Short Title

RECAPTURE OF ACETYLCHOLINE AND ITS METABOLIC PRODUCTS

ABSTRACT

THE RECAPTURE OF ACETYLCHOLINE AND ITS METABOLIC PRODUCTS

BY A SYMPATHETIC GANGLION

Ph.D. Thesis

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The recapture of acetylcholine (ACh) and its metabolic products, acetate and choline, was studied in the superior cervical ganglion of the cat. Ganglia that were perfused with ^{14}C-ACh accumulated exogenous ACh but this ACh was not available for release by unlabelled ACh or by high K^+, or by ortho- or antidromic nerve stimulation; accumulation of exogenous ACh was not increased during increased transmitter turnover and exogenous ACh could not be used to maintain ACh stores depleted by nerve stimulation in the absence of added choline. ACh accumulation was not blocked by atropine, hexamethonium or hemicholinium-3, and was the same in acutely or chronically decentralized ganglia. These results suggest that neuronally released ACh is not recaptured for re-use by preganglionic fibres. Exogenous ACh released surplus ACh, but the mechanism of this release was shown not to be a process of ACh exchange. Exogenous acetate was taken up by ganglia, but was not used for ACh synthesis, suggesting that the recapture of acetate from hydrolyzed released ACh is unlikely. When releasable ACh was radioactively labelled by in situ synthesis from labelled choline, the release of radioactivity by nerve stimulation was always greater in the presence of a drug that blocked choline recapture (directly or indirectly) than it was in the drug's absence; this difference was balanced by extra radioactive ACh in the ganglion perfused with medium allowing choline recapture. The results suggest that approximately 50% of the choline made available by hydrolysis of released ACh is recaptured for ACh synthesis.

RESUME

MODALITES DE REUTILISATION DE L'ACh PRÉALABLEMENT LIBÉRÉE AU NIVEAU DU GANGLION CERVICAL SUPERIEUR DU CHAT

Thèse pour l'obtention du degré de Docteur en Philosophie

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Le ganglion cervical supérieur du chat synthétise et libère de l'ACh. Le présent travail est une analyse de la possibilité d'une réutilisation de l'ACh liberée, ou directement sous cette forme ou sous la forme de l'un de ses métabolites: choline et acétate. Le ganglion cervical supérieur du chat, après perfusion à l'ACh marquée, accumule de l'ACh exogène. Cependant l'ACh ainsi accumulée n'est liberée ni par des stimulations ortho- ou antidromiques, ni par des perfusions avec un milieu contenant de l'ACh ou enrichi en ions K^+. De même, l'accumulation d'ACh exogène n'est pas accrue par une accélération de la synthèse et de la dégradation du transmitteur et elle ne peut pas compenser, en l'absence d'un apport externe de choline, la dépletion par stimulation soutenue, des réserves d'ACh. Des agents cholinergiques, tels que l'atropine, l'héxamethonium ou l'hémecholinium-3, ou des dénervations "chroniques" ou "aigues" ne modifient pas cette accumulation d'ACh exogène. Nous pouvons donc conclure que l'ACh préalablement libérée par les fibres préganglionnaires n'est pas directement utilisée sous cette forme par la suite. Il est montré aussi que la libération d'ACh "surplus" par de l'ACh exogène ne relève pas d'un processus d'échange. D'autre part, l'utilisation de l'acétate liberé par l'hydrolyse de l'ACh ne peut représenter qu'un processus secondaire puisque seules de très faibles proportions d'acétate exogène marqué ont eté retrouvées incorporées sous forme d'ACh. Finalement, après marquage in situ de l'ACh à partir de choline exogène ^3H, la fraction radioactive recueillie par stimulation nerveuse est accrue par toute substance réduisant la réutilisation de choline; cependant, dans un milieu où la capture de choline est possible, le ganglion presente relativement une teneur

superieure en ACh radioactive. En consequence, il semble que la choline provenant de l'hydrolyse de l'ACh liberée puissent être utilisée à la resynthèse d'ACh et ce dans une proportion de 50%.

THE RECAPTURE OF ACETYLCHOLINE AND ITS METABOLIC PRODUCTS BY A SYMPATHETIC GANGLION

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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August, 1972.

לזכר אבי היקר
איש תם וישר
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ACKNOWLEDGEMENTS

The problem discussed in this thesis was suggested by my research director, Dr. Brian Collier. I am thankful for his assistance during the course of the research and during the writing of this thesis; to Dr. Collier I dedicate the ";". I am also thankful to Dr. Mark Nickerson, Chairman, for the opportunity of studying Pharmacology in his department.

I acknowledge with thanks the excellent technical assistance of Peter Poon, and of Tim Lynch.

My special thanks go to my wife, Lorna F., for typing the rough version of this manuscript, and to my wife and parents for consistent encouragement during the course of my studies. I am grateful to Lorna and Saleh Saleh for proof-reading the manuscript, which was typed with care by Tziporah Cohen.

The figures presented in this thesis were drawn by Donna Morgan, and photographed by Roy Raymond.

The "thesis-room" was often a very stimulating environment. To Fred Vickerson I dedicate the number 43.33 and to Bruce Harfield I dedicate an egg for photography.

This work was supported by a grant from the Medical Research Council of Canada.

STATEMENT OF THE PROBLEM

Acetylcholine (ACh) is now accepted to be a neurotransmitter in sympathetic ganglia, but detailed knowledge of its turnover is still incomplete; this thesis contains the results of experiments that were designed to provide some of this knowledge.

It was not known with certainty whether ACh and its metabolic products, acetate and choline, can be recaptured and re-used by the superior cervical ganglion of the cat. Previous experiments suggested that neuronally released ACh cannot be recaptured by the ganglion, and cannot therefore substitute for ACh synthesis from precursor choline. This was first suggested by Perry (1953) from indirect experiments and the suggestion was supported by the results of Collier and MacIntosh (1969), but the results of these experiments were not unequivocal and the study was not extensive. Therefore, the present experiments re-examined ACh uptake by the superior cervical ganglion and the possibility that released ACh might be re-used for transmission. It is shown that the recapture of neuronally released ACh is unlikely; exogenous ACh is accumulated but not into the releasable transmitter store. These results are discussed with regard to the termination of action of transmitter.

Surplus ACh is the extra ACh which accumulates in ganglia exposed to an anti-cholinesterase (anti-ChE) agent and provided with a supply of choline (Birks and MacIntosh, 1961). Exogenous ACh can release surplus ACh (Collier and Katz, 1970; Katz, 1970) and the present experiments tested whether the exogenous ACh that accumulates in ganglia exchanges with endogenous surplus ACh; the results suggest that ACh does not exchange with surplus ACh.

The recapture of choline from hydrolyzed released ACh was also examined in the present study. Other workers (Perry, 1953; Collier and MacIntosh, 1969; Potter, 1970) have demonstrated that the release of ACh from cholinergic nerve endings is greater in the presence of an anti-ChE agent than is the release of choline in the absence of an anti-ChE agent; this was interpreted as evidence that choline is recaptured. However, these earlier experiments did not test whether the choline that is recaptured re-enters the presynaptic nerve endings or whether this choline is used for ACh synthesis. The present experiments directly demonstrated that choline, produced from released ACh, is taken up by the ganglion where it is acetylated to ACh.

Although several studies have shown that exogenous acetate is a poor precursor for ACh synthesis by brain, no corresponding work has been done to determine whether acetate can be taken up and synthesized into ACh by ganglia. Kahlson and MacIntosh (1939) showed that acetate cannot substitute for glucose in preserving synaptic transmission, but since respiration is impaired in the absence of glucose, the experiments of Kahlson and MacIntosh (1939) were somewhat unclear. The present experiments demonstrated the uptake of acetate by ganglia, but show that this acetate is not used for ACh synthesis. This suggests that acetate produced by breakdown of released ACh, is not used for ACh synthesis.

This thesis begins with a review of the literature pertinent to its subject; this is not a comprehensive review of ACh as a synaptic transmitter because such literature is extensive and has been reviewed on numerous occasions (see e.g., Hebb and Krnjević, 1962; MacIntosh, 1963;

Eccles, 1964; Volle, 1966; Cooper, Bloom and Roth, 1970; Phillis, 1970; Hubbard, 1971; Koelle, 1971; Potter, 1972). The methods used in this investigation are then described; the results are presented and discussed in relation to relevant work by others. The experiments, except where it is stated explicitly, are original as far as the author is aware.

I. INTRODUCTION

A. Acetylcholine as a Neurotransmitter in the Peripheral Nervous System

The presence of a specialized process of nerve-muscle transmission was first suggested by the experiments of Claude Bernard (1856) which showed that curare can interfere with neuromuscular transmission without having a direct effect on the nerve or on the muscle. Dubois-Reymond (1875) later suggested that nerve stimulation induces muscular contraction through the release of some chemical mediator. This concept of chemical transmission was not expanded until Elliott (1904) suggested that sympathetic nerve endings liberate adrenaline during transmission. This idea was based largely on the observation that the effects of injected adrenaline closely parallel those of sympathetic nerve stimulation (Langley, 1901; Elliott, 1904, 1905). Evidence in support of this idea came from experiments by Loewi (1921) which showed that stimulation of sympathetic nerves to the heart released an "accelerator" chemical, and from experiments by Cannon (see Cannon and Rosenblueth, 1937) which suggested that sympathetic nerves release an adrenaline-like material. von Euler (1946) later demonstrated, however, that noradrenaline, and not adrenaline, more closely reproduces the pharmacological actions of purified extracts of spleen, and suggested that noradrenaline is the transmitter at sympathetic nerve terminals.

Dixon (1906, 1907) postulated that in the parasympathetic nervous system, vagal stimulation releases a muscarine-like substance, "inhibitin", which is responsible for cardiac arrest. The component of ergot extract which has muscarine-like activity was identified as ACh by Ewins (1914); ACh had earlier been shown to produce a profound fall in blood pressure (Hunt

and Taveau, 1906). Dale (1914) made a detailed study of the pharmacological actions of ACh and showed that the effects of injected ACh (in small amounts) are very similar to the effects of stimulating parasympathetic nerves. Dale also observed that when this parasympathomimetic activity (or "muscarinic" action of ACh, as he called it) had been abolished by atropine, another action of ACh could be revealed which closely resembles that of nicotine as a stimulant of autonomic ganglia and of skeletal muscle. Dale suggested that the transient action of injected ACh may be due to its inactivation by an esterase.

It was, however, the classical experiments of Loewi (1921) on the frog's heart which provided unequivocal evidence for neurohumoral transmission. He showed that stimulating parasympathetic cardiac nerves released an ACh-like substance which he termed "vagusstoff"; this material affected a second denervated heart as if its own parasympathetic nerve had been stimulated. Both "vagusstoff" and ACh had similar properties (Loewi and Navratil, 1926a, 1926b): the activity of both compounds on test preparations was destroyed by pretreating the compounds with alkali, but the activity re-appeared on subsequent acetylation of both the alkali treated "vagusstoff" and ACh; atropine blocked the pharmacological activity of both compounds, and eserine enhanced their activity. It was also shown that "vagusstoff" had ACh-like effects on other tissues, such as the frog's stomach (Brinkman and van Dam, 1922).

That ACh might be acting as a neurotransmitter was strengthened by the discovery that ACh is a constituent of animal tissue. The demonstration that horse spleen contains large amounts of ACh (Dale and Dudley, 1929) led

others to study the distribution of choline esters in the mammalian nervous system. Chang and Gaddum (1933) isolated ACh from horse sympathetic ganglia, and Feldberg and Gaddum (1934) showed the release of ACh from the cat's superior cervical sympathetic ganglion when its preganglionic nerve was stimulated; Feldberg and Gaddum (1934) used the technique of ganglion perfusion developed by Kibjakow (1933). The substance released from the electrically stimulated ganglion was identified as ACh by a series of parallel biological assays, by its ease of destruction by alkali, by the ability of atropine or curare to block its effects on appropriate test organs, and by the fact that the presence of eserine in the perfusion fluid was essential to preserve the released ACh. Antidromic activation of the ganglion did not release ACh (Feldberg and Vartiainen, 1934), and injection of exogenous ACh to the superior cervical ganglion was shown to stimulate the ganglion (Feldberg and Vartiainen, 1934; Paton and Perry, 1953). Thus the evidence that ACh is a transmitter in sympathetic ganglia is fairly complete; the evidence that ACh is a neurotransmitter in parasympathetic ganglia is mostly indirect (Emmelin and Muren, 1950; Perry and Talesnik, 1953).

To differentiate between the autonomic fibres which release ACh and the autonomic fibres which release an adrenaline-like material, Dale (1933) suggested the terminology of "cholinergic" and "adrenergic" fibres, respectively. By this classification, all preganglionic fibres are cholinergic; all postganglionic parasympathetic fibres and some anatomically sympathetic fibres which release ACh (e.g., fibres which innervate the sweat glands (Dale and Feldberg, 1934a)) are cholinergic. All other postganglionic fibres are adrenergic.

The observation that ACh is a transmitter substance at postganglionic parasympathetic nerve endings and autonomic ganglia was extended to the neuromuscular junction by the experiments of Dale and Feldberg (1934b), Dale, Feldberg and Vogt (1936) and Brown, Dale and Feldberg (1936). These authors demonstrated: a) the release of ACh from perfused skeletal muscle, b) the stimulant effect of ACh on skeletal muscle when ACh was injected close-arterially and c) the reduced effect of both injected ACh and of nerve stimulation on muscular contraction in the presence of curare.

The evidence that ACh is a neurotransmitter was reviewed by Feldberg (1945) who emphasized the following criteria: a) the presence of ACh in certain tissues together with the enzymes for its synthesis and destruction, b) the reproduction of physiological events by properly applied exogenous ACh, c) the potentiation of the effects of applied and released ACh by an anti-ChE agent, d) the block of transmission and of the action of ACh by certain drugs at specific loci, e) the release of ACh during nerve stimulation. These criteria are the basic pre-requisites of a neurotransmitter (see e.g., Feldberg, 1945; McLennan, 1963; Phillis, 1970), and for the superior cervical ganglion, ACh satisfies all of them except c). The poor potentiation by eserine of ganglionic synaptic potentials (Eccles, 1944) is most easily explained by supposing that the transmitter action is terminated by diffusion of ACh from the receptor with subsequent hydrolysis, rather than by immediate destruction of the mediator by acetylcholinesterase (AChE) (Emmelin and MacIntosh, 1956; see also Ogston, 1955). The evidence from light microscopic histochemical studies has suggested that AChE in the superior cervical ganglion of the cat is located presynaptically (Koelle,

1951, 1957; Koelle and Koelle, 1959), and thus it is possible that the AChE in ganglia is poorly placed for the immediate termination of transmitter action. However, preliminary experiments with electron microscopic examination of the superior cervical ganglion has suggested that most of the enzyme is located postsynaptically (Koelle, 1971).

B. Storage of Acetylcholine

a) Storage of acetylcholine in vesicles. One of the requirements that is expected of a possible neurotransmitter is that it must be stored ready for use. Electrophysiological experiments that studied electrical events at the amphibian or mammalian neuromuscular junction have suggested that ACh is stored in preformed multimolecular packages (Fatt and Katz, 1952; Del Castillo and Katz, 1954; Liley, 1956a, 1956b, 1956c; Hubbard and Schmidt, 1963). In these experiments, spontaneous miniature end-plate potentials (miniature e.p.p.'s) were recorded from resting nerve-muscle preparations. The miniature e.p.p.'s are small randomly-occurring depolarizations and under normal conditions are always about the same size. They have all the characteristics of the end-plate potential (e.p.p.) produced by nerve stimulation, except for their size (Fatt and Katz, 1952). Both the miniature e.p.p. and the e.p.p. are reduced in size by tubocurarine but are potentiated and prolonged by anti-ChE agents (Fatt and Katz, 1952; Liley, 1956a). The frequency of miniature e.p.p.'s is increased in a high K^+ medium (Liley, 1956c; Birks, Huxley and Katz, 1960). ACh produces a graded response when it is applied directly to the end-plate region of the muscle (Fatt and Katz, 1952) and thus miniature e.p.p.'s must be the result of multimolecular discharges of ACh from the nerve terminal. The e.p.p. that can be measured when

the nerve is stimulated was shown to be the sum of many synchronous miniature e.p.p. discharges (Del Castillo and Katz, 1954; Martin, 1955; Boyd and Martin, 1956; Liley, 1956b); a miniature e.p.p. response, due to the action of many molecules, represents the smallest unit or "quantum" release (Del Castillo and Katz, 1954; Liley, 1956c).

Electrical events similar to those recorded from the neuromuscular junction have been demonstrated in autonomic ganglia. Spontaneous miniature synaptic potentials have been recorded from frog sympathetic ganglia (Nishi and Koketsu, 1960; Blackman, Ginsborg and Ray, 1963a; Hunt and Nelson, 1965). Their amplitude is reduced by tubocurarine and their frequency is increased by raising external K^{+}; Ca^{++} is required for release, and the action of this ion is antagonized by an elevated Mg^{++} concentration. Miniature synaptic potentials disappear several days after ganglionic denervation (Hunt and Nelson, 1965) suggesting that the ACh that generates the miniature synaptic potentials is released from nerve endings. The excitatory postsynaptic potential recorded during preganglionic nerve stimulation was shown to be composed of multiples of the miniature synaptic potentials (Blackman, Ginsborg and Ray, 1963b) and this has been demonstrated for avian (Martin and Pilar, 1964) and mammalian ganglia (Blackman and Purves, 1969).

About the same time as the studies of electrophysiological events at the neuromuscular junction, electron microscopic examination of different synapses revealed the presence of small spherical structures that were clustered near the terminals of the presynaptic membrane. These "synaptic vesicles" (De Robertis and Bennett, 1954) have been found in most synaptic regions, including the neuromuscular junction (Reger, 1958), the central

nervous system (Palay, 1956; Gray, 1959a, 1959b), frog sympathetic ganglion (De Robertis and Bennett, 1954, 1955; Taxi, 1961), ciliary ganglion of the chick (De Lorenzo, 1960) and the superior cervical ganglion of the cat (Elfvin, 1963). It is unlikely that these vesicles are tubules seen in cross-section (Palay, 1956). It was suggested that these synaptic vesicles are the subcellular structures in which ACh is stored and from which ACh is released, a single vesicle containing one quantum and its release giving rise to one miniature e.p.p. (De Robertis and Bennett, 1955; Del Castillo and Katz, 1956, 1957; Katz, 1962).

