowed to stand with 1·0 ml. of a mixture of chloroform and methanol (2:1) containing hydrochloric acid (0·25%) in order to extract phospholipid.

Bio-assay of ACh. The ACh content of ganglion extracts was determined by bioassay on the blood pressure of the eviscerated cat (MacIntosh & Perry, 1950). The vasodepressor effect of the unknown sample was compared with that of authentic ACh chloride. The effect of both the unknown sample and of authentic ACh was always enhanced by eserine, and was abolished by pretreating the cat with atropine or by treating the sample with alkali.

Separation of radioactive compounds. Labelled ACh was separated from other labelled material (choline and phosphorylcholine) by the sequential reineckate and gold precipitation procedure described earlier (Collier & Katz, 1971; Katz, Salehmoghaddam & Collier, 1973). The partition of authentic labelled choline and labelled ACh was determined routinely each time the procedure was used.

Determination of radioactivity. Radioactivity was determined by liquid scintillation spectrometry (Picker Nuclear Liquimat 330, or Nuclear Chicago Mark I) in a solvent system consisting of 1000 ml. toluene, 1000 ml. ethanol, 1000 ml. dioxane, 240 g naphthalene, 15 g PPO and 187 mg POPOP. Quench correction was made by adding internal standards.

Chronic decentralization of ganglia. The cats were anaesthetized with sodium pentobarbitone (I.P., 35 mg/kg) and a segment (2–3 mm) of the right preganglionic sympathetic nerve was removed under aseptic conditions. The animals were given (I.M.) 50,000 u. of procaine penicillin and 50,000 u. benzathine penicillin, allowed to recover, and were used for experiment 14–16 days after the operation.

RESULTS

1. *The lack of effect of neostigmine on ACh release.* In ten experiments, ganglionic ACh was labelled by perfusing for 45 min with Krebs solution containing [^{3}H]choline during preganglionic nerve stimulation (5 Hz); this was followed by a 16 min washout with Krebs solution containing unlabelled choline. In five experiments, perfusion was then switched to Krebs solution containing choline and neostigmine, the preganglionic nerve was stimulated (5 Hz) for 20 min and the release of [^{3}H]ACh was

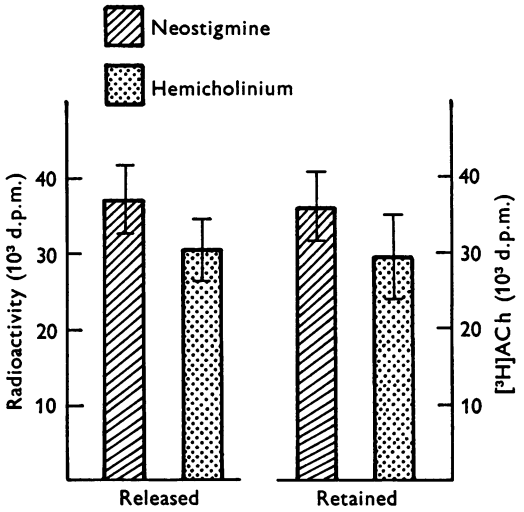

Fig. 1. The extra radioactivity released by nerve stimulation (5 Hz for 20 min) and the amount of [^{3}H]ACh retained by ganglia at the end of the experiment in ganglia perfused with either neostigmine–choline–Krebs or with hemicholinium–Krebs. The ganglia had been perfused with [^{3}H]choline–Krebs for 45 min (5 Hz stimulation throughout) and then for 16 min with choline–Krebs. Each column represents the mean ± S.E. of five experiments.

measured. In the other five experiments, perfusion during the 20 min test stimulation was with Krebs solution containing hemicholinium and the release of [^{3}H]choline during nerve stimulation (5 Hz) was measured. In all experiments, the amount of total ACh and of [^{3}H]ACh retained by the ganglion at the end of the experiment was determined. Fig. 1 summarizes the results of these experiments; there was no significant difference ($P > 0{\cdot}3$) between the release of [^{3}H]ACh into medium containing neostigmine and the release of [^{3}H]choline into medium containing hemicholinium. The amount of [^{3}H]ACh retained by the ganglia was similar, and this demonstrates that the labelling procedure was as efficient for the

experiments that tested release in the presence of neostigmine as it was for those that tested release in the presence of hemicholinium. As expected, total ACh in ganglia stimulated in the presence of hemicholinium was partly depleted, and ACh content of these ganglia was $47 \pm 6\%$ of that of their controls. Neostigmine was chosen as the anticholinesterase agent for these experiments because a 20 min exposure to neostigmine does not alter total ACh content (Collier & Katz, 1971); this was confirmed in three of the present experiments in which total ACh content of the test ganglia was $108 \pm 6\%$ of control.