The suggestion that ACh is stored in vesicles was supported by the demonstration that ACh can be recovered from synaptic vesicles isolated from brain (Whittaker, 1959; Gray and Whittaker, 1962; De Robertis, Rodriguez de Lores Arnaiz, Salganicoff, Pellegrino de Iraldi and Zieher, 1963; Whittaker, Michaelson and Kirkland, 1964; Whittaker and Sheridan, 1965), from the mammalian sympathetic ganglion (Wilson and Cooper, 1971) and from the electric organ of Torpedo (Israël, Gautron and Lesbats, 1970).

The suggestion that ACh is stored in and released from synaptic vesicles is thus supported by electrophysiological, morphological and biochemical data. Evidence for the vesicle hypothesis is still not complete although there is much circumstantial evidence in its favour. Some strong evidence comes from experiments that attempted to change the number of vesicles in synaptic regions by processes that alter transmitter turnover. Hubbard and Kwanbumbumpen (1968) and Jones and Kwanbumbumpen (1968, 1970) have demonstrated that bathing the rat phrenic nerve-diaphragm preparation in 20 mM K^+, or nerve stimulation in the presence of hemicholinium-3 (HC-3, a drug which prevents ACh synthesis by blocking the choline uptake mechanism;

MacIntosh, Birks and Sastry, 1956; Gardiner, 1957; MacIntosh, 1961), significantly reduces the number of vesicles that can be counted on electron micrographs; nerve stimulation in the absence of HC-3 has no such effect. The frequency of miniature e.p.p.'s recorded from the frog neuromuscular junction increases dramatically when the muscles are bathed in Ca^{++}-free hypertonic sucrose solution or in solution containing black widow spider venon, and then miniature e.p.p. frequency decreases to less than control values (Longenecker, Hurlbut, Mauro and Clark, 1970) at which time the nerve terminals contain almost no vesicles (Clark, Mauro, Longenecker and Hurlbut, 1970). The superior cervical ganglion of the cat provided with choline and stimulated at 60 Hz, is partially depleted of its ACh content within the first few minutes of stimulation (Bourdois, McCandless and MacIntosh, 1970; Friesen and Khatter, 1971b), and there is a concomitant decrease in vesicle numbers (Friesen and Khatter, 1971a). If, however, stimulation is at 10-20 Hz and choline is present, there is no depletion of ACh content (Birks and MacIntosh, 1961), and no reduction in vesicle numbers (Párducz and Fehér, 1970; Párducz, Fehér and Joó, 1971); preganglionic stimulation in the absence of choline or in the presence of HC-3 reduces ACh content (Birks and MacIntosh, 1961) and vesicle population (Párducz and Fehér, 1970; Párducz et al., 1971). Partial depletion of vesicle numbers has also been reported for the superior cervical ganglion of the rat after stimulation in the absence of choline (Perri, Sacchi, Raviola and Raviola, 1972).

The parallel between ACh content and vesicle numbers is not always seen. Rodriguez de Lores Arnaiz, Zieher and De Robertis (1970) demonstrated that the

subarachnoidal administration of HC-3 reduces ACh in nerve endings of rat cerebral cortex, but does not reduce the number of vesicles. Although high frequency stimulation depletes both ACh and vesicles from the superior cervical ganglion of the cat, the return of ACh to steady state levels is not parallelled by a concomitant increase in the number of vesicles (Friesen and Khatter, 1971a).

Dunant, Gautron, Israël, Lesbats and Manaranche (1971) have reported that stimulation of the electric organ of the Torpedo until no evoked post-synaptic discharge can be recorded depletes non-vesicular ACh but not vesicular ACh (as isolated biochemically; see p. 18); the number of vesicles before and after stimulation is the same. There is evidence for electrical transmission at some synapses. For example, in the avian ciliary ganglion Martin and Pilar (1963) have shown that pre- and postganglionic elements are electrically bi-directionally coupled; chemical mediation through the action of ACh is also present. The electrical "coupling potential" is independent of the membrane polarization and it is not abolished by tubo-curarine. Landmesser and Pilar (1972) showed that during the early stages of development of transmission in the chick ciliary ganglion, few synaptic vesicles are present when transmission is purely chemical; with the onset of electrical coupling, however, the number of vesicles greatly increases.

b) Pools of acetylcholine in ganglia. In the presence of glucose and choline, prolonged electrical stimulation of the superior cervical ganglion of the cat does not deplete ganglionic ACh content (Kahlson and MacIntosh, 1939; Birks and MacIntosh, 1961); prolonged preganglionic stimulation in the presence of HC-3 releases about 85% of the total extractable ACh of the ganglion (Birks and MacIntosh, 1961). HC-3 has no effect upon ACh release,

but prevents the synthesis of the transmitter by inhibiting the entry of choline into nerve endings (MacIntosh et al., 1956; Gardiner, 1957; MacIntosh, 1961). Thus a measure of depletion in HC-3's presence is a measure of the ganglion's releasable pool of ACh. This releasable ACh, which amounts to about 220 ng in an average ganglion, was called "depot" ACh by Birks and MacIntosh (1961). When ganglia are stimulated at a high frequency (60 Hz) and are then allowed to rest, the amount of ACh in the depot pool increases (Rosenblueth, Lissak and Lanari, 1939; Bourdois et al., 1970; Friesen and Khatter, 1971b) and this may indicate an increased formation of storage sites. Birks and MacIntosh (1961) showed that the rate of ACh release from a ganglion whose synthesis is prevented by HC-3 can be resolved into two distinct exponential components. Based on this data, Birks and MacIntosh (1961) considered the depot ACh pool to be divided into two subfractions: a smaller pool, which contains transmitter that is more readily released by nerve impulses, and a larger less readily releasable pool. The relationship between these two sub-fractions is not clear, but Birks and MacIntosh (1961) suggested that they exist in "series" such that ACh from the larger must first pass through the smaller before being released. The finding that there seems to be a preferential release of newly-synthesized transmitter in the superior cervical ganglion of the cat (Collier and MacIntosh, 1969; Collier, 1969) suggests that "new" ACh enters into and is released from the more readily available sub-fraction before it can mix with the bulk of the depot pool. Those vesicles that are seen in electron micrographs to be closely aligned to the presynaptic terminals (Elfvin, 1963) may correspond to the readily releasable sub-fraction. The concept of two fractions of the

depot ACh pool has recently been questioned. By measuring the amplitude of excitatory postsynaptic potentials recorded from guinea-pig superior cervical ganglia stimulated in vitro, Bennett and McLachlan (1972a) demonstrated that the release of ACh follows a single exponential decay in the presence of HC-3; these authors concluded that when ACh synthesis is prevented, the release of ACh is from a single pool. It is clear that depot ACh in the superior cervical ganglion of the cat must be located in nerve terminals, but the 15% of the transmitter substance that is not available for release by nerve stimulation, called "stationary" ACh by Birks and MacIntosh (1961), may be located in the intraganglionic axon.

Birks and MacIntosh (1961) showed that another pool of ACh appears when a resting ganglion is perfused with a medium containing a source of choline and an anti-ChE agent such as eserine; under these conditions ganglia synthesize and store extra ACh which was called "surplus" ACh. The accumulation of surplus ACh in the presence of an anti-ChE agent shows that ACh is continually being made by the resting ganglion; in the absence of an anti-ChE agent, this ACh must be quickly destroyed. The formation of surplus ACh is dependent upon the presence of both an anti-ChE agent and a source of choline and its accumulation doubles the ACh content of ganglia within one hour. If exposure to an anti-ChE agent and choline is continued after the full complement of surplus ACh has formed, no further increase in content is observed (Birks and MacIntosh, 1961; Katz, 1970; Collier and Katz, 1971) although surplus ACh still continues to turn over (Katz, 1970; Collier and Katz, 1971). The accumulation of extra ACh up to a limiting value has been observed in other nervous tissue, e.g., in skeletal

muscle (Potter, 1970), and in brain, either studied in vitro or in vivo (see p. 21).

Birks and MacIntosh (1961) presumed that surplus ACh is synthesized and stored in nerve endings, and this was confirmed by experiments which showed that chronically decentralized ganglia do not accumulate surplus ACh (Katz, 1970; Collier and Katz, 1971). Surplus ACh must exist intracellularly since it does not leak into the perfusate, and Birks and MacIntosh (1961) suggested that surplus ACh might exist free in nerve ending cytoplasm.

There is now some confusion as to the location of AChE in the superior cervical ganglion of the cat. Koelle (1971) recently demonstrated that most of the AChE is located postsynaptically, although his earlier work suggested otherwise. Koelle (1951) used histochemical techniques to study the distribution of AChE in the ganglion and showed that most of this enzyme is located in presynaptic terminals since chronically decentralized ganglia do not stain for AChE. Koelle (1957) and Koelle and Koelle (1959) later demonstrated that part of this AChE is intracellular and part of it is extracellular and suggested that internal (or "residual") AChE is synthesized within the endoplasmic reticulum at the perikaryon and is then transported within the axon to its external (or "functional") sites; external AChE may be postsynaptic. External AChE (and not residual AChE) is responsible for hydrolyzing neuronally released ACh, since the preservation of ACh released from the stimulated superior cervical ganglion is the same in lipid-soluble anti-ChE agents as it is in poorly lipid-soluble anti-ChE agents (Burgen and Chipman, 1952; Emmelin and MacIntosh, 1956; Katz, 1970; Collier and

Katz, 1971). However, since the accumulation of surplus ACh is delayed in the presence of poorly lipid-soluble anti-ChE drugs (Katz, 1970; Potter, 1970; Collier and Katz, 1971), it seems likely that it is the internal AChE which must be inactivated before surplus ACh can accumulate. The most likely explanation of the formation of surplus ACh is that the synthesis of ACh is continually occurring in the resting ganglion probably within the nerve ending cytoplasm (see p. 33); during accelerated transmitter turnover, this newly-synthesized ACh is packaged into the vesicles of the depot pool and used for transmission, but in the absence of a "vesicular need", the transmitter is destroyed by the cytoplasmic or residual pool of AChE. The inhibition of residual AChE by a lipid-soluble anti-ChE agent allows the accumulation of surplus ACh.

The depot or surplus ACh pool of the superior cervical ganglion can be labelled radioactively (Collier and MacIntosh, 1969; Collier, 1969; Collier and Katz, 1970; Katz, 1970; Collier and Katz, 1971). Perfusing stimulated ganglia with ^{3}H-choline in the absence of an anti-ChE agent labels the depot (or releasable) ACh pool; since an anti-ChE is not present, surplus ACh does not accumulate. If the sympathetic nerve is not stimulated, and the perfusion fluid contains an anti-ChE and ^{3}H-choline, then the surplus ACh pool is labelled, as well as a fraction of the depot pool (the slight labelling of depot ACh is due to the spontaneous turnover of releasable ACh (Collier and MacIntosh, 1969)). The radioactive labelling of surplus ACh was used to demonstrate that there is little or no mixing of surplus ACh with depot ACh (Collier and Katz, 1971). In these experiments, ganglia were perfused with medium containing ^{3}H-choline in the presence or in the absence of eserine. Ganglia which were exposed to eserine doubled their

ACh content by accumulating labelled surplus ACh, yet the release of total and of labelled ACh by subsequent nerve stimulation from these preparations is the same as the release of total and of radioactive ACh from the preparations which had not been exposed to eserine during the exposure to radioactive choline and had therefore not accumulated labelled surplus ACh.

Exposure to high K^+ releases both depot and surplus ACh (Brown and Feldberg, 1936a; Katz, 1970; Collier and Katz, 1971); surplus ACh cannot accumulate in ganglia perfused with high K^+ (Birks, 1963). This suggests that surplus ACh may be held within the nerve terminal cytoplasm as a result of the existing membrane potential.

Depot ACh is available for release by nerve stimulation, but cannot be released by exogenous ACh or by carbachol unless very high concentrations are used (Collier, Vickerson and Varma, 1969; Collier and Katz, 1970). Surplus ACh, which is not available for release by nerve stimulation, can be released by exogenous ACh or by carbachol (Collier and Katz, 1970; Katz, 1970). This release of surplus ACh by ACh clarified the interpretation of certain experiments by other workers on the presynaptic actions of ACh. Koelle (1961, 1962) had suggested that the amount of ACh released by a nerve impulse is not enough to propagate the action potential across the cholinergic synapse, but may induce the release of enough ACh from presynaptic terminals to effect transmission. This idea was strengthened by the findings of McKinstry, Koenig, Koelle and Koelle (1963) and of McKinstry and Koelle (1967) that injected carbachol can induce the release of ACh from the perfused superior cervical ganglion of the cat. These authors, because of technical limitations, could not test ACh itself for

its ability in releasing ACh. In the experiments of McKinstry and her colleagues, the ganglia were perfused with eserine and choline and would therefore accumulate surplus ACh. The experiments of Collier, Vickerson and Varma (1969), Collier and Katz (1970) and Katz (1970) suggested that the whole of the ACh released by carbachol in the McKinstry experiments was surplus ACh. Thus, these experiments provide no evidence in support of Koelle's theory.

The mechanism of release of surplus ACh by ACh is not clear, but Katz (1970) suggested that the cation exchange of exogenous ACh with surplus ACh might be of importance. In the present experiments, the uptake of exogenous ACh by ganglia into the surplus ACh pool was tested. It was found that exogenous ACh cannot be accumulated into the surplus ACh pool, and the release of ACh by ACh is not by exchange.

c) Pools of acetylcholine in brain. The subcellular distribution of ACh in mammalian brain has been studied by numerous workers (see e.g., Whittaker, 1959; De Robertis et al., 1963; Whittaker et al., 1964; Marchbanks, 1968; Beani, Bianchi, Megazzini, Ballotti and Bernardi, 1969; Chakrin and Whittaker, 1969; Takeno, Nishio and Yanagiya, 1969; Richter and Marchbanks, 1971b; Collier, Poon and Salehmoghaddam, 1972). The pinched-off nerve endings which are formed when brain is homogenized in isotonic sucrose (Gray and Whittaker, 1962) were termed "synaptosomes" by Whittaker et al. (1964). These structures have a diameter of about 0.5 μ and they contain synaptic vesicles and small mitochondria within their cytoplasm. When brain is homogenized in eserine-sucrose, about 20% of total brain ACh appears free in a high speed supernatant; this is called "free" ACh and mostly represents

ACh that is outside nerve endings. "Bound" ACh is present in synaptosomes and appears in two forms when the synaptosomes are broken by hypo-osmotic shock: a "stable" form, which is associated with the synaptic vesicles and a more "labile" form found in the cytoplasm of the synaptosomes. These three pools of ACh are not artifacts of the homogenization procedure. Chakrin and Whittaker (1969) injected labelled choline directly into the exposed cerebral cortex of guinea-pigs, and measured the specific activity of the subcellular fractions prepared from the homogenized cerebral cortex. The three ACh pools had not been equally labelled; the specific activity of labile ACh was the highest, followed by stable ACh, while free ACh had the lowest specific activity. If the ACh in brain is held within a single store, and the three pools of ACh isolated after fractionation are artifacts of the homogenization procedure, then similar specific activities of ACh for each pool would be expected.

The labile ACh that appears free in the nerve ending cytoplasm can only be recovered in the presence of an anti-ChE but stable ACh in vesicles is protected from AChE. Stable ACh is released only by procedures which destroy the integrity of vesicles, suggesting that there might be a chemical bond between ACh and the vesicle matrix. Whittaker (1971) has recently reported that the osmotic rupture of vesicles isolated from the electric organ of Torpedo releases a low molecular weight protein; this substance, which he termed "vesiculin", represents about one-half of the total protein in the vesicle. Although the function of vesiculin is unclear, Whittaker (1971) suggested that it may be involved in the packaging of ACh in vesicles.

The vesicle fraction may not be homogeneous. The electric organ of Torpedo or cerebral cortex of guinea-pig can synthesize labelled ACh from precursor choline; some of this labelled ACh is present in the vesicle fraction. However, when these vesicles are passed through an iso-osmotic Sephadex column, the unlabelled ACh-containing vesicles pass through, but with little, if any, of the radioactive ACh-containing vesicles (Marchbanks and Israël, 1971; Richter and Marchbanks, 1971b). Thus a population of vesicles exists which turns over its ACh more rapidly than the rest of the vesicles. When nervous tissue is homogenized according to the procedure of Whittaker et al. (1964) and the disrupted synaptosomes are placed on a sucrose density gradient, synaptic vesicles can be isolated in two phases of the gradient; these are the "D" (collected from 0.4 M sucrose) and "H" (collected from between 1.0 M - 1.2 M sucrose) vesicle fraction. It had been presumed (Whittaker et al., 1964) that the H vesicles were similar to the D vesicles. However, Whittaker (1971) demonstrated that ACh in H vesicles was labelled to a higher specific activity than the specific activity of D vesicles after ^{3}H-choline was injected intraventricularly into guinea-pigs and cerebral cortex then homogenized for the subcellular fractionation.

In the cerebral cortex, as in the superior cervical ganglion, newly synthesized ACh is preferentially released (Molenaar, Nickolson and Polak, 1971; Richter and Marchbanks, 1971a). It is not yet clear whether ACh which is preferentially released is coming from the Marchbanks "hot" pool of vesicles, or from the Whittaker "hot" pool of vesicles, or indeed from vesicles at all. In electric organ of Torpedo or guinea-pig

cerebral cortex incubated with labelled choline, the specific activity of ACh released by stimulating the tissue approximates the specific activity of cytoplasmic ACh and not the specific activity of vesicular ACh. The "hot" pool of vesicles in brain may correspond to the population of vesicles rapidly turning over in the superior cervical ganglion from which newly synthesized transmitter is preferentially released (Collier and MacIntosh, 1969; Collier, 1969).