In these experiments, the specific radioactivity of ganglionic ACh at the time that nerve stimulation was started could be calculated by adding the amount of radioactivity released by nerve stimulation to the amount of [^{3}H]ACh retained by the ganglion at the end of the experiment, and dividing this value by the calculated amount of total ACh in the ganglion at the time nerve stimulation was started. In the ganglia exposed to neostigmine, the total ACh content of the ganglion at the onset of nerve stimulation was taken to be equal to the final ACh content measured; in ganglia exposed to hemicholinium, the total ACh content of the ganglion at the onset of nerve stimulation was assumed to be equal to that of the contralateral control ganglion. The calculated specific activity of ACh in ganglia at the onset of nerve stimulation was compared to the measured specific activity of ACh in ganglia at the end of the experiment. The specific activity of ACh in ganglia perfused with HC-3 did not change during nerve stimulation, but the specific activity of ACh in ganglia perfused with neostigmine was reduced by $51 \pm 5\%$.

2. *Choline uptake by chronically decentralized ganglia.* To test whether choline uptake by ganglia is specifically into cholinergic nerve terminals, the accumulation of [^{3}H]choline by chronically decentralized ganglia was compared to that by acutely decentralized ganglia. In each experiment, both ganglia (not stimulated) were perfused for 12 min with Krebs solution containing [^{3}H]choline (5×10^{-7} M, or $1{\cdot}4 \times 10^{-6}$ M or 10^{-5} M) and then washed by perfusing for 2 min with medium containing the same concentration of unlabelled choline. The ganglia were removed, and assayed for total ACh, [^{3}H]ACh, [^{3}H]choline, and [^{3}H]phospholipid. The results of these experiments (Table 1) show that the decentralized ganglia accumulated as much [^{3}H]choline as did intact ganglia, and this indicates that, at rest, ganglia do not selectively take up choline into preganglionic nerve terminals. The chronically decentralized ganglia had, as expected, lost most of their transmitter store, and, in contrast to the innervated ganglia, they did not synthesize a measurable amount of [^{3}H]ACh. The decentralized ganglia consistently incorporated more [^{3}H]choline into phospholipid than did the control tissue, but the significance of this is not obvious.

TABLE 1. The accumulation of [^{3}H]choline by chronically decentralized ganglia (D) and by acutely decentralized ganglia (N)

		Ganglion content (p-mole)											
		[^{3}H]choline			[^{3}H]ACh			[^{3}H]phospholipid			Total ACh		
[^{3}H]choline content	Expt.	D	N	D/N × 100	D	N	D/N × 100	D	N	D/N × 100	D	N	D/N × 100
5×10^{-7} M	1	10·2	8·3	123	< 0·15	0·43	< 35	1·5	0·71	211	71	989	7
5×10^{-7} M	2	13·3	13·8	96	< 0·26	0·72	< 36	3·3	2·6	127	< 82	1154	< 7
5×10^{-7} M	3	10·7	11·7	91	< 0·20	1·1	< 18	7·2	3·4	212	< 55	1236	< 4
$1{\cdot}4 \times 10^{-6}$ M	4	36·7	44·2	83	< 0·73	4·9	< 15	11·5	3·6	319	98	1230	8
1×10^{-5} M	5	176	213	83	< 3·5	16·1	< 22	52	21	248	< 71	1319	< 5
1×10^{-5} M	6	325	324	100	< 6·0	28·1	< 21	35	30	117	< 130	1099	< 12
	Mean ± S.E.			96 ± 6			< 24			206 ± 31			

3. *The recapture of choline from hydrolysed ACh.* In six experiments, both of the cat's superior cervical ganglia were perfused for 45 min with [^{3}H]choline–Krebs solution during preganglionic nerve stimulation (5 Hz). At the end of this perfusion, nerve stimulation was stopped and ganglia were washed by perfusing for 16 min with Krebs solution containing unlabelled choline. In each experiment, perfusion of one ganglion was continued with Krebs solution containing choline and the other ganglion was perfused with either Krebs solution containing choline (one experiment) or with Krebs solution containing neostigmine and choline (three experiments) or, with Krebs solution containing hemicholinium (two experiments). The effluent was collected from these ganglia in 2 min aliquots for 40 min; for the first 10 min the ganglia were not stimulated, then the preganglionic trunk was stimulated (5 Hz) for 20 min, and this period of stimulation was followed by a 10 min rest. (In the experiments that used neostigmine, the last 10 min perfusion was with Krebs solution containing choline so that accumulation of surplus ACh was minimized). The release of extra radioactivity during nerve stimulation and the amount of [^{3}H]ACh retained by the ganglia was measured. The radioactivity released by nerve stimulation was [^{3}H]choline in the absence of neostigmine, and was [^{3}H]ACh in the presence of neostigmine.

One experiment (experiment 1 of Table 2) demonstrated that paired ganglia behave similarly under similar test conditions. This experiment measured the release of radioactivity from the left and the right ganglion when both were perfused with Krebs solution containing choline. The release of extra radioactivity during stimulation was about the same from the two ganglia and there was no large difference in the amount of [^{3}H]ACh retained by the ganglia at the end of the experiment.

Five experiments (expts. 2–6 of Table 2) demonstrated the recapture of choline produced by the hydrolysis of released transmitter and showed that this choline was synthesized to ACh. In these experiments, the amount of radioactivity collected when perfusion was with Krebs solution containing choline was compared to that collected when perfusion was with medium containing neostigmine or hemicholinium. In all experiments, the amount of radioactivity collected during perfusion with choline–Krebs was less than the amount of radioactivity collected in the presence of either neostigmine or of hemicholinium; release in the absence of drug (neostigmine or hemicholinium) was 39 ± 5 % of release in the presence of drug, and this demonstrates the recapture of choline. The ganglia perfused with choline–Krebs always contained more [^{3}H]ACh at the end of the experiment than did the contralateral ganglia that had been perfused with neostigmine- or with hemicholinium-medium. The lesser amount of radioactivity collected (20, 360 ± 2382 d.p.m.) in the absence of a drug was

almost accounted for ($85 \pm 6\%$) by the greater amount of [^{3}H]ACh (17, 731 ± 3049 d.p.m.) contained in these ganglia at the end of the experiment. Thus most of the recaptured choline was synthesized to ACh. The amount of free [^{3}H]choline in ganglia perfused with choline–Krebs was not different ($114 \pm 12\%$) from that in ganglia perfused with neostigmine or with hemicholinium.

TABLE 2. Radioactivity collected during nerve stimulation (5 Hz for 20 min) and radioactivity remaining in ganglia at the end of the experiment; the ganglia were perfused with choline–Krebs, neostigmine–choline–Krebs or hemicholinium–Krebs during nerve stimulation

	Experiment	^{3}H-label collected (d.p.m.)	[^{3}H]ACh retained (d.p.m.)	S.A.* of ACh onset of stim. (d.p.m./ p-mole)	S.A.* of ACh end of expt. (d.p.m./ p-mole)
1. (*a*)	Choline	14,103	50,593	990	792
(*b*)	Choline	12,402	49,941	952	743
	Difference (*a*–*b*)	+1,701	+652	+38	+49
2. (*a*)	Neostigmine–Choline	42,349	31,213	1799	797
(*b*)	Choline	16,922	58,366	1842	1424
	Difference (*a*–*b*)	+25,427	−27,153	−43	−627
3. (*a*)	Neostigmine–Choline	40,797	39,831	1479	665
(*b*)	Choline	16,822	59,788	1403	1094
	Difference (*a*–*b*)	+23,975	−19,957	+76	−429
4. (*a*)	Neostigmine–Choline	28,551	23,984	902	352
(*b*)	Choline	8,272	41,549	858	715
	Difference (*a*–*b*)	+20,279	−17,565	+44	−363
5. (*a*)	Hemicholinium	26,921	30,622	1265	1298
(*b*)	Choline	15,170	39,085	1194	858
	Difference (*a*–*b*)	+11,751	−8,463	+71	+440
6. (*a*)	Hemicholinium	29,346	12,995	968	1067
(*b*)	Choline	8,977	28,540	858	654
	Difference (*a*–*b*)	+20,369	−15,545	+110	+413

* Specific activity.