Brain tissue exposed to an anti-ChE agent and choline increases its ACh content, but only to a limiting value. Minced or sliced brain incubated in a suitable medium increases its ACh content to a maximum level at which it remains constant (Stedman and Stedman, 1937; Mann, Tennenbaum and Quastel, 1939; Bhatnagar and MacIntosh, 1967; Polak, 1969; Sharkawi and Schulman, 1969); in vivo, brain ACh content increases in certain situations, but again there seems to be a ceiling level beyond which it does not increase further (Tobias, Lipton and Lepinat, 1946; Torda, 1953; Giarmanand Pepeu, 1962; Crossland and Slater, 1968). Collier et al. (1972) have recently shown that free and labile ACh, but not vesicular ACh, increases in content when brain slices are incubated in the presence of eserine and choline. This increase in ACh content may be analogous to the accumulation in the cytoplasm of surplus ACh in ganglia. Bourdois and Szerb (1972) have recently demonstrated that the increase in ACh content in prisms of rat cerebral cortex incubated in Krebs solution for one hour is not significantly enhanced by the addition of an anti-ChE drug during the second hour of incubation. The authors suggested that cerebral cortex cannot accumulate surplus ACh.

C. Acetylcholine Uptake by Brain and by Ganglia

The early experiments on ACh uptake by brain demonstrated only a small and limited accumulation of exogenous ACh in the presence of eserine (Mann, Tennenbaum and Quastel, 1938; Brodkin and Elliott, 1953), but it is now known that eserine as well as several other drugs, including atropine, HC-3 and hexamethonium block ACh uptake competitively (Polak and Meeuws, 1966; Schuberth and Sundwall, 1967; Liang and Quastel, 1969b; Polak, 1969). A common feature of these competitive blocking drugs is that at physiological pH all possess a positive charge and it has been suggested that these drugs prevent ACh accumulation by binding anionic sites (Polak, 1969; Liang and Quastel, 1969b). The uptake of ACh by brain is not inhibited when organophosphorus anti-ChE agents are used (Polak and Meeuws, 1966; Schuberth and Sundwall, 1967; Heilbronn, 1969; Liang and Quastel, 1969a; Polak, 1969). This uptake of ACh in the presence of an organophosphorus anti-ChE agent is by an active process since its accumulation is against a concentration gradient, blocked by drugs which inhibit glycolysis, blocked by drugs which uncouple oxidative phosphorylation, and blocked by drugs which interfere with the Na^+-K^+ pump. It is possible that at low ACh concentrations in the incubation medium, the concentrative uptake of ACh by brain may be by exchange diffusion, but this seems unlikely when exogenous ACh concentrations are increased (Liang and Quastel, 1969a).

Some of the ACh transported by brain slices is retained in the nerve endings (Schuberth and Sundwall, 1968); it is not yet entirely clear whether this accumulated ACh can be released, but recent experiments (Salehmoghaddam, Collier and Harfield, 1970; Katz, Salehmoghaddam and Collier, in

preparation) suggest that it is not. ACh accumulated by brain may be entering a surplus ACh pool analogous to surplus ACh in ganglia. As with surplus ACh in ganglia, accumulated ACh by brain may also be held in the cytoplasm as the result of the existing membrane potential, since uptake of ACh into brain slices is inhibited by high concentrations of K^+ (Polak, 1969; Liang and Quastel, 1969a).

The physiological significance of ACh uptake by brain is still unclear. Two possible functions have been postulated. Neuronally released ACh might be recaptured and re-used for transmission (Schuberth and Sundwall, 1968), much like the recapture and re-use of transmitter at non-cholinergic synapses (see Iversen, 1967, 1971). Alternatively, the accumulation of exogenous ACh may represent a mode of termination of action of transmitter without this ACh being re-used for transmission.

ACh uptake by ganglia has not been studied extensively. Perry (1953) demonstrated that in the absence of choline, the release of ACh by nerve stimulation is smaller in the second of two test periods of stimulation when eserine is present during both periods of stimulation. Perry (1953) interpreted this experiment as suggesting that neuronally released ACh cannot be recaptured and re-used by preganglionic fibres for transmission. These experiments, however, did not indicate whether ACh had accumulated into the surplus ACh pool from where it would not be available for release by nerve stimulation (Birks and MacIntosh, 1961; Katz, 1970; Collier and Katz, 1971). Collier and MacIntosh (1969) perfused ganglia with ^{3}H-ACh in the presence of eserine during which time the preganglionic nerve was stimulated. They found that only small amounts of ACh accumulate and concluded that the recapture of neuronally released ACh by ganglia is

unlikely. However, ACh uptake in the experiments of Perry (1953) and of Collier and MacIntosh (1969) might have been blocked by the eserine present in the perfusion medium since eserine blocks ACh uptake by brain (Polak and Meeuws, 1966; Schuberth and Sundwall, 1967; Liang and Quastel, 1969b; Polak, 1969). Furthermore, Collier and MacIntosh (1969) used ACh labelled on the N-methyl group, and the labelled ACh measured in the ganglion at the end of the experiment might have come, in part, from the labelled choline formed from the slight breakdown of labelled ACh during perfusion through the ganglion. They argued that as much as 60% might have come from this choline on the basis of phospholipid labelling (see Collier and Lang, 1969). Finally, Collier and MacIntosh (1969) did not test whether the ACh accumulated by ganglia mixed with depot ACh, or with surplus ACh or with neither of these ACh pools. The present experiments extended the previous studies and avoided the difficulties associated with the experiments of Collier and MacIntosh (1969); ACh uptake was examined in the presence of diisopropylfluorophosphate (DFP) which does not inhibit ACh uptake by brain (Polak, 1969) and the experiments used acetyl-1-^{14}C-choline which if broken down would yield labelled acetate, and not labelled choline; acetate is a poor precursor for ACh synthesis in brain (Cheng, Nakamura and Waelsch, 1967; Browning and Schulman, 1968; Heilbronn, 1970; Nakamura, Cheng and Naruse, 1970; Sollenberg and Sörbo, 1970) and in ganglia (present experiments). The present experiments suggest that neuronally released ACh is not available for recapture by preganglionic fibres and that there is no preferential accumulation of ACh by preganglionic fibres. The relevance of this uptake of ACh by ganglia is

discussed.

D. Synthesis of Acetylcholine

The synthesis of ACh from choline and acetyl coenzyme A (acetyl CoA) requires the enzyme choline acetyltransferase (ChAc); the acetylation of coenzyme A (CoA) requires ATP and a source of active acetate.

a) Coenzyme A and active acetate. CoA (discovered by Lipmann in 1945) is involved in the acetylation of choline (Lipmann and Kaplan, 1946). It is a ubiquitous component of nerve cells, and its supply is not limiting in ACh synthesis except, perhaps, during pantothenic acid deficiency (Novelli, 1953); pantothenic acid is structurally part of CoA (Lipmann, Kaplan, Novelli, Tuttle and Guirard, 1947). Because of its active involvement in numerous metabolic cycles, CoA can be derived from protein, carbohydrate and fat dietary sources. Its absence in circulating plasma (although precursors are present) suggests that each CoA-containing tissue can synthesize its own supply of CoA as needed (Novelli, 1953).

Active acetate is also readily obtained from protein, carbohydrate and fat (Novelli, 1953). Glucose is necessary for ACh synthesis in brain slices or in perfused superior cervical ganglia; lactate or pyruvate can substitute for glucose, but acetate, acetoacetate or succinate cannot (Quastel, Tennenbaum and Wheatly, 1936; Kahlson and MacIntosh, 1939; Browning and Schulman, 1968). Experiments on brain have demonstrated that in the presence of glucose, exogenous acetate is a poor precursor of ACh synthesis; important acetyl sources are glucose, lactate, pyruvate (Cheng et al., 1967; Heilbronn, 1970; Nakamura et al., 1970) and possibly

citrate (Sollenberg and Sörbo, 1970; but see Tuček, 1970).

In the experiments of Kahlson and MacIntosh (1939), ganglia were perfused with glucose-free solution; ACh release was reduced and total ganglionic ACh at the end of the period of nerve stimulation was depleted suggesting that synthesis of ACh could not match its release. Acetate had no restorative effect on ACh synthesis. However, the depletion of ACh in these experiments in the absence of glucose was the same as the depletion of ACh when glucose was present (see Birks and MacIntosh, 1961).

The metabolism of glucose through the Embden-Meyerhoff pathway yields acetyl groups for ACh synthesis, but glucose is also involved in maintaining adequate levels of respiration in nervous tissue. Thus conduction fails in presynaptic fibres when glucose is omitted (Larrabee and Bronk, 1952; Krnjević and Miledi, 1958; Larrabee, 1958; Larrabee and Klingman, 1962). Impaired release of ACh in the absence of glucose can therefore be interpreted as due to either or both conduction failure and reduced ACh synthesis. Thus, in the absence of glucose, the failure of acetate to restore synthesis in the superior cervical ganglion need not necessarily be interpreted as the inability of exogenous acetate to provide acetyl groups for ACh synthesis, since adequate levels of respiration may not be present. The experiments described in this thesis examined more closely acetate uptake by ganglia in ganglia in which synthesis and release of ACh were not impaired, and allowed conclusions to be reached as to whether acetate, derived from the hydrolysis of neuronally released ACh, can serve as a source of acetyl groups.

b) Choline. For optimal synthesis and release of ACh, the ganglion must be provided with a source of extracellular choline. Brown and Feldberg

(1936b) perfused ganglia with choline-free Locke and showed that when ACh release from the stimulated ganglion begins to decrease, the addition of choline to the perfusion fluid enhances both the ACh output and the nictitating membrane contraction. Birks and MacIntosh (1961) confirmed that the output of ACh from ganglia is maintained at a higher level when perfusion is with choline in eserine-Locke than it is when perfusion is with eserine-Locke containing no choline. In the presence of choline, synthesis of ACh matches its release, and a ganglion's ACh content turns over about four times during one hour's stimulation at 20 Hz (Birks and MacIntosh, 1961; Matthews, 1966). Cat's plasma contains about 10^{-5} M free choline and this level is maintained remarkably constant (Bligh, 1952); it is unlikely that choline becomes deficient under physiological conditions.

Although the release of ACh is less when choline is absent than it is when choline is present, the stimulated superior cervical ganglion, perfused in the absence of externally added choline, releases more ACh than it initially contained (Brown and Feldberg, 1936b; Kahlson and MacIntosh, 1939; Perry, 1953; Birks and MacIntosh, 1961; Matthews, 1963). Therefore some ACh synthesis occurs in the absence of added choline. This synthesis of ACh in the absence of added choline is not sufficient, however, to maintain ACh stores, since ganglionic ACh is depleted by 53% within 60 minutes of stimulation at 20 Hz (Birks and MacIntosh, 1961). It is still not entirely clear where the choline for this ACh synthesis comes from. The presynaptic pool of choline in ganglia (as measured by Friesen, Ling and Nagai, 1967) may provide some of this choline for ACh synthesis. The

large amounts of choline that appear in the effluent from resting ganglia perfused with choline-free solution (Perry, 1953) may provide some of the choline necessary for ACh synthesis, but the origin of this choline is not known. Nervous tissue contains large amounts of bound choline in phospholipid and it is possible that some of this is available for ACh synthesis. The turnover of phospholipid-choline, as measured by Collier and Lang (1969), would release only 2 ng/minute and if this is the source of choline for ACh synthesis in ganglia perfused without choline, all of it would have to be available for ACh synthesis. Perhaps a ganglion which lacks exogenous choline can break down bound choline more rapidly than can a normal preparation.

A ganglion perfused with an anti-ChE agent in the absence of choline will accumulate approximately 60 ng of surplus ACh within the first few minutes, presumably from the ganglion's endogenous supply of choline, but will not synthesize more surplus ACh if perfusion is continued. Thus, the choline which is available for synthesis of releasable ACh in the absence of added choline appears to be unavailable for the synthesis of surplus ACh. It may be that the ganglion's endogenous choline is used for the synthesis of releasable ACh and not for the synthesis of surplus ACh when ganglia are stimulated in the presence of choline-free Locke containing eserine.

A way of limiting the supply of choline for ACh synthesis became available with the synthesis of HC-3 (Schueler, 1955). This substance inhibits ACh synthesis by intact cells (see e.g., MacIntosh et al., 1956; Birks and MacIntosh, 1961; Gardiner, 1961) by competing with choline for carrier sites (MacIntosh et al., 1956; Gardiner, 1957; MacIntosh, 1961).

The experiments of MacIntosh et al., (1956), Gardiner (1957) and Gardiner (1961) suggest that HC-3 has little, if any, inhibitory effect on ChAc but it now appears that in addition to competing with choline for uptake sites into nervous tissue, HC-3 can compete with choline for acetylation by ChAc (Rodriguez de Lores Arnaiz et al., 1970; Hemsworth, 1971); the acetylation of choline is more efficient, however, than is the acetylation of HC-3 (Hemsworth, 1971). In the presence of HC-3, ACh release from the superior cervical ganglion is not maintained and the ganglion's releasable transmitter is depleted. The addition of choline antagonizes the action of HC-3 (Birks and MacIntosh, 1961; Matthews, 1966). HC-3 also prevents the formation of surplus ACh by starving the ganglion of the choline necessary for the synthesis of surplus ACh (Birks and MacIntosh, 1961).

The manner in which choline gains access into nerve endings is not yet entirely clear. Carrier uptake against a concentration gradient of choline has been described for a variety of tissues: into kidney slices (Sung and Johnstone, 1965), erythrocytes (Askari, 1966; Martin, 1967, 1968), squid giant axon (Hodgkin and Martin, 1965), and brain slices (Schuberth, Sundwall, Sörbo and Lindell, 1966; Schuberth, Sundwall and Sörbo, 1967); facilitated diffusion has been described for choline uptake into synaptosomes (Marchbanks, 1968b; Potter, 1968; Diamond and Kennedy, 1969). Whether transport of choline into these tissues is against the electrochemical gradient is not known. The active uptake of choline into brain slices (Schuberth et al., 1967) or red blood cells (Martin, 1967, 1968) is Na^+-dependent, but the effects of Na^+ on the facilitated transport of choline into synaptosomes is contradictory (Marchbanks, 1968b; Potter, 1968;

Diamond and Kennedy, 1969). The synthesis of ACh by the superior cervical ganglion (Birks, 1963) or by brain (Bhatnagar and MacIntosh, 1967) is reduced in low Na^+ medium and this may be the result of decreased choline transport.

Although plasma is an important source of choline for ACh synthesis, indirect experiments have suggested that choline formed from the hydrolysis of released ACh can be re-used by the ganglion. This was first suggested by Perry (1953) from experiments which showed that the evoked release of ACh during long periods of stimulation in the presence of eserine is greater than the release of choline in the absence of eserine. Perry's suggestion was strengthened by the experiments of Collier and MacIntosh (1969) which showed that ganglia whose depot ACh pool had been labelled with ^{3}H-ACh release twice as much labelled material upon preganglionic nerve stimulation when eserine is present than when eserine is absent. These experiments were interpreted as evidence that choline formed from the hydrolysis of ACh in the absence of eserine can effectively compete with plasma choline for transport into nerve endings; the greater release in the presence of eserine was interpreted as being due to the drug preventing ACh hydrolysis and therefore indirectly preventing choline recapture. The alternative explanation that eserine increased the release of transmitter could not be entirely eliminated. Furthermore, these experiments did not test whether choline is recaptured by preganglionic fibres; postganglionic elements of the superior cervical ganglion contain large amounts of choline (Friesen et al., 1967) and might therefore take up the choline made available from ACh. The experiments of Perry (1953) and of Collier

and MacIntosh (1969) did not indicate the fate of the recaptured choline. The experiments described in this thesis demonstrated that choline, derived from neuronally released ACh, can be recaptured by preganglionic fibres and acetylated to ACh.

Recent neurophysiological experiments have suggested that choline from hydrolyzed neuronally released ACh represents the main source of precursor for ACh synthesis in the isolated guinea-pig superior cervical ganglion (Bennett and McLachlan, 1972b). In these experiments, postganglionic potentials evoked by stimulation of the preganglionic nerve were maintained in the absence of choline added to the medium. This implied that guinea-pig ganglia contain or recapture or can synthesize adequate supplies of choline. However, when eserine was added, and therefore uptake of released choline prevented indirectly, ganglionic potentials were not maintained, and the decline of the size of ganglionic potentials was similar to that when ACh synthesis was inhibited by the addition of HC-3.

c) Choline acetyltransferase and the subcellular location of acetylcholine synthesis. ChAc was first isolated by Nachmansohn and Machado (1943) from extracts of brain and from electric organ of the eel and was later isolated from the mammalian nervous system (Nachmansohn and John, 1944, 1945). The enzyme transfers acetyl groups from acetyl CoA to choline and although it is fairly specific it can acetylate substances structurally similar to choline (Burgen, Burke and Desbarats-Schonbaum, 1956; Hemsworth and Smith, 1970), and it can synthesize choline esters other than ACh (Berman, Wilson and Nachmansohn, 1953; Berry and Whittaker, 1959). ChAc is present in cholinergic neurons (Cohen, 1956; Hebb and Silver, 1956) and

it appears to be synthesized in the cell body and transported to the periphery by axoplasmic flow (Hebb and Waites, 1956; Hebb and Silver, 1961; Frizell, Hasselgren and Sjöstrand, 1970). Hebb and Waites (1956) demonstrated that decentralized sheep or cat superior cervical ganglia lose much of their ChAc; only about 10% of the enzyme remains one week after the nerve is cut. Decentralization also results in the parallel loss of the ganglion's ACh (MacIntosh, 1938; Feldberg, 1943; Banister and Scrase, 1950; Friesen et al., 1967).

The exact location of ChAc within the nerve ending is not yet entirely clear. The first attempts to employ the techniques of differential centrifugation to brain showed that approximately 60% of the tissue's ChAc activity was associated with mitochondria (Hebb and Smallman, 1956), but with more refined techniques it was shown that this is not so (Hebb and Whittaker, 1958). De Robertis et al. (1963) isolated synaptosomes from rat or rabbit brain homogenates and showed the association of both ACh and ChAc with synaptic vesicles; they suggested that vesicles are the site of storage and synthesis of ACh. However, Whittaker et al. (1964) who used homogenates of pigeon or of guinea-pig brain demonstrated that ChAc is a soluble cytoplasmic enzyme. These conflicting results were due partly to a species difference and partly to the different techniques used in isolating the subcellular fractions (McCaman, Rodriguez de Lores Arnaiz and De Robertis, 1965). Fonnum (1966, 1967) showed that the procedures used by Whittaker and those used by De Robertis favoured the results they obtained in their respective species. Many low molecular weight proteins are known to be relatively insoluble in media of low ionic strength, and

they therefore appear to be membrane bound (see e.g., Schneider, 1963). Fonnum showed this to be true for the rat brain ChAc. At the pH and ionic strength likely to be found in the intact nerve ending, 80% of the ChAc appears free in the cytoplasm but at the low ionic strengths used in earlier experiments the enzymes can be adsorbed onto membranes; this adsorption of ChAc varies with species and is low in those used by Whittaker et al. and is high in those used by De Robertis et al. Potter, Glover and Saelens (1968) have confirmed Fonnum's findings on rat brain ChAc.

The evidence from the subcellular fractionation studies of ChAc led to the suggestion that ACh synthesis takes place in the cytoplasm and the transmitter is then packaged into vesicles (Fonnum, 1967; Beani et al., 1969). However, Kása, Mann and Hebb (1970), using histochemical procedures to localize the ChAc in spinal neurones, suggested ChAc to be both free in cytoplasm and associated with vesicles. If this is so, then two sites of ACh synthesis, cytoplasmic and vesicular, would be expected and this has been suggested by Ritchie and Goldberg (1970).

II. METHODS AND MATERIALS

Some of the procedures used have already been described (Collier and Lang, 1969; Collier and MacIntosh, 1969; Collier and Katz, 1970; Katz, 1970; Collier and Katz, 1971).

A. Induction of Anaesthesia

All acute animal experiments were performed on cats of either sex weighing 1.8 to 3.0 kg. Anaesthesia was induced by ethyl chloride followed by ether and maintained by chloralose (i.v., 80 mg/kg). Sodium pentobarbital (3 mg/kg) was administered when required to maintain surgical anaesthesia throughout the experiment. The animal's trachea was cannulated.

B. Ganglion Perfusion

The superior cervical ganglion was prepared for perfusion by Kibjakow's (1933) procedure as modified by Feldberg and Gaddum (1934). A mid-line incision was made in the neck, the transverse vein was cut between double ties, the cervical lymph node was removed and all branches of the common carotid artery, except those supplying the ganglion, were tied off. The external carotid artery, the lingual artery and, if present, the internal carotid artery were tied and cut, and the occipital artery was prepared for ligation.

Blood leaves the ganglion by the transverse prevertebral vein and in some cats by the internal jugular vein as well. If an internal jugular vein was present, it was prepared for cannulation and all its branches were tied off except the one from the ganglion; in these animals the transverse prevertebral vein was tied off. In the absence of an internal jugular

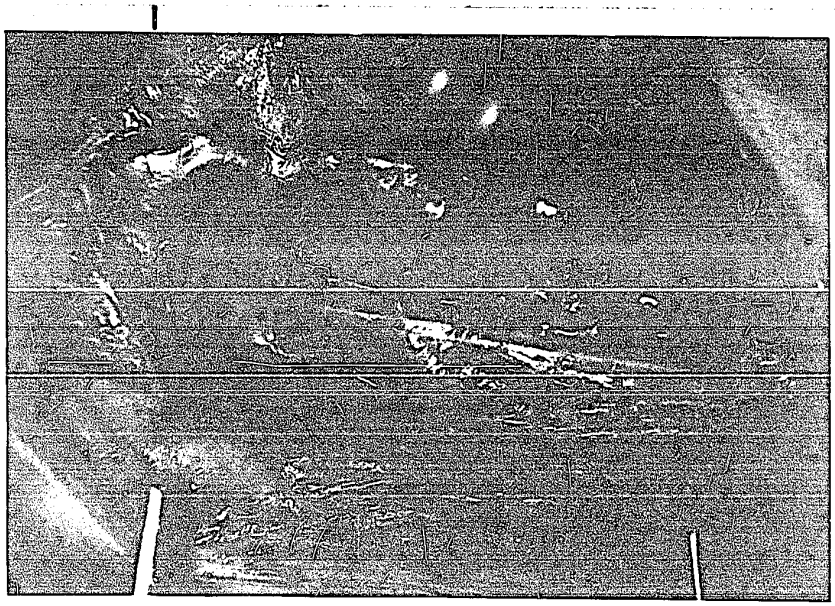
vein, the transverse prevertebral vein was made ready for cannulation. Arterial twigs alongside the postganglionic fibres were tied carefully and a thread was tied around all tissue rostral to the superior cervival ganglion, except for the postganglionic sympathetic fibres. The occipital artery was tied and the carotid artery was cannulated with a small glass cannula that was inserted slightly caudal to the superior cervical ganglion. The internal jugular vein or the transverse prevertebral vein was cannulated (volume of the cannula was 0.1 ml) and the effluent was collected into chilled test tubes. Photographs of the surgical procedure are shown in Fig. 1.

Perfusion was with filtered Krebs solution (mM: NaCl 120, KCl 4.6, $CaCl_2$ 2.4, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 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. When perfusion was with a high K^+ medium, the KCl concentration was increased to 46 mM, and in a low glucose solution, the glucose concentration was reduced to 1.98 mM; with these solutions, isotonicity was maintained by altering the NaCl concentration appropriately.

The perfusion fluid contained one or more of the following compounds: choline chloride (10^{-5} M) either unlabelled or methyl-^{3}H-labelled, acetylcholine chloride (2.8×10^{-6} M - 16.8×10^{-6} M) either unlabelled, acetyl-1-^{14}C-labelled or acetyl-^{3}H-labelled, methyl-^{3}H-inulin (2.5×10^{-6} M), neostigmine bromide (3.2×10^{-5} M), diisopropylfluorophosphate (DFP, 4.8×10^{-5} M), hemicholinium-3 dibromide (HC-3, 8.5×10^{-5} M), atropine sulphate (4×10^{-5} M - 5×10^{-5} M), hexamethonium chloride (2×10^{-5} M - 10^{-4} M). The concentration of choline used was close to the physiological level of choline in cat plasma (Bligh, 1952). In some experiments, either unlabelled ACh or acetyl-1-^{14}C-labelled-choline was dissolved in

Fig. 1. Surgical procedure in the preparation of the superior cervical ganglion for perfusion.

a) A mid-line incision has been made in the neck, and the transverse vein has been cut between double ties. tc = tracheal cannula.



b) Connective fascia has been cleared exposing the vagus nerve (vn), common carotid artery (cca) and cervical lymph node (cln).

Fig. 1. Surgical procedure in the preparation of the superior cervical ganglion for perfusion.

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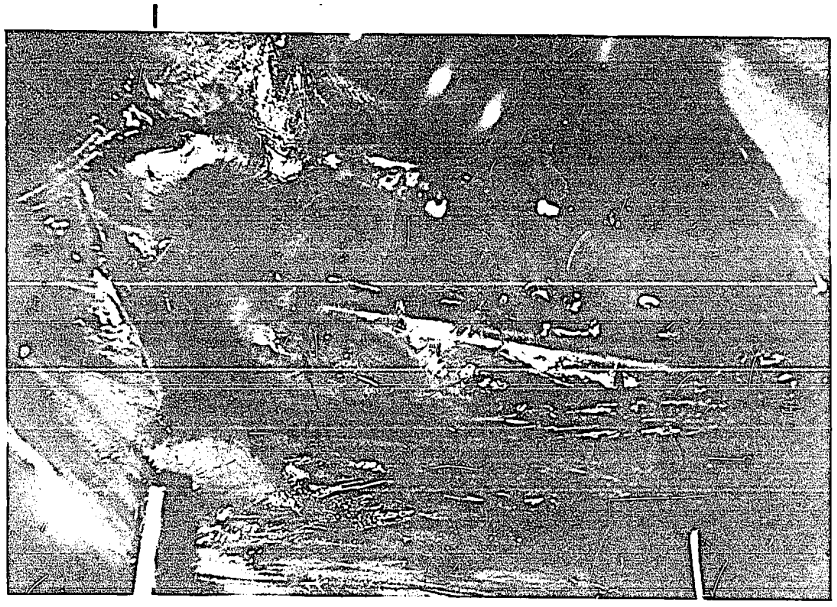
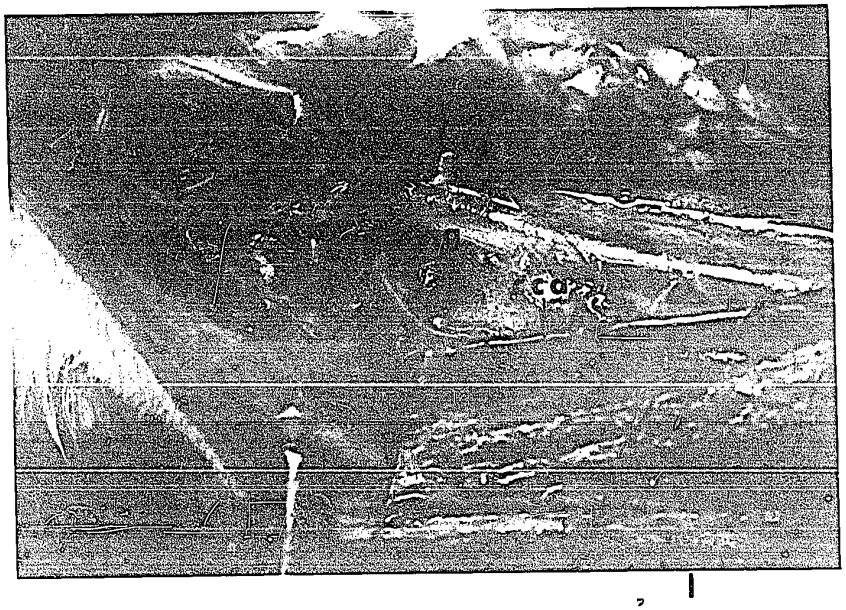
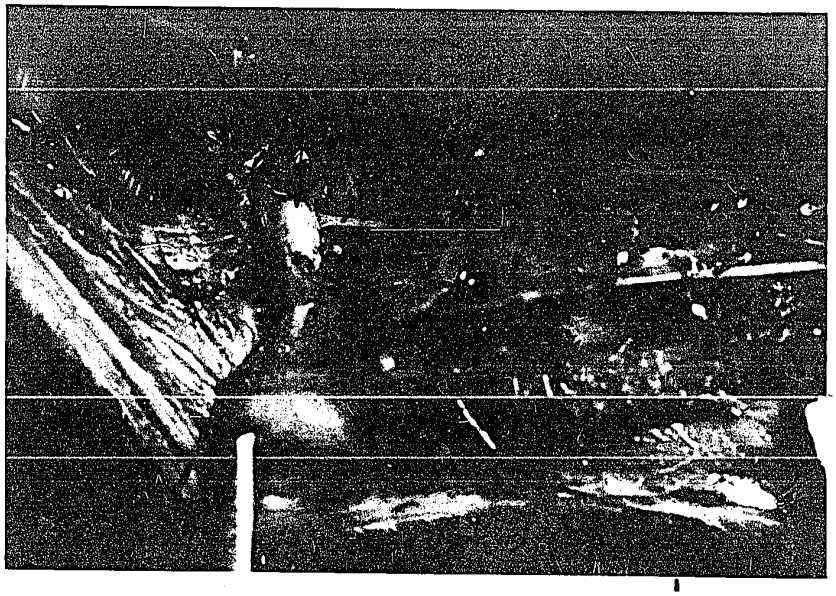


b) Connective fascia has been cleared exposing the vagus nerve (vn), common carotid artery (cca) and cervical lymph node (cln).

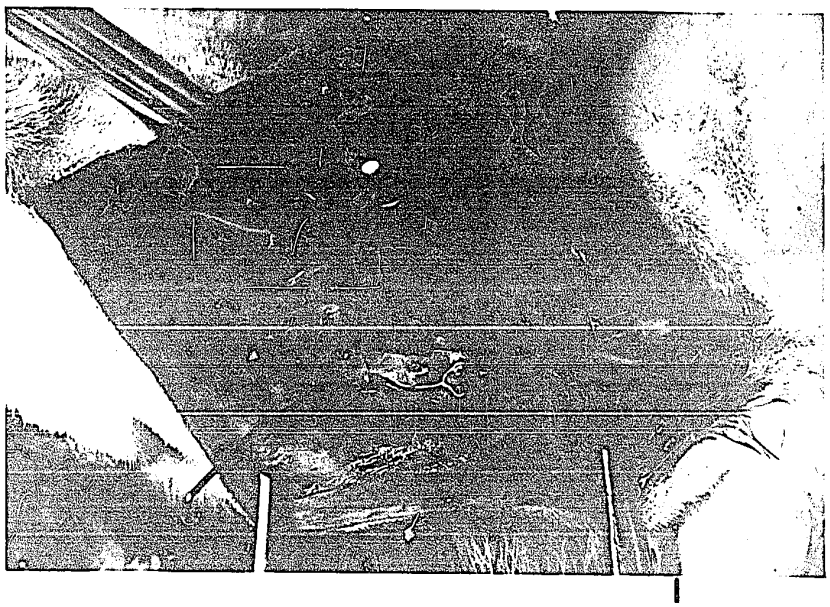
c) The cervical lymph node has been removed, the hypoglossal nerve (hn) exposed, and the common carotid artery (cca) further cleared from connective fascia.



d) The lingual artery and nerve, and the external carotid artery have been cut between double ties. The superior cervical sympathetic ganglion (scsg) and the superior cervical sympathetic nerve (scsn) have been exposed. cca = common carotid artery.



e) Tissue rostral to the superior cervical sympathetic ganglia (scsg) has been tied, except for the postganglionic fibres. The occipital artery has been ligated. The superior cervical sympathetic nerve (scsn) has been cut and cleared from the vagus nerve. Perfusate is introduced through a small glass arterial cannula (a. can) inserted into the common carotid artery (cca); perfusate leaves the superior cervical sympathetic ganglion by the transverse prevertebral vein (tpv).



f) The venous cannula (v. can) has been inserted and tied into the transverse prevertebral vein; the superior cervical sympathetic nerve (scsn) has been placed on the electrode (e). a. can = arterial cannula; vn = vagus nerve.

0.2 ml Krebs solution and injected directly into the arterial cannula.

Each solution to be used as a perfusion fluid was placed in a separate 250 ml conical bottle which was connected through polyethylene tubing to a 22 gauge needle; the needle was pushed through a rubber stopper into the arterial cannula. The perfusion medium could readily be switched from one solution to the next by turning the appropriate stopcock, the dead space being only that of the arterial cannula (0.1 ml). The tubes carrying solutions from the reservoir to the cannula were passed up the cat's oesophagus to an exit just below the level of the ganglion; thus the solutions were warmed to the cat's body temperature (Emmelin and MacIntosh, 1956) which was maintained at 37° C by a heating pad placed under the animal. The perfusion pressure was controlled by connecting the perfusion bottles through a reservoir (20 litres) to a cylinder containing 5% CO_2 in O_2. The rate of flow of perfusion medium through the ganglion was maintained at approximately 0.3 ml/minute by adjusting the perfusion pressure over the range 30-120 mm Hg.

C. Experimental Procedure

In experiments where one ganglion was perfused, the control contralateral ganglion was removed before starting to perfuse the test ganglion; the ACh content of this control ganglion was measured. In other experiments, both ganglia were perfused. In all experiments, perfusion for the first 15 minutes was with Krebs solution containing choline. This 15 minutes allowed time for the unhurried cannulation of the vein, for adjustments of perfusion pressure and for a short test stimulation of the preganglionic nerve. After this initial 15-minute perfusion, the experimental protocol differed for the

different experiments performed and each procedure will be described in "Results".

D. Chronic Decentralization of Superior Cervical Ganglion

The cats were anaesthetized by sodium pentobarbital (35 mg/kg, i.p.); the skin of the neck was washed with a 70% ethanol solution containing 2% potassium iodide and 2% iodine and was allowed to dry. All surgical instruments were washed thoroughly in boiling water and placed in a 70% ethanol solution. A mid-line incision was made in the neck and either one or both of the superior cervical sympathetic nerves were exposed carefully by blunt dissection; the nerve was cut, and approximately 5 mm of nerve was removed. The wound was washed with sterile isotonic NaCl, and sutured with sterile surgical thread. All animals were given 200,000 i.u. of procaine penicillin G and 0.25 g streptomycin sulphate (Fortimycin-^{1}/2 suspension, Ayerst Laboratories) as an i.m. injection immediately after the operation. The cats were allowed to recover; loss of tone in the nictitating membrane indicated successful denervation. The animals were used for experiment 7 days later.

E. Stimulation of the Preganglionic Sympathetic Nerve

The superior cervical sympathetic nerve was separated carefully from the vagus nerve and from connective tissue, and was cut low in the neck whether it was to be stimulated or not. The nerve was placed on platinum wire electrodes, bathed in paraffin oil and stimulated when necessary with supramaximal rectangular pulses (5 V, 0.3 msec duration, 5-20 Hz) delivered by a Grass SD-5 stimulator. When the nerve was stimulated for long periods

of time, the electrode was moved a few mm proximally along the nerve every 5 minutes. A thread was tied to the nictitating membrane and attached to a Grass force transducer so that contractions of the membrane could be monitored on a Grass pen recorder.

F. Ganglion Extracts

Perfusion of the ganglion was continued until its removal at the end of the experiment. The ganglion was removed quickly, dipped briefly in 0.9% NaCl and the tissue was minced finely in 2.0 ml ice cold 10% trichloroacetic acid (TCA) solution. This procedure extracts all choline, ACh and phosphorylcholine (Chang and Gaddum, 1933; Collier and Lang, 1969).

The tissue suspension was allowed to stand at 0^{o} C for 90 minutes; the supernatant was decanted, the residue was washed with 1.0 ml of the TCA solution, and the wash was combined with the original supernatant. The TCA was removed by shaking the extract 5 times with 5 volumes of water-saturated ether, the aqueous solutions (pH 4) were then aerated briefly to remove residue ether, and, when necessary, the volume was made up to 3.0 ml with 0.9% NaCl. In all experiments, a blank containing only 10% TCA was carried through the same procedure. An aliquot of the blank solution rarely produced a response in the bio-assay for ACh; if it did, blank and test solutions were aerated for an additional few minutes to remove any ether still remaining.

Aliquots of the final extract were used for bio-assay, for liquid scintillation counting, or for the separation of radioactive ACh from other labelled material.