The specific radioactivity of the ganglion's ACh at the start of nerve stimulation in these experiments was estimated by adding the amount of radioactivity released to the amount of [^{3}H]ACh retained by the ganglion and dividing by the ganglion's total ACh content. For the purpose of the calculation, the total ACh content of each ganglion at the time nerve stimulation was started, was assumed to be equal to the final ACh content

of the ganglion that had been perfused with choline–Krebs solution. It is clear (Table 1) that in all experiments both ganglia of any pair had been labelled to a similar specific radioactivity; the difference between paired ganglia was < 13%. Except for the ganglia perfused with HC-3, the specific radioactivity of ganglionic ACh at the start of nerve stimulation

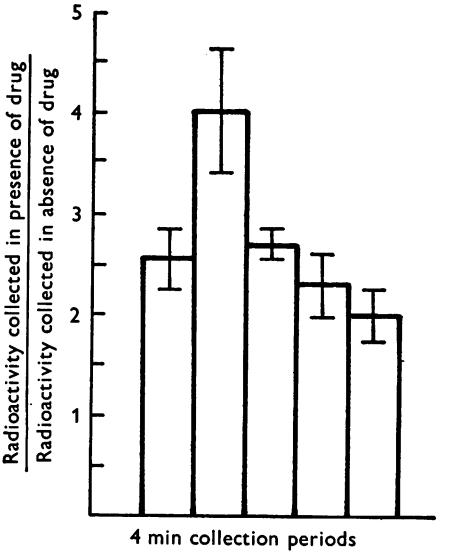

Fig. 2. Ratio of the amount of radioactivity collected during nerve stimulation (5 Hz for 20 min) in the presence of drug (neostigmine or hemicholinium) to the amount of radioactivity collected in the absence of drug. The ganglia were first perfused for 45 min with [^{3}H]choline–Krebs (5 Hz stimulation throughout) and then with choline–Krebs for 16 min. Each column represents a 4 min collection period and is the mean ± S.E. of five experiments.

was greater than the measured specific radioactivity of ACh in the ganglia at the end of the experiment. The decrease of specific radioactivity of ACh as the result of nerve stimulation in the absence of hemicholinium or of neostigmine was 22 ± 1%. Thus the dilution of the ganglion's radioactive ACh during nerve stimulation was greater in the presence than in the absence of neostigmine, and this is consistent with the hypothesis that [^{3}H]choline produced by hydrolysis of released [^{3}H]ACh is re-used for ACh synthesis.

It has been suggested (Perry, 1953; Emmelin & MacIntosh, 1956) that there is a delay of about 5 min after the onset of stimulation before choline recapture can be demonstrated. This was not so in the present experiments. Fig. 2 shows the ratio of the amount of extra radioactivity collected during

nerve stimulation when perfusion was with neostigmine- or with hemicholinium-medium to the amount of extra radioactivity collected when perfusion was without either of these drugs. The recapture of choline was apparent from the onset of stimulation, although the ratio was somewhat greater in the second 4 min of stimulation than it was in the first 4 min of stimulation.

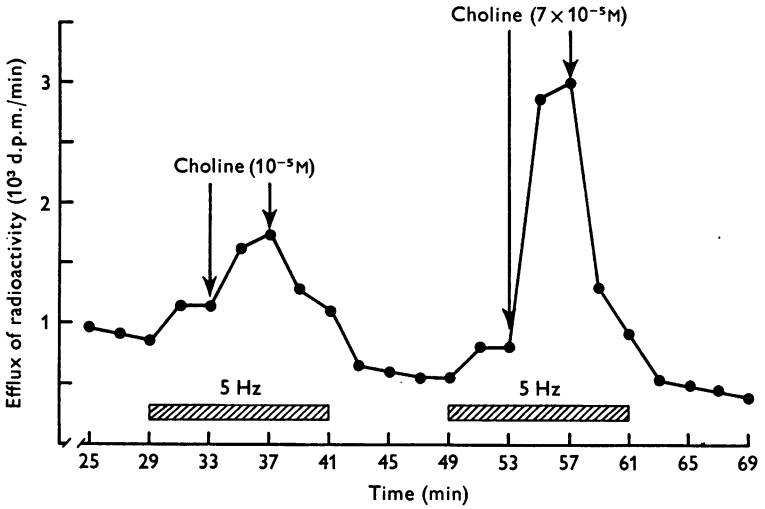

Fig. 3. The effect of exogenous choline (10^{-5} M or 7×10^{-5} M, where indicated) on the efflux of radioactivity released by nerve stimulation (5 Hz, during period indicated by horizontal bar). The ganglion was perfused with Krebs solution containing no anticholinesterase agent; it had previously been perfused with [^{3}H]choline–neostigmine–Krebs for 40 min during preganglionic nerve stimulation (5 Hz), and then for 25 min with Krebs solution (no choline or neostigmine).