G. Bio-assay

The ACh content of ganglion extracts or effluents was estimated by bio-assay on the blood pressure of the eviscerated cat (MacIntosh and Perry, 1950). Cats (1.5 - 2.5 kg) were anaesthetized and a mid-line abdominal incision was made. In the following order: the rectum, the inferior and superior mesenteric arteries, the coeliac artery, the oesophagus and the portal vein were cut between double ties. The gastrointestinal tract together with the spleen was then removed by cutting connective fascia. The abdomen was closed after checking for internal bleeding and after moistening internal structures with isotonic saline.

Twenty ml of 0.9% NaCl was then injected slowly into the femoral vein. The carotid artery was cannulated and connected to a Statham pressure transducer by polyethylene tubing filled with heparinized saline. Blood pressure was recorded on a Grass pen recorder. When necessary, blood pressure was maintained at approximately 100-120 mm Hg by the intermittent infusion of noradrenaline (1 µg/ml) through the arterial cannula (Quastel, 1962). The reservoir containing noradrenaline had a pressure head of 100-120 mm Hg, and noradrenaline was therefore only infused when the blood pressure fell below this level. If breathing was irregular and resulted in blood pressure fluctuations, the vagus nerves were cut and the cat was artificially respired.

The assay-cat was tested for sensitivity to ACh by injecting the drug i.v.; if it did not respond to 3 ng, eserine sulphate (0.05 mg/kg) was injected to increase the sensitivity to ACh. ACh (as the chloride) was diluted in a solution of similar composition as the material to be assayed,

and its concentration was adjusted to approximate that of the assay material so that similar volumes of both could be injected. Doses of the test sample and known doses of ACh were always injected alternately at 2-minute intervals and were washed into the animal with 1.5 ml of 0.9% NaCl. The concentration of the test sample was estimated by comparing its depressor effects to those produced by known amounts of ACh, and an attempt was always made to assay at more than one dose level. All values of ACh are given as their chlorides.

The depressor effects of both the test sample and authentic ACh were always abolished by treating the sample with alkali, or by pretreating the cat with atropine (0.1 mg/kg).

H. Separation of Radioactive ACh from other Labelled Material

a) Experiments that used ^{3}H-choline. Perfusing ganglia with radioactive choline labels ganglionic stores of ACh, phosphorylcholine and phospholipids as well as leaving unchanged choline (Collier and Lang, 1969). Labelled ACh was separated from other TCA-soluble radioactive material (choline and phosphorylcholine) in ganglion extracts by the procedure described by Collier and Katz (1971). These methods are modifications of procedures described earlier (Collier and Lang, 1969). Standard ^{3}H-ACh (120,000 DPM), standard ^{3}H-choline (130,000 DPM) and an aliquot (1.5-2.0 ml) of the ganglion extract were placed into separate test tubes; the standards were diluted to the same volume as that of the ganglion extract. Carrier ACh (1.0 mg) and carrier phosphorylcholine (0.1 mg) were added, followed by 4.0 ml of a saturated aqueous solution of ammonium reineckate. The precipitate was allowed to form for 30 minutes at room temperature and was

collected by centrifugation; it contained ACh and choline (>90%, see Table 1A), but not phosphorylcholine (<5%). The phosphorylcholine in the test ganglion was not precipitated by ammonium reineckate and therefore the % of the initial radioactivity that was recovered in the reineckate precipitate was smaller from the ganglion extract than it was from the standards. The reineckate precipitate was suspended in 1.5 ml water, and the bases were recovered as chlorides from the reineckate salts by shaking the suspension vigorously with 400 mg of the anion exchange resin Dowex AG 2-X8 (chloride form, 100-200 mesh, Bio-Rad Laboratories); recovery was complete when the solution was colourless. Radioactivity in an aliquot (0.1 ml) of the colourless solution containing ACh and choline was determined, and 1.0 ml of this colourless solution was transferred to clean test tubes for further separation. The test tubes were cooled on ice, carrier ACh (1.0 mg) and 0.4 ml of a 10% (w/v) solution of gold chloride were added; the precipitate was allowed to form for 30 minutes at 0^{o} C. Most of the ACh was precipitated by the gold chloride, but most of the choline remained in solution (Table 1B). The precipitate was collected by centrifugation, the supernatant was decanted into clean test tubes and was decolourized by shaking vigorously with 200 mg of silver metal; radioactivity was determined in an aliquot (0.5 ml) of this colourless solution. The gold precipitate was suspended in 1.0 ml water, decolourized by shaking with 100 mg of silver metal and an aliquot (0.5 ml) was used for liquid scintillation counting. This method for recovering ACh from its chloroaurate using silver metal was described by Dudley (1929). The recovery of the standards and of radioactivity in the test ganglion in this procedure was >88%. The proportion of labelled ACh in the ganglion extract was calculated from the partition

Table 1. The separation of standard ACh and standard choline, and a test ganglion extract by sequential reineckate and gold precipitation in 4 typical experiments.

Experiment	(A) % Radioactivity Recovered Reineckate Precipitation	(B) % Radioactivity Recovered by Gold: Gold Supernatant	(B) % Radioactivity Recovered by Gold: Gold Precipitate	(B) % Recovery from Gold	% ACh in Test
1. ACh	94	6	94	88	
Choline	90	85	15	98	
Test extract	74	42	58	89	54
2. ACh	94	6	94	89	
Choline	91	77	23	96	
Test extract	69	23	77	90	76
3. ACh	97	4	96	89	
Choline	95	74	26	88	
Test extract	91	31	69	94	61
4. ACh	91	6	94	94	
Choline	93	81	19	91	
Test extract	77	37	63	89	59
MEAN ± S.E.					
ACh	94 ± 1	6 ± 0.5	94 ± 0.5	90 ± 1	
Choline	92 ± 1	79 ± 2	21 ± 2.4	93 ± 2	
Test extract				90 ± 1	

(A) Precipitation by reineckate. (B) Reineckate precipitate resuspended, decolourized and separated further by gold chloride. In these experiments, ganglia were perfused with ^{3}H-choline-Krebs during stimulation (5 Hz) for 45 minutes and then perfused for 16 minutes with choline-Krebs.

of standard ^{3}H-choline and standard ^{3}H-ACh between the gold supernatant and precipitate.

b) Experiments that used ^{14}C-ACh or ^{14}C-acetate. In experiments in which ganglia were perfused with acetyl-1-^{14}C-choline or with ^{14}C-acetate, ^{14}C-ACh and ^{14}C-acetate were separated as follows. Standard ^{14}C-ACh (54,000 DPM) and standard ^{14}C-acetate (54,000 DPM) and an aliquot (1.5 - 2.0 ml) of the ganglion extract were placed into separate test tubes; the standards were diluted to the same volume as that of the ganglion extract. Carrier ACh (1.0 mg) and carrier acetate (1.0 mg) were added followed by 4.0 ml of a saturated aqueous solution of ammonium reineckate. The precipitate was allowed to form for 30 minutes at room temperature and the precipitate was collected by centrifugation. Radioactivity in an aliquot (0.5 ml) of the supernatant was determined. The precipitate was suspended in 1.0 ml water and decolourized by shaking the suspension vigorously with the anion exchange resin Dowex AG 2-X8, and radioactivity was determined in an aliquot (0.5 ml) of this colourless solution. The proportion of labelled ACh in the ganglion extract was calculated from the partition of standard ^{14}C-acetate and standard ^{14}C-ACh between the reineckate supernatant and precipitate. The partition of these standards and of some test ganglia are summarized in Table 2. More than 94% of the ACh was precipitated, while >96% of the acetate remained in solution.

I. Measurements of Radioactivity

Radioactivity in aliquots of ganglion extracts, effluents collected from ganglia, or in samples from the separation procedure was determined by liquid scintillation spectrometry (Picker Nuclear, Liquimat-330); a

Table 2. The separation of standard ACh and standard acetate, and a test ganglion extract by reineckate precipitation in 4 typical experiments.

Experiment	% Total Radioactivity			% ACh in Test
	Reineckate Supernatant	Reineckate Precipitate	% Recovery	
1. ACh	6	94	97	
Acetate	96	4	99	
Test extract	96	4	104	0
2. ACh	4	96	97	
Acetate	99	1	109	
Test extract	98	2	99	1
3. ACh	6	94	96	
Acetate	98	2	98	
Test extract	15	85	101	90
4. ACh	5	95	106	
Acetate	99	1	98	
Test extract	22	78	97	82
MEAN ± S.E.				
ACh	5 ± 0.5	95 ± 0.5	99 ± 2	
Acetate	98 ± 0.7	2 ± 0.7	101 ± 3	
Test extract			100 ± 1	

In these experiments, ganglia 1 and 2 were perfused with ^{14}C-acetate in DFP-Krebs for 90 minutes, and ganglia 3 and 4 were perfused with ^{14}C-ACh-DFP-Krebs for 90 minutes.

minimum of 3500 counts was accumulated so that the counting error was 5% or less. The solvent system consisted of: 1 litre toluene, 1 litre ethanol, 1 litre dioxane, 240 g naphthalene, 15 g 2,5-diphenyloxazole (PPO) and 187.5 mg p-bis[2-(5-phenyloxazolyl)]-benzene (POPOP). This mixture would incorporate up to 10% water; when a 0.2 ml aliquot of an aqueous solution was added to 15 ml of this mixture, ^{3}H-activity could be counted with an efficiency of 15-20%, and ^{14}C-activity could be counted with an efficiency of 50-55%. This solvent system was not used when counting both ^{3}H and ^{14}C in the same aliquot since at the double-label spectrometer settings used, 40-45% of the counts appearing in the channel set to count ^{14}C would spill into the ^{3}H-channel. This was too large a spill to allow for accurate determination of ^{3}H-radioactivity. In some experiments, radioactivity was counted in "Aquasol" (New England Nuclear) to improve counting efficiency at either single or double-label settings, to reduce ^{14}C to ^{3}H isotope spill, or to count greater volumes of aqueous sample. "Aquasol" would readily incorporate >25% water. Efficiency for single-isotope determination was 25-30% for ^{3}H and 65-70% for ^{14}C when 0.2 ml aqueous sample was added to 15 ml of "Aquasol". For double-isotope counting, ^{3}H-efficiency was 20-25%, and ^{14}C-efficiency was 35-40%, with 15-20% spill of ^{14}C into ^{3}H-counting channel. Samples were corrected for quench and for isotope spill in the double-isotope experiments by adding internal standards.

J. Materials

The following drugs and chemicals were used in these experiments:

a) Radio-isotopes:

^{14}C-acetate (13.7 mCi/m-mole) was obtained from the hydrolysis of acetyl-1-^{14}C-choline.

Acetyl-1-^{14}C-choline chloride (13.7 mCi/m-mole, Amersham/Searle)

Acetyl-^{3}H-choline chloride (250 mCi/m-mole, Amersham/Searle)

Methyl-^{3}H-choline chloride (100 mCi/m-mole, 110 mCi/m-mole, New England Nuclear)

Methyl-^{3}H-inulin (250 mCi/m-mole, Amersham/Searle)

b) Others:

Acetylcholine chloride (Lematte-Boinot Laboratories)

Acetylcholinesterase (110 u/mg; 1 u hydrolyses 1 µ-mole ACh/minute, Schwartz/Mann)

Ammonium reineckate (J.T. Baker Chemical Co.)

Atropine sulphate (British Drug Houses Ltd.)

Chloralose (kindly provided by Hoffmann-LaRoche Ltd.)

Choline chloride (British Drug Houses Ltd.)

Diisopropylfluorophosphate (DFP, kindly provided by Dr. W.D. Dorian of Merck, Sharp and Dohme, Canada)

Gold chloride (J.T. Baker Chemical Co.)

Hemicholinium #3 dibromide (HC-3, kindly provided by Dr. V.B. Haarstad, Department of Pharmacology, Tulane University)

Heparin sodium (Nutritional Biochemicals Corp.)

Hexamethonium chloride (Nutritional Biochemicals Corp.)

Neostigmine bromide (Sigma Chemical Co.)

Noradrenaline hydrochloride (Nutritional Biochemicals Corp.)

Penicillin G procaine in aqueous suspension with streptomycin sulphate (Fortimycin-^{1}/2 suspension, Ayerst Laboratories)

Trichloroacetic acid (TCA, J.T. Baker Chemical Co.)

All other chemicals used were Baker Analysed Quality.

K. Hydrolysis of Acetyl-1-^{14}C-choline

^{14}C-acetate was obtained by hydrolyzing acetyl-1-^{14}C-choline. An aliquot of ^{14}C-ACh was dissolved in 1.0 ml Krebs solution and hydrolyzed with NaOH; the pH was titrated back to 7.4 with HCl. Separation of an aliquot of the resultant solution by the reineckate precipitation test described above showed that >99% of the ^{14}C-ACh had been hydrolyzed; no ACh was detected when the medium was assayed biologically.

L. Statistical Analysis

Results are expressed as mean $\pm$ S.E. In the statistical evaluation of the results, Student's "t" test for unpaired data was used as a test of significance. Two-tailed significance tests were used throughout.

III. RESULTS

SECTION I

THE RECAPTURE OF ACh BY PREGANGLIONIC FIBRES

A. ^{14}C-ACh Accumulation by Ganglia

To test whether exogenous ACh can be accumulated by the superior cervical ganglion, 6 ganglia were perfused at rest (the preganglionic nerve was cut, but was not stimulated) for 90 minutes with Krebs solution containing acetyl-1-^{14}C-choline (2.8×10^{-6} M), DFP (4.8×10^{-5} M) and ^{3}H-inulin (2.5×10^{-6} M) as an extracellular marker. At the end of the 90-minute perfusion, the ganglia were removed for extraction by TCA, and the ^{3}H- and ^{14}C-radioactivity in the extract was measured. The ratio of ^{3}H to ^{14}C in the perfusion medium was compared to the ^{3}H to ^{14}C ratio in the final tissue extract. The results of these experiments are summarized in Table 3. A ratio of ^{3}H to ^{14}C in the tissue extract similar to the ratio of ^{3}H to ^{14}C in the perfusion fluid would have suggested no ACh accumulation. However, the ^{3}H to ^{14}C ratio measured in the tissue extract was significantly smaller ($P<0.01$) than the isotope ratio in the perfusion medium and this demonstrates that more ACh was accumulated than can be accounted for by extracellular trapping. These ganglia accumulated 3.2 ± 0.2 (mean $\pm$ S.E.) times as much ACh as inulin and the amount of ^{14}C-ACh accumulated, $2,272 \pm 385$ DPM (mean $\pm$ S.E.) of ^{14}C-ACh, was equivalent to 14 ng; this represents about 6% of a ganglion's endogenous ACh content.

The greater accumulation of ^{14}C-ACh than ^{3}H-inulin might have been the result of intracellular accumulation of ACh or of extracellular binding of ACh. If the labelled ACh was accumulated by extracellular binding, it might be expected that it would be readily displaced by transient perfusion

Table 3. ^{3}H-inulin : ^{14}C-ACh in the perfusion medium and in the extracts of ganglia that had been perfused with ^{14}C-ACh and ^{3}H-inulin.

	DPM ^{3}H : DPM ^{14}C		
Experiment	Medium (A)	Tissue (B)	A : B
1	13.0	5.0	2.6
2	14.3	5.2	2.7
3	12.5	4.5	2.7
4	21.7	5.3	4.0
5	13.5	3.7	3.6
6	14.0	3.7	3.7
Mean $\pm$ S.E.			3.2 $\pm$ 0.2

Cat superior cervical ganglia were perfused for 90 minutes with Krebs solution containing DFP (4.8×10^{-5} M), ^{14}C-ACh (2.8×10^{-6} M) and ^{3}H-inulin (2.5×10^{-6} M); the tissue was then removed and extracted with TCA.

with unlabelled ACh and this was tested in 3 experiments. In these experiments ganglia were first perfused for 90 minutes with acetyl-^{3}H-choline (2.8×10^{-6} M) of high specific activity (250 mCi/m-mole) in DFP-containing Krebs solution; perfusion was then changed to DFP-Krebs and effluent was collected for 50 minutes in 2-minute samples. Between the 24th and the 28th minute and between the 36th and the 40th minute of this washout perfusion, the perfusion medium was changed to one containing unlabelled ACh (5.6×10^{-6} M) in DFP-Krebs. Figure 2 illustrates the results from one of these experiments; transient perfusion with ACh did not increase the efflux of ^{3}H-ACh, although contraction of the nictitating membrane showed that the perfused ACh effectively stimulated the ganglion. Similar results were obtained in the other 2 experiments. At the end of these experiments that attempted to release accumulated ^{3}H-ACh by unlabelled ACh, the perfused ganglion was removed and a TCA extract was made. Total ACh content and the amount of labelled ACh remaining in the ganglion was determined. These values are summarized in Table 4. Even after 50 minutes of the washout, enough ^{3}H-ACh (7,043 $\pm$ 813 DPM, mean $\pm$ S.E.) remained in the ganglia for its release to have been detected. This amount of ^{3}H-ACh left in the ganglia was equivalent to 2-3 ng; if 14 ng accumulated in the 90 minutes loading perfusion (see above), 78-85% of the accumulated labelled ACh was lost from the ganglion by spontaneous efflux during the 50-minute washout perfusion.