4. *Competition between exogenous choline and endogenously produced choline.* All the experiments described above measured the recapture of choline formed from released ACh in the presence of exogenous choline (10^{-5} M); this concentration of choline is close to the normal value for cat's plasma (Bligh, 1952). Other experiments measured release in the absence of added choline and tested whether exogenous choline could compete for uptake with the choline produced by hydrolysis of released transmitter. In three experiments, ganglionic ACh was labelled by perfusing stimulated (5 Hz) ganglia for 40 min with [^{3}H]choline–Krebs solution containing neostigmine, stimulation was stopped and ganglia were washed by perfusion with choline-free-Krebs solution (no neostigmine) for 25 min. Effluent was then collected in 2 min aliquots and the preganglionic nerve was stimulated (5 Hz) for 12 min; during the 4th–8th min of

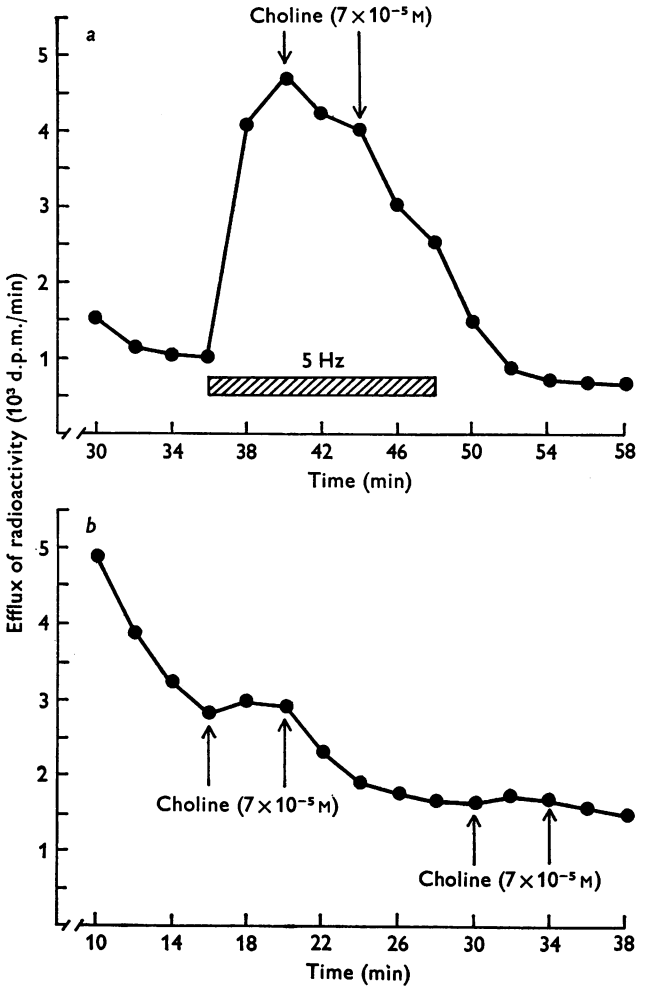

Fig. 4. *a*, The effect of choline (7×10^{-5} M, where indicated) on the efflux of radioactivity released by nerve stimulation (5 Hz, during period indicated by horizontal bar). The ganglion was perfused with neostigmine–Krebs throughout the experiment; it had previously been perfused with [^{3}H]choline–neostigmine–Krebs for 40 min during preganglionic nerve stimulation (5 Hz) and then for 30 min with neostigmine–Krebs solution.

b, The effect of choline (7×10^{-5} M, where indicated) on the spontaneous efflux of radioactivity. The ganglion was perfused with Krebs solution; it had previously been perfused for 40 min with [^{3}H]choline–neostigmine–Krebs and then with Krebs solution for 10 min.