Other experiments were designed to test the effect of nicotinic and muscarinic blocking drugs, as well as a drug which interferes with choline uptake, on the accumulation of labelled ACh by ganglia. These drugs, hexamethonium, atropine and HC-3, effectively block ACh uptake by brain by at least 50% in the concentrations used in these experiments (Liang and Quastel,

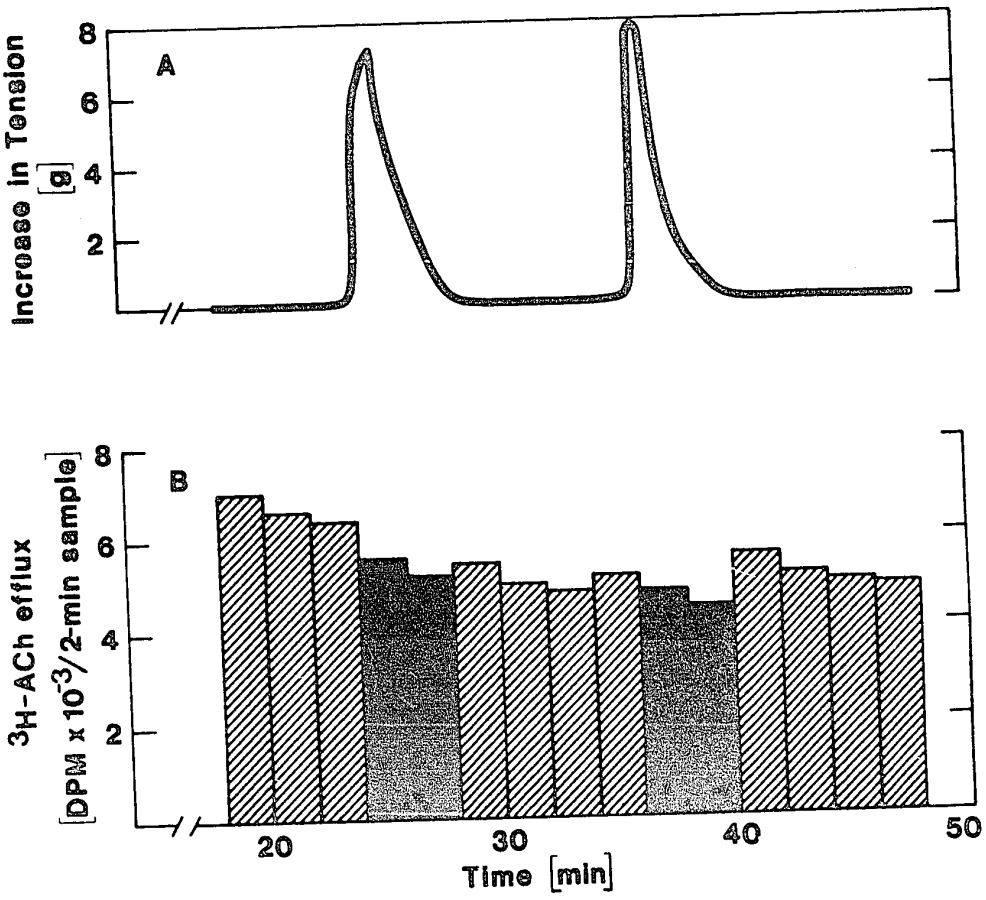


FIG. 2. The effect of perfused ACh (5.6×10^{-6} M, during the collection periods indicated by the black columns) upon (A) contraction of the nictitating membrane and upon (B) the efflux of radioactivity from a cat's superior cervical ganglion perfused with DFP-Krebs. The ganglion had previously accumulated ^{3}H-ACh during perfusion for 90 minutes with ^{3}H-ACh (2.8×10^{-6} M)-DFP-Krebs.

Table 4. Labelled ACh and total ACh in ganglia at the end of the experiment in which the release of accumulated ^{3}H-ACh was tested by transient perfusion with unlabelled ACh.

Experiment	^{3}H-ACh (DPM)	Total ACh Content (ng)
1	7,278	390
2	8,319	450
3	5,532	375
Mean ± S.E.	7,043 ± 813	405 ± 23

Ganglia were first perfused for 90 minutes with ^{3}H-ACh (2.8×10^{-6} M)-DFP-Krebs and then for 50 minutes with DFP-Krebs during which the release of ^{3}H-ACh by unlabelled ACh (5.6×10^{-6} M) was tested; at the end of this time, the tissue was removed and extracted with TCA.

1969b). The results of these experiments are summarized in Table 5. In initial experiments ((A) of Table 5), hexamethonium or HC-3 was added to the perfusion medium containing ^{14}C-ACh (2.8×10^{-6} M) and DFP, perfusion was for 90 minutes and the amount of ^{14}C-ACh accumulated was compared to the amount accumulated by ganglia perfused in the absence of the drug ($2,272 \pm 385$ DPM, mean $\pm$ S.E.; see above). There appeared to be only a small reduction of uptake in the presence of these drugs. In order to more accurately determine if these drugs affect ACh uptake, double perfusion experiments were done ((B)of Table 5). In these experiments, both ganglia of the cat were perfused with Krebs solution containing DFP and ^{14}C-ACh (2.8×10^{-6} M); a drug was added to the medium perfusing the test side and no drug was added to the medium perfusing the control contralateral ganglion. ACh uptake was then directly compared in the 2 ganglia. HC-3, atropine or a combination of hexamethonium and atropine did not dramatically reduce ACh uptake. These experiments suggest that ACh is not accumulating in ganglia by binding to nicotinic or muscarinic receptors in the ganglion; they further suggest that ACh accumulation is not related to the choline uptake mechanism.

B. Does Increased Transmitter Turnover Alter ACh Accumulation?

The measurement of ACh uptake described in section A above was obtained from ganglia that had a low ACh turnover (the preganglionic nerve was cut, but was not stimulated). These ganglia contained their full complement of releasable depot ACh, and it might seem unreasonable to expect any further accumulation (although ganglia perfused with an anti-ChE and choline (not ACh) accumulate 200-300 ng of surplus ACh). Experiments were therefore designed to test whether increasing transmitter turnover by stimulating the preganglionic

Table 5. The effect of hexamethonium, atropine and HC-3 on the uptake of labelled ACh by the superior cervical ganglion of the cat.

	Drug	Concentration (M)	^{14}C-ACh Accumulated (DPM)
A.	None (Mean ± S.E.)	-	2,272 ± 385
	Hexamethonium	2.0×10^{-5}	1,860
	Hexamethonium	1.0×10^{-4}	1,938
	HC-3	6.0×10^{-5}	1,788
B.	None	-	1,870
	HC-3	8.5×10^{-5}	1,848
	None	-	1,926
	Atropine	4.0×10^{-5}	1,797
	None	-	1,880
	Atropine	5.0×10^{-5}	1,663
	None	-	2,011
	Atropine + Hexamethonium	5.0×10^{-5} + 1.0×10^{-4}	1,784

Ganglia were perfused for 90 minutes with Krebs solution containing DFP and ^{14}C-ACh (2.8×10^{-6} M). Experiments in (A) were single-perfusion experiments; the ganglion was perfused in the absence or in the presence of the drug. Experiments in (B) were double-perfusion experiments; the drug(s) was (were) added to the medium perfusing the test side and no drug was added to the medium perfusing the control contralateral ganglion.

nerve would alter labelled ACh uptake by ganglia; choline was deliberately omitted from the perfusion medium in these experiments.

Ganglia were perfused for 90 minutes with ^{14}C-ACh (2.8×10^{-6} M) in DFP-Krebs and the preganglionic nerve was either stimulated throughout (20 Hz) or was at rest throughout. At the end of the perfusion, the ganglia were removed, extracted, and their content of radioactive ACh was measured. The total ACh in the extract of the test ganglion was determined by bio-assay and this value was compared to the ACh content of the control contralateral ganglion (which was not perfused). The results of these experiments are summarized in Table 6. Nerve stimulation did not affect the amount of ^{14}C-ACh accumulated; there was no significant difference in the amount of labelled ACh extracted from the stimulated or the non-stimulated preparations ($P>0.5$). The ganglia whose preganglionic nerve was not stimulated increased their total ACh content by $53 \pm 5\%$ (mean $\pm$ S.E.) and this was partly due to the accumulation of labelled ACh and partly due to the formation of surplus ACh (resting ganglia will synthesize some surplus ACh when exposed to an anti-ChE agent even in the absence of added choline (Birks and MacIntosh, 1961) presumably from the presynaptic pool of endogenous choline; in the present experiments there was probably, in addition, a little free unlabelled-choline available from the slight breakdown of the acetyl-1-^{14}C-choline added to the perfusion medium). However, the ganglia whose preganglionic nerve was stimulated continuously during the experiment lost approximately $29 \pm 5\%$ (mean $\pm$ S.E.) of their ACh content; this loss must be attributed to the failure of synthesis to match release in the choline-deficient medium. Because similar amounts of labelled ACh were accumulated by the stimulated and the non-stimulated ganglia, but total ACh was partially depleted in the stimulated ganglia, the

Table 6. The effect of preganglionic nerve stimulation (20 Hz) on ^{14}C-ACh accumulation and on total ACh content of ganglia perfused with ^{14}C-ACh (2.8×10^{-6} M)-DFP-Krebs for 90 minutes.

Experiment	Perfused Ganglion			Control Ganglion	Change in ACh Content (%)
	^{14}C-ACh (DPM)	Total ACh Content (ng)	Specific Activity (DPM/ng)	Total ACh Content (ng)	
A. Preganglionic Nerve not Stimulated					
1	2,078	420	5	270	+56
2	3,172	240	13	150	+60
3	1,713	390	4	270	+44
Mean ± S.E.	2,321 ± 438	350 ± 56	7.3 ± 2.5	230 ± 40	+53 ± 5
B. Preganglionic Nerve Stimulated					
4	2,191	150	15	240	-37
5	1,539	105	15	150	-30
6	2,307	165	14	210	-21
Mean ± S.E.	2,012 ± 239	140 ± 18	14.6 ± 0.3	200 ± 26	-29 ± 5

final specific activity of ACh in the stimulated ganglia was almost twice that in the non-stimulated ganglia. These experiments clearly show that exogenous ACh cannot be used to replace transmitter stores depleted by neuronal activity in the absence of added choline.

C. Can Accumulated ACh be Released by Nerve Impulses or by K^+?

The failure of nerve stimulation to alter the amount of ^{14}C-ACh accumulated by ganglia (section B) suggested that exogenous ACh does not mix readily with releasable transmitter. This was confirmed by experiments which tested the release of previously accumulated ^{3}H-ACh.

In 3 of these experiments, the ganglia were first perfused for 90 minutes with ^{3}H-ACh (2.8×10^{-6} M) of high specific activity (250 mCi/m-mole) in DFP-Krebs. After this loading procedure, perfusion was switched to DFP-Krebs and perfusion was continued for an additional 50-minute period during which time effluent was collected in 2-minute samples. The preganglionic nerve was stimulated (20 Hz) between the 24th and the 28th minute and between the 36th and the 40th minute of this washout perfusion. Total radioactivity and total ACh were determined for each sample. Figure 3 illustrates the results of a typical experiment which tested whether nerve stimulation releases accumulated ^{3}H-ACh. Nerve stimulation did not affect the efflux of labelled ACh, but released unlabelled ACh as determined by bio-assay. Similar results were obtained in the other 2 experiments. In 1 further experiment, the ganglion was perfused with ^{3}H-ACh during intermittent stimulation (20 Hz, 5 minutes stimulation, 5 minutes rest, for a total of 90 minutes), stimulation was then stopped and perfusion was switched to DFP-Krebs; subsequent nerve

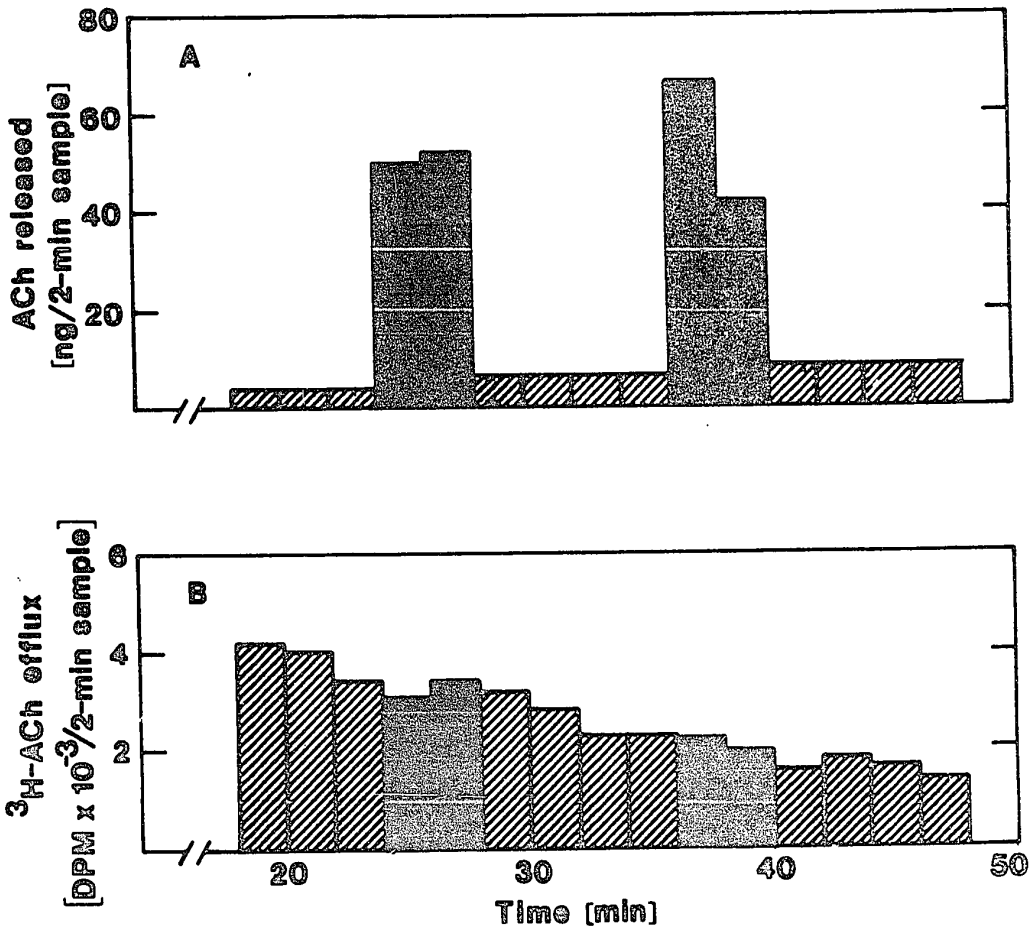


FIG. 3. The effect of nerve stimulation (20 Hz, during the collection periods indicated by the black columns) upon (A) the release of ACh and upon (B) the efflux of radioactivity from a cat's superior cervical ganglion perfused with DFP-Krebs. The ganglion had previously accumulated ^{3}H-ACh during perfusion for 90 minutes with ^{3}H-ACh (2.8×10^{-6} M)-DFP-Krebs.

stimulation (20 Hz) released only unlabelled ACh. These results demonstrate that the accumulated ^{3}H-ACh is not available for release by nerve impulses and confirmed that exogenous ACh does not mix with the releasable transmitter store.

Exposing tissue to a raised K^{+} concentration is often used to release neurotransmitters and this procedure releases endogenous ACh from ganglia (Brown and Feldberg, 1936a). Three experiments tested the release of accumulated ^{3}H-ACh by high K^{+} (46 mM). Ganglia were first perfused for 90 minutes with ^{3}H-ACh (2.8×10^{-6} M) in Krebs solution containing DFP. Perfusion was then switched to DFP-Krebs, effluent was collected in 2-minute samples for 50 minutes, and the ganglia were stimulated to release ACh by 2 separate periods (each 4 minutes) of perfusion with high K^{+}-DFP-Krebs. The results of a typical experiment are illustrated in Fig. 4; transient exposure to high K^{+} released only unlabelled ACh and the results in the other 2 experiments were similar. These experiments confirmed those described above that showed that exogenous ACh does not mix with releasable transmitter.

At the end of all these experiments that attempted to release accumulated labelled ACh by nerve stimulation or by high K^{+}, the perfused ganglion was removed and a TCA extract was made. Total ACh content and the amount of labelled ACh remaining in the ganglion was determined. These values are summarized in Table 7. The amount of ^{3}H-ACh left in the ganglia at the end of the experiment was 7,413 $\pm$ 916 DPM (mean $\pm$ S.E.); this amounted to between 2-3 ng of labelled ^{3}H-ACh left in the ganglia after the 50-minute washout perfusion with DFP-Krebs, and this value is similar to that reported above in the experiments that attempted to release accumulated ^{3}H-ACh by ACh (section A).

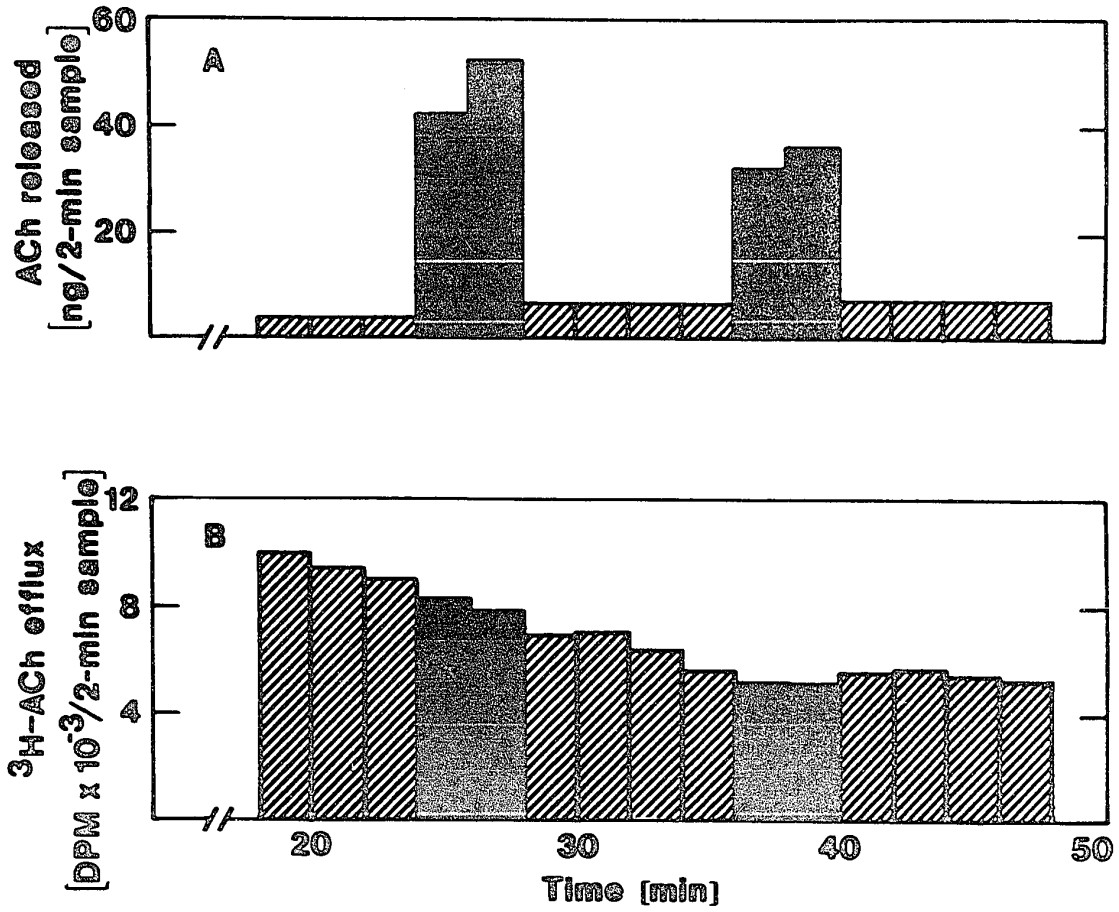


FIG. 4. The effect of high K^+ (46 mM, during the collection periods indicated by the black columns) upon (A) the release of ACh and upon (B) the efflux of radioactivity from a cat's superior cervical ganglion perfused with DFP-Krebs. The ganglion had previously accumulated ^{3}H-ACh during perfusion for 90 minutes with ^{3}H-ACh (2.8×10^{-6} M)-DFP-Krebs.