the 12 min stimulation, perfusion was switched to Krebs solution containing choline (10^{-5} M or 7×10^{-5} M). Fig. 3 illustrates one of these experiments; it is clear that exogenous choline increased the amount of radioactivity collected and that 7×10^{-5} M choline was more effective than was 10^{-5} M choline. Similar results were obtained in the other two experiments. The results of these experiments suggest that exogenous choline can compete with choline produced from hydrolysed transmitter for capture by the ganglion. Alternatively the results of these experiments could be explained if choline increased ACh release, or if exogenous choline displaced radioactive choline, but these possibilities were eliminated by two further experiments (three tests). These were similar to that in Fig. 3 except that neostigmine was present in the perfusion medium throughout the experiment. The results of a typical experiment is illustrated by Fig. 4*a* which shows that choline (7×10^{-5} M) did not enhance the release of radioactivity in the presence of the anticholinesterase agent. The lack of effect

TABLE 3. The release of [^{3}H]ACh (d.p.m. per impulse) at different frequencies of stimulation

Expt.	5 Hz	20 Hz
1	10·8	12·4
2	11·2	12·1
3	7·5	7·2
4	14·1	14·6
Mean ± S.E.	10·9 ± 1·3	11·6 ± 1·5

of choline upon the release of radioactivity in the presence of neostigmine also demonstrates that exogenous choline releases little [^{3}H]choline from perfused structures by displacement of extracellular choline or by exchange diffusion with intracellular choline. A small displacement of radioactivity by exogenous choline could be demonstrated (Fig. 4*b*) when the rate of efflux of residual [^{3}H]choline was high, but this was too small to account for the effect of choline on the collection of radioactivity during nerve stimulation shown in Fig. 3.

5. *Release of transmitter at 5 Hz and 20 Hz stimulation.* Four experiments confirmed the finding of Birks & MacIntosh (1961) that ACh release/impulse is the same at differing rates of stimulation. In these experiments, ganglia were first perfused with [^{3}H]choline during preganglionic nerve stimulation (20 Hz) for 40 min to label the releasable store of transmitter. The release of [^{3}H]ACh by 600 impulses was then measured during nerve stimulation at 5 or 20 Hz; perfusion was with Krebs solution containing choline and neostigmine. In all experiments release was tested first at 20 Hz, then at 5 Hz and then again at 20 Hz, and each period of stimulation was followed by an 8 min period of rest. The release of [^{3}H]ACh at

20 Hz was obtained from the mean of the two tests, and the release/impulse was compared to that measured during 5 Hz stimulation (Table 3). There was no difference between the release/impulse at either frequency of stimulation.

DISCUSSION

In the presence of an anticholinesterase agent, ACh released from cholinergic nerve terminals appears not to be recaptured and re-used as a neurotransmitter (Perry, 1953; Collier & MacIntosh, 1969; Potter, 1970; Katz *et al.* 1973). However, the amount of ACh that can be collected in the presence of eserine from a cat's superior cervical ganglion when its preganglionic nerve is stimulated is greater than the amount of choline that can be collected from a ganglion stimulated in the absence of eserine (Perry, 1953; Collier & MacIntosh, 1969). This difference might be explained in either of two ways: it could indicate an effect of the anticholinesterase upon transmitter release or it could indicate that the ganglion can recapture choline formed from the hydrolysis of ACh. The present experiments used radioactive tracer techniques to distinguish between these two possibilities.

In these experiments, the ganglion's endogenous ACh store was replaced with [^{3}H]ACh and the amount of [^{3}H]ACh released by preganglionic nerve stimulation in the presence of neostigmine was shown to be about the same as the amount of [^{3}H]choline released by stimulation in the presence of hemicholinium. Hemicholinium blocks choline uptake (e.g. MacIntosh, Birks & Sastry, 1956; Schuberth, Sundwall, Sörbo & Lindell, 1966; Marchbanks, 1968; Diamond & Milfay, 1972) but has little effect on ACh release (Birks & MacIntosh, 1961; Matthews, 1966; Takagi, Kojima, Nagata & Kuromi, 1970). It seems likely, therefore, that under the conditions of the present experiments, neostigmine does not enhance ACh release.