Table 7. Labelled ACh and total ACh in ganglia at the end of the experiment in which the release of accumulated ^{3}H-ACh was tested by (A) transient nerve stimulation or (B) transient perfusion with high K^{+}.

Experiment	^{3}H-ACh (DPM)	Total ACh Content (ng)
A. Nerve Stimulation		
1	4,570	130
2	9,714	360
3	8,835	360
4*	5,190	337
Mean ± S.E.	7,077 ± 1,287	296 ± 58
B. High K^{+}		
1	9,854	210
2	8,908	270
3	4,820	197
Mean ± S.E.	7,860 ± 1,545	225 ± 22

*Load was during intermittent stimulation (see text).

Ganglia were first perfused for 90 minutes with ^{3}H-ACh (2.8×10^{-6} M)-DFP-Krebs and then for 50 minutes with DFP-Krebs during which the release of ^{3}H-ACh by (A) nerve stimulation (20 Hz) or (B) high K^{+} (46 mM) was tested.

D. Does Exogenous ACh Exchange with Surplus ACh?

The experiments described in sections B and C clearly show that exogenous ACh does not readily exchange with the ganglion's endogenous transmitter stores. When a ganglion is exposed to a suitable anti-ChE agent and choline, surplus ACh accumulates (Birks and MacIntosh, 1961); this extra "pool" of ACh appears not to be available for release by nerve impulses (Katz, 1970; Collier and Katz, 1971), but can be released by exogenous ACh (Collier and Katz, 1970; Katz, 1970). One possible mechanism for this release of surplus ACh by ACh is that exogenous ACh exchanges with ACh in the surplus pool, and the experiments described in this section were designed to test this possibility.

i) ^{14}C-ACh uptake in the presence or the absence of surplus ACh. ACh uptake by ganglia was compared in the presence and in the absence of surplus ACh. The effect of ACh to release surplus ACh is transient, but reappears after a short period of perfusion with ACh-free medium (Collier and Katz, 1970; Katz, 1970). It was therefore decided to perfuse labelled ACh through the ganglion intermittently during the 90-minute loading procedure; intervals of 5-minute exposure to ACh and 5-minute ACh-free perfusion were chosen.

In these experiments, both ganglia of the cat were perfused. The test ganglion was first perfused for 60 minutes with DFP-choline-Krebs so that it accumulated surplus ACh; the control ganglion was first perfused for 60 minutes with choline-Krebs (no anti-ChE, therefore no surplus ACh forming). Both ganglia were then perfused with DFP-Krebs containing ^{14}C-ACh for 5 minutes, followed by a 5-minute perfusion with DFP-Krebs, and perfusion with these 2 media was altered every 5 minutes for a total of 90 minutes. At the end of this time, the ganglia were removed and total ACh as well as labelled ACh was measured. The results of these experiments are summarized in Table 8. Ganglia

Table 8. ^{14}C-ACh uptake by ganglia that had (test) or had not (control) previously accumulated surplus ACh.

Experiment	^{14}C-ACh (DPM)			Total ACh Content (ng)		
	(A) Test Ganglion	(B) Control Ganglion	(A-B)	(A) Test Ganglion	(B) Control Ganglion	(A-B)
1	478	511	-33	400	280	+120
2	909	969	-60	500	300	+200
3	608	596	+12	330	270	+60
Mean ± S.E.	665 ± 128	692 ± 141	-27 ± 21	410 ± 49	283 ± 9	127 ± 41

Both ganglia of the cat were perfused. The test ganglion was first perfused with DFP-choline-Krebs for 60 minutes to accumulate surplus ACh; the control contralateral ganglion was first perfused with choline-Krebs for 60 minutes. Both ganglia were then perfused with ^{14}C-ACh (2.8×10^{-6} M)-DFP-Krebs and DFP-Krebs in alternate 5-minute intervals for a total of 90 minutes.

that had previously accumulated surplus ACh did not accumulate a greater amount of ^{14}C-ACh than did ganglia which did not first accumulate surplus ACh. The total amount of ACh in the test ganglia at the end of the experiment was greater than the total ACh content in the control ganglia and this difference (44 $\pm$ 13%, mean $\pm$ S.E.) was due to the presence of surplus ACh. These experiments suggest that ACh uptake is not related to surplus ACh release and this was confirmed by more direct experiments.

ii) Direct measurement of exchange of exogenous ACh with surplus ACh. More direct experiments were done to determine if exogenous ACh exchanges with surplus ACh by comparing directly the amount (ng) of surplus ACh released to the amount (ng) of exogenous ACh taken up. For these experiments, surplus ACh could be labelled by perfusing ganglia (unstimulated) for 60 minutes with ^{3}H-choline in DFP-Krebs; this procedure labels the surplus ACh and a fraction of the releasable ACh (Collier and Katz, 1971). Labelled ACh in the depot pool is not released by ACh but is releasable by nerve stimulation and surplus ACh is not released by nerve stimulation, but can be released by exogenous ACh (Collier and Katz, 1970; Katz, 1970). If the exogenous ACh used to release ^{3}H-surplus ACh was ^{14}C-ACh, DPM ^{14}C-ACh in the ganglion at the end of the experiment could be converted to the total amount (ng) of exogenous ACh accumulated by knowing the specific activity of the ^{14}C-ACh used. Radioactivity of released ^{3}H-surplus ACh could be converted to the total amount (ng) surplus ACh released if the specific activity of ^{3}H-surplus ACh could be measured.

Two preliminary experiments, using unlabelled ACh, were done to confirm that exogenous ACh releases surplus ACh and to find a way to quantify

the amount (ng) of surplus ACh released. In these experiments surplus ACh was labelled by perfusing ganglia at rest for 60 minutes with Krebs containing DFP and ^{3}H-choline (10^{-5} M; 100 mCi/m-mole). At the end of this loading procedure, perfusion was continued with DFP-choline-Krebs. Two-minute samples were collected and radioactivity was determined in each. ACh injected into the arterial cannula was to be tested for its ability to release ^{3}H-ACh, and the tubing leading from the syringe containing the ACh was first flushed out with Krebs solution to remove any labelled choline which might have diffused into its tip during the loading procedure; the effectiveness of this wash was tested by injecting 0.2 ml Krebs solution (blank injection, see Fig. 5). After the ganglion had been perfused with DFP-choline-Krebs for 28 minutes, the preganglionic nerve trunk was stimulated for 2 minutes (10 Hz) to measure the release of depot ACh and then ACh (10 µg in 0.2 ml Krebs solution) was injected to test the effect of ACh on releasing surplus ACh. Figure 5 illustrates the effect of blank injection, of nerve stimulation (10 Hz), and of injected ACh (10 µg) on the efflux of radioactivity in a typical experiment. A total of 4,000 DPM were released from depot ACh by a 2-minute period of nerve stimulation, and injected ACh released 6,000 DPM from surplus ACh in the 2-minute sample immediately following the injection. The result of the other experiment was similar, and in this experiment the extra DPM released by ACh was identified as labelled ACh by the selective precipitation tests (see Methods). At the end of these experiments, the ganglia were removed, extracted with TCA, and the extract was assayed for total ACh and for ^{3}H-ACh (after separation from other ^{3}H-products). The analyses of the test and the control ganglia, as well as the DPM released by ACh, and DPM and total ACh released by nerve stimulation are shown in Table 9(a).

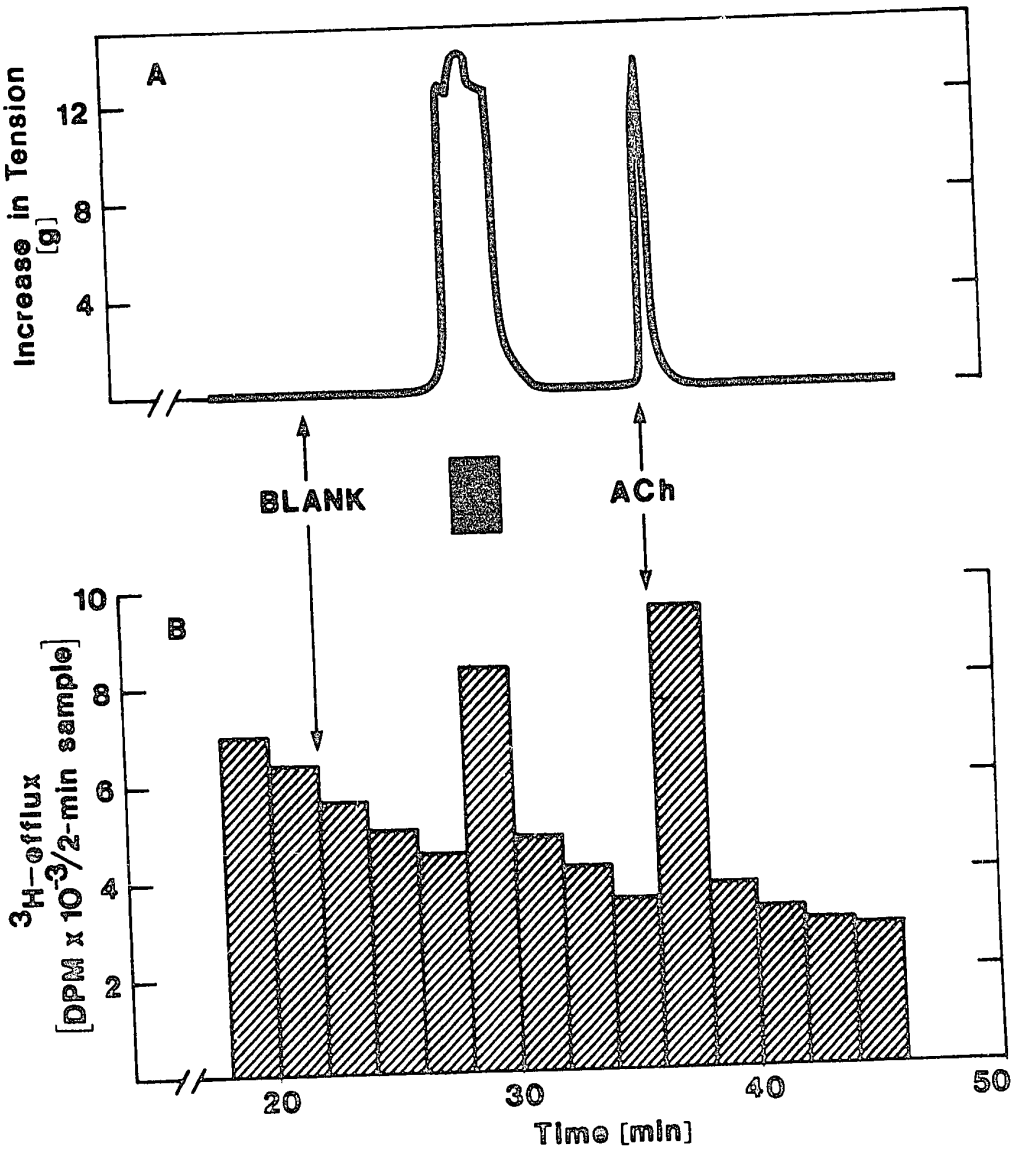


FIG. 5. The effect of Krebs solution (0.2 ml indicated by "blank"), of nerve stimulation (10 Hz, during the 2-minute period indicated by the black bar), and of injected ACh (10 µg in 0.2 ml Krebs where indicated) upon (A) contraction of the nictitating membrane and upon (B) the efflux of radioactivity from a cat's superior cervical ganglion perfused with DFP-choline-Krebs. The ganglion had previously accumulated labelled surplus ACh during perfusion with ^{3}H-choline-DFP-Krebs for 60 minutes.

In these experiments, radioactivity released by ACh is coming only from the surplus pool (Collier and Katz, 1970; Katz, 1970). To convert the amount of radioactivity released by ACh (DPM) to the amount (ng) of surplus ACh released by ACh, it was necessary to estimate the specific activity of the ACh in the surplus pool. This was done as follows. (a) The measured specific activity of ACh released by nerve stimulation (DPM/ng) gave the specific activity of depot ACh; this is a reasonable estimate if the period of stimulation is short (Collier, 1969). (b) The ACh content (ng) of the contralateral ganglion (not perfused) was used as a measure of the total amount (ng) of depot ACh in the perfused ganglion, and multiplying this value by the specific activity of depot ACh (from (a)) yielded a measure of the total radioactivity (DPM) of ACh in the depot pool of the test ganglion (this assumes that both ganglia of the same cat contain an approximately equal amount of ACh (see, e.g., Brown and Feldberg, 1936b; Birks and MacIntosh, 1961). (c) Subtracting this value (DPM) for the total radioactivity on depot ACh from the total radioactivity (DPM) of ACh that was measured in the test ganglion at the end of the experiment, gave the radioactivity (DPM) of surplus ACh. (d) Dividing this value for the radioactivity (DPM) of surplus ACh by the total amount (ng) of surplus ACh present (total ACh content of the test ganglion minus total ACh content of the control ganglion) gave the specific activity (DPM/ng) of ACh in the surplus pool. (e) The amount of radioactivity (DPM) released by ACh divided by the specific activity (DPM/ng) of surplus ACh gave the amount (ng) of surplus ACh released. These calculations are summarized in Table 9(b). Table 9 shows that in these 2 experiments, 6,000 DPM and 5,300 DPM were released by ACh, and this was equivalent to a release of about 55 ng and 65 ng, respectively, of surplus ACh.

Table 9. The amount (DPM and ng) of surplus ACh released by ACh.

a)

Experiment	Test Ganglion (A) ACh Content (ng)	(B) ACh Content (DPM)	(C) ACh Released by Nerve Stimulation (DPM)	(D) ACh Released by Nerve Stimulation (ng)	(E) ACh Released by ACh (DPM)	Control Ganglion (F) ACh Content (ng)
1	520	56,768	4,000	37	6,000	250
2	580	43,866	2,800	40	5,300	300

Calculated by	(G) Depot ACh *S.A. (DPM/ng) → (C/D)	(H) Depot ACh (DPM) (G x F)	(I) Surplus ACh (DPM) (B - H)	(J) Surplus ACh (ng) (A - F)	(K) *S.A. (DPM/ng) (I/J)	(L) Surplus ACh Released (ng) (E/K)
b) Experiment						
1	108	27,027	29,741	270	110	55
2	70	21,000	22,866	280	82	65

Test ganglia were first perfused for 60 minutes with ^{3}H-choline-DFP-Krebs; this labelled the surplus ACh pool and a fraction of the depot ACh pool. Perfusion was then switched to DFP-choline-Krebs and after 28 minutes of washout perfusion, the release of ^{3}H-ACh by nerve stimulation (10 Hz for 2 minutes) and by ACh (10 µg in 0.2 ml Krebs injected into the arterial cannula) was measured; the release of total ACh by nerve stimulation was also measured. At the end of the experiment, the ganglia were removed and extracted with TCA. Calculations are shown in (b).

*S.A. = specific activity.

This kind of experiment was then used to test whether exogenous ACh is taken up in exchange for surplus ACh. In these experiments, acetyl-1-^{14}C-choline was used to release ^{3}H-surplus ACh as described above for unlabelled ACh. If exogenous ACh exchanges with endogenous surplus ACh, then the amount (ng) of ^{14}C-ACh accumulated would be about the same as the amount (ng) of ^{3}H-ACh released. The following parameters had to be determined. (a) Radioactivity of ^{14}C-ACh in the ganglion at the end of the experiment. (b) Specific activity of ^{3}H-surplus ACh in the ganglion; this was calculated as described above. Since the ^{3}H-radioactivity in the ganglion extract was considerably higher than the ^{14}C-radioactivity, double-isotope counting was possible; spill of ^{14}C into ^{3}H-counting channel did not represent more than 0.1% of the counts in the ^{3}H-channel, and, therefore, the values for each isotope could readily be determined. The amount of ^{14}C-ACh accumulated (ng) was calculated from the specific activity of the added ACh. (c) The release of ^{3}H-ACh into the effluent by ^{14}C-ACh injected to the ganglion. Standard double-isotope counting could not be used for this measurement because there was a relatively small amount of ^{3}H-ACh released compared to the large amount of ^{14}C-ACh used and it was not possible to count the effluent accurately for ^{3}H-radioactivity at double-isotope settings on the spectrometer for it was masked by the spill of ^{14}C into the ^{3}H-counting channel. This problem was overcome by hydrolyzing both the ^{3}H-ACh and the ^{14}C-ACh in the effluent by acetylcholinesterase (AChE); since the ^{3}H-surplus ACh released had been synthesized <u>in situ</u> from N-methyl-^{3}H-choline, hydrolysis yielded ^{3}H-choline but the ^{14}C-ACh used was labelled on the acetate moiety, and hydrolysis yielded ^{14}C-acetate. The ^{3}H-choline was then precipitated by reineckate, leaving >99% of the acetate in solution and the ^{3}H-choline could be counted without interference from ^{14}C spill. The DPM of

surplus ACh released was thus determined by the extra ^{3}H-choline measured. This assumes that the extra ^{3}H-choline represents ^{3}H-ACh released by ACh. This assumption is valid since the earlier experiments using unlabelled ACh demonstrated that the only radioactivity released by ACh is ACh.