The similar release of transmitter in the presence of neostigmine or of hemicholinium contrasts with the results of similar measurements made on strips of cerebral cortex by Szerb & Somogyi (1973). These authors demonstrated that anticholinesterase agents decrease the amount of ACh released by electrical stimulation of the cortical strips, probably by allowing accumulation of ACh which inhibits further release. The present experiments suggest that this inhibitory phenomenon does not occur in the sympathetic ganglion stimulated at 5 Hz. ACh-induced inhibition of transmitter release in ganglia could occur under other conditions, but the similar ACh release/impulse at 5 and 20 Hz suggests that this is not so even at an unphysiologically high frequency of stimulation; in addition,

previous experiments (Collier & Katz, 1970) showed that ACh (10^{-4} M) perfused through the ganglion did not inhibit release of transmitter.

The present experiments clearly demonstrated that much of the choline that is recaptured by the ganglion is used for ACh synthesis. In the presence of neostigmine or of hemicholinium, the amount of radioactivity collected during preganglionic stimulation of a ganglion whose ACh store had been labelled was greater than the amount of radioactivity collected under similar conditions but in the absence of neostigmine or of hemicholinium. This difference in the amount of radioactivity collected was almost fully accounted for by a greater amount of [^{3}H]ACh retained by the ganglia that had been allowed to recapture choline. Choline acetyltransferase is located in preganglionic nerve endings (Hebb & Silver, 1956; reviewed by Hebb, 1963, 1972), and therefore choline recapture must be into the cholinergic nerve endings. This suggests that during nerve stimulation, preganglionic nerve endings selectively accumulate choline, as if they have a more efficient choline transport system than have non-cholinergic neurones. However, this difference between choline uptake into cholinergic and non-cholinergic structures could not be demonstrated in non-stimulated ganglia. Ganglia that had been preganglionically denervated for 2 weeks, and had presumably lost their cholinergic nerve endings (Hámori, Láng & Simon, 1968), accumulated as much choline as did innervated ganglia. Thus, nerve stimulation appears to accelerate choline uptake by cholinergic nerve endings (see also Collier & MacIntosh, 1969), and this might be important for the physiological regulation of ACh synthesis.

The similar uptake of choline by denervated and by normal ganglia agrees with the results obtained by Diamond & Milfay (1972), who demonstrated equal accumulation of choline by synaptosomes prepared from brain areas comparatively rich in cholinergic nerve endings and by synaptosomes from areas of brain comparatively sparse of cholinergic terminals. However, other evidence suggests that cholinergic synaptosomes possess a more efficient choline transport system than do non-cholinergic synaptosomes. Kuhar, Sethy, Roth & Aghajanian (1973) have demonstrated that degeneration of cholinergic neurones in rat hippocampus selectively reduces choline uptake by isolated synaptosomes. In addition, Whittaker, Dowdall & Boyne (1972) and Yamamura & Snyder (1972) have described two kinetically distinct processes for choline uptake into synaptosomes; the process with high affinity appears to be associated with ACh synthesis (Yamamura & Snyder, 1972; Guyenet, Lefresne, Rossier, Beaujouan & Glowinski, 1973).

Although choline produced by ACh hydrolysis can be re-used for ACh synthesis, it is clear from the present experiments that exogenous choline can compete with endogenously produced choline for use in ACh synthesis,

and this is consistent with the technique used to label the ganglion's ACh store. The present experiments suggest that ganglia, stimulated at 5 Hz and perfused with a physiological concentration (10^{-5} M) of choline, recapture about 50–60 % of the choline made available from ACh hydrolysis. Thus endogenously produced choline is at least as important as is circulating choline for maintaining ACh synthesis. The present estimate of the proportion of choline formed from released ACh that is recaptured by ganglia is very similar to the value estimated by Collier & MacIntosh (1969), who also perfused ganglia with 10^{-5} M choline but stimulated the sympathetic nerve at 20 Hz. The release of ACh/impulse at 20 Hz is the same as at 5 Hz and therefore the concentration of choline from hydrolysed ACh should be the same at either frequency, and a similar proportion of endogenously produced and exogenous choline would be used for ACh synthesis. The present experiments also demonstrated that choline recapture is more efficient when exogenous choline is absent than when exogenous choline is present. This observation is consistent with the results of Bennettt & McLachlan (1972) who showed that the isolated sympathetic ganglion of guinea-pig can maintain ACh synthesis in the absence of added choline, provided that an anticholinesterase agent is not present.

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