Before using the AChE in a release experiment, it was necessary to determine its activity in the presence of an anti-ChE; DFP was present throughout the experiment except when the ^{14}C-ACh was injected to the ganglion and its concentration in the collected effluent must therefore have been $<4.8 \times 10^{-5}$ M; the concentration of ^{14}C-ACh in the collected sample was calculated to be less than 5.6×10^{-5} M. Table 10 summarizes the results of experiments which measured the hydrolysis of ^{14}C-ACh by AChE. Various concentrations of ^{14}C-ACh were used in the presence or in the absence of DFP; AChE was added in concentrations 0-100 times the amount needed to hydrolyze the ACh as calculated from the enzyme's specific activity. The samples were shaken for 30 minutes and the reaction was stopped by adding 0.1 ml of 1.0 N HCl. Carrier ACh (1.0 mg) and carrier acetate (1.0 mg) were added followed by 3 ml of ammonium reineckate solution. The precipitate was allowed to form for 30 minutes at room temperature, and was collected by centrifugation. An aliquot of the supernatant was counted for radioactivity, and the precipitate was washed 3 times with 5 ml of reineckate to remove excess ^{14}C-acetate. The precipitate was suspended in water, decolourized and counted for radioactivity. Table 10 shows that in the absence of AChE, no ^{14}C-ACh was hydrolyzed. However, when AChE was added in the concentration needed to hydrolyze ACh (calculated from the specific activity of the AChE) almost all of the ^{14}C-ACh added at the different concentrations was hydrolyzed. In the presence of DFP (4.8×10^{-5} M), 100 times the normal amount of AChE was required before

Table 10. The hydrolysis of ^{14}C-ACh by acetylcholinesterase.

ACh Concentration (M)	DFP Concentration (M)	AChE Concentration (u/ml)	X Normal Amount AChE Needed	Hydrolysis (%)
2.8×10^{-6}	0	0	0	0
"	0	0.0027	1	97
"	0	0.027	10	99
"	4.8×10^{-5}	0.0027	1	5
"	"	0.027	10	67
"	"	0.135	50	89
"	"	0.270	100	100
1.7×10^{-5}	0	0	0	0
"	0	0.016	1	98
"	0	0.16	10	100
"	4.8×10^{-5}	0.016	1	6
"	"	0.16	10	61
"	"	0.80	50	85
"	"	1.6	100	100
2.8×10^{-5}	0	0	0	0
"	0	0.027	1	100
"	0	0.27	10	100
"	4.8×10^{-5}	0.027	1	9
"	"	0.27	10	53
"	"	1.35	50	84
"	"	2.70	100	100
5.6×10^{-5}	0	0	0	0
"	0	0.054	1	100
"	0	0.54	10	100
"	4.8×10^{-5}	0.054	1	16
"	"	0.54	10	70
"	"	2.7	50	99
"	"	5.4	100	100

hydrolysis was complete. Since the concentration of ^{14}C-ACh in the collected effluent in the experiments designed to measure exchange might approach, but not reach, a concentration in the effluent of 5.6×10^{-5} M, and since DFP was omitted from the perfusion medium during the injection of ^{14}C-ACh, its concentration was $<4.8 \times 10^{-5}$ M, it was therefore decided to use 5.4 u AChE/ml effluent (see Table 10). The addition of AChE did not interfere with the precipitation of choline (Table 11); choline was precipitated equally well by reineckate in the absence or in the presence of AChE (5.4 u/ml). Thus the use of AChE allowed the calculation of DPM of ^{3}H-ACh released by ^{14}C-ACh, and from the specific activity of surplus ACh (see (b) above), the amount (ng) of surplus ACh released was calculated.

Figure 6 shows the effect of nerve stimulation (10 Hz) and of injecting ^{14}C-ACh (10 µg in 0.2 ml Krebs) on the efflux of ^{3}H-radioactivity from a ganglion that had been first perfused with ^{3}H-choline as described earlier. Nerve stimulation released 3,000 DPM in the 2-minute sample; ^{14}C-ACh released 4,800 DPM in the 2 minutes following the injection. At the end of the experiment, the ganglion was removed for analysis of ^{3}H-ACh and ^{14}C-ACh; total ACh content was determined for both the test and the control ganglion. The results from 2 such experiments (experiments 1 and 2) are shown in Table 12(a) and the calculations in Table 12(b) show how these results were used to compute the amount (ng) of ^{3}H-surplus ACh released by ^{14}C-ACh. In a third experiment (experiment 3 in Table 12(b)), a different method was used to estimate the specific activity of depot ACh. The test ganglion was treated in the same way as in the other 2 experiments, except that the preganglionic nerve was not stimulated. The contralateral ganglion was perfused with ^{3}H-choline-Krebs for 60 minutes (no DFP, therefore no ^{3}H-surplus ACh) and then with choline-Krebs solution for 28 minutes. The amount of ^{3}H-ACh in this ganglion was used

Table 11. The effect of acetylcholinesterase (5.4 u/ml) on the precipitation of ^{3}H-choline by ammonium reineckate.

Experiment	AChE Absent		AChE Present	
	Supernatant % Total Radioactivity	Precipitate % Total Radioactivity	Supernatant % Total Radioactivity	Precipitate % Total Radioactivity
1	4	96	6	94
2	5	95	5	95
3	8	92	5	95
4	8	92	7	93
Mean ± S.E.	6 ± 1	94 ± 1	6 ± 0.5	94 ± 0.5

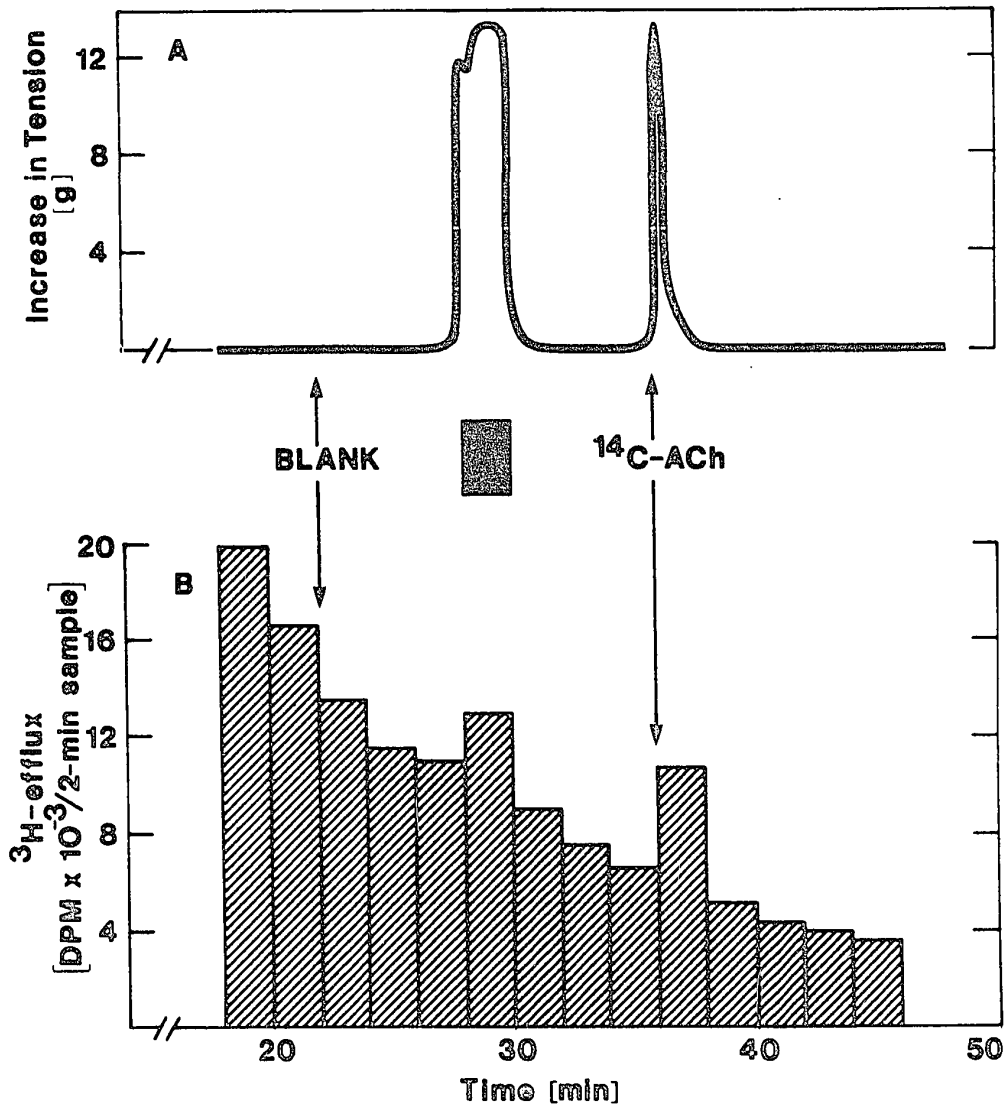


FIG. 6. The effect of Krebs solution (0.2 ml indicated by "blank"), of nerve stimulation (10 Hz, during the 2-minute period indicated by the black bar), and of injected ^{14}C-ACh (10 µg in 0.2 ml Krebs where indicated) upon (A) contraction of the nictitating membrane and upon (B) the efflux of radioactivity from a cat's superior cervical ganglion perfused with DFP-choline-Krebs. The ganglion had previously accumulated labelled surplus ACh during perfusion with ^{3}H-choline-DFP-Krebs for 60 minutes.

Table 12. The amount (DPM and ng) of surplus ACh released by ^{14}C-ACh, and the amount (ng) ^{14}C-ACh accumulated.

a)

	Test Ganglion						Control Ganglion
	(A)	(B)	(C)	(D)	(E)	(F)	(G)
Experiment	ACh Content (ng)	ACh Content (^{3}H-ACh DPM)	ACh Content (^{14}C-ACh DPM)	ACh Released by Nerve Stim. (DPM)	ACh Released by Nerve Stim. (ng)	ACh Released by ^{14}C-ACh (DPM)	ACh Content (ng)
1	487	37,016	170	2,180	32	4,132	240
2	640	46,349	382	3,000	34	4,800	330
3	333	26,337	216	not determined	not determined	5,600	180
MEAN ± S.E.	486 ± 89	36,567±5,781	256 ± 64			4,844 ± 424	250 ± 44

b)

	(H) Depot ACh	(I)	(J) Surplus ACh	(K)	(L)	(M)	(N)
Calculated by ⟶	*S.A. (DPM/ng) (D/E)	(DPM) (GxH)	(DPM) (B-I)	(ng) (A-G)	*S.A. (DPM/ng) (J/K)	Surplus ACh released (ng) (F/L)	^{14}C-ACh Accumulated (ng) (C/S.A.* of ^{14}C-ACh)
Experiment							
1	68	16,320	20,696	247	84	50	1
2	88	29,117	17,231	310	56	86	2
3		14,441**	11,896	153	78	72	1
MEAN ± S.E.		19,959±4,610	16,607±2,559	236±46	73 ± 9	69 ± 11	1.3 ± 0.3

Test ganglia were first perfused for 60 minutes with ^{3}H-choline-DFP-Krebs; this labelled the surplus ACh pool and a fraction of the depot ACh pool. Perfusion was then switched to DFP-choline-Krebs and after 28 minutes of washout perfusion the release of ^{3}H-ACh by nerve stimulation (10 Hz for 2 minutes) and by ^{14}C-ACh (10 µg in 0.2 ml Krebs injected into the arterial cannula) was measured; the release of total ACh by nerve stimulation was also measured. At the end of the experiment, the ganglia were removed and extracted with TCA. Calculations are shown in (b).

*S.A. = specific activity.

**Measured directly (see text).

as an estimate of the amount of ^{3}H-depot ACh in the test ganglion.

The amount of surplus ACh released by ACh in these 3 experiments was 69 $\pm$ 11 ng (mean $\pm$ S.E.), a value similar to the release measured in the experiments above which used unlabelled ACh. However, only about 1-2 ng ^{14}C-ACh accumulated in these ganglia (256 $\pm$ 64 DPM (mean $\pm$ S.E.) of ^{14}C-ACh.) Thus 35-70 times more ACh was released than ACh accumulated. These results clearly show that the release of surplus ACh from ganglia by ACh is not by ACh exchange.

E. The Non-Specificity of ACh Uptake

The results presented above demonstrate that exogenous ACh does not readily mix or exchange with presynaptic ACh pools; experiments were therefore designed to test whether ACh was entering cholinergic nerve endings or was accumulating in postsynaptic structures. In 3 experiments, the accumulation of ^{14}C-ACh in chronically decentralized (7 days) ganglia was compared to the accumulation in acutely (30 minutes) decentralized ganglia. Perfusion was for 90 minutes with ^{14}C-ACh (2.8×10^{-6} M) in DFP-Krebs. The ganglia were then removed and the DPM of ^{14}C-ACh and total ACh content were determined. The results of all these experiments are summarized by Fig. 7. There was no significant difference in the amount of ACh accumulated by either preparation ($P>0.8$). As expected, the chronically decentralized ganglia had lost about 85% of their stored ACh. Thus the final specific activity of ACh in the chronically decentralized preparations (85 DPM/ng) was significantly greater than the specific activity of ACh in acutely decentralized preparations (7 DPM/ng; $P<0.01$). In 1 further experiment, both ganglia of the cat were perfused; 1 ganglion had been chronically decentralized. The amount of ^{14}C-ACh

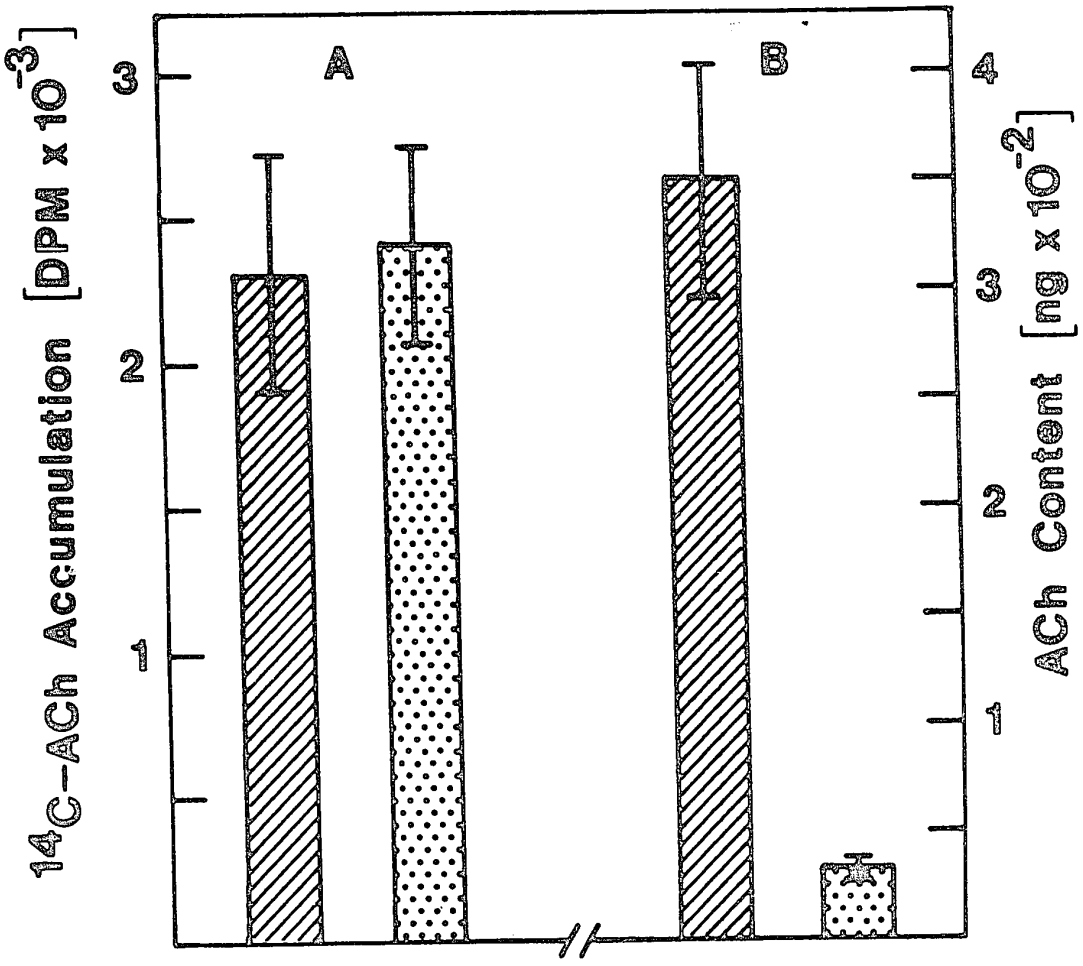


FIG. 7. (A) The accumulation of ^{14}C-ACh and (B) the total ACh content in ganglia perfused for 90 minutes with DFP-Krebs containing ^{14}C-ACh (2.8×10^{-6} M). Control ganglia (hatched columns) had their sympathetic nerves cut at the time of the experiment; decentralized ganglia (stippled columns) had their sympathetic nerves cut seven days before the experiment. Each column represents the mean $\pm$ S.E. of 3 experiments.

accumulated by either ganglion was similar (2,196 DPM in the intact preparation and 1,968 DPM in the decentralized ganglion); the total ACh content was greater in the intact ganglion (380 ng) than it was in the decentralized ganglion (45 ng). These findings suggest that ACh is not preferentially accumulated into cholinergic nerve endings.

One experiment studied the effect of stimulating the postganglionic fibres on the release of accumulated ACh from the ganglion. The ganglion was first loaded by perfusing for 90 minutes with ^{3}H-ACh (2.8×10^{-6} M) in DFP-Krebs and perfusion was then switched to DFP-Krebs. An electrode was placed around the postganglionic nerve trunk, and the ganglion was stimulated antidromically (20 Hz). The result of antidromic nerve stimulation on the spontaneous efflux of ^{3}H-ACh is illustrated by Fig. 8. No labelled ACh was released by stimulation. The effluent collected during stimulation also did not contain a measurable amount of ACh when assayed biologically. This was expected, since antidromic nerve stimulation is known to be ineffective in releasing ACh (Feldberg and Vartiainen, 1934). The sensitivity of the assay-cat in this experiment was too low to pick up the spontaneous output of ACh which must have been <2 ng/minute.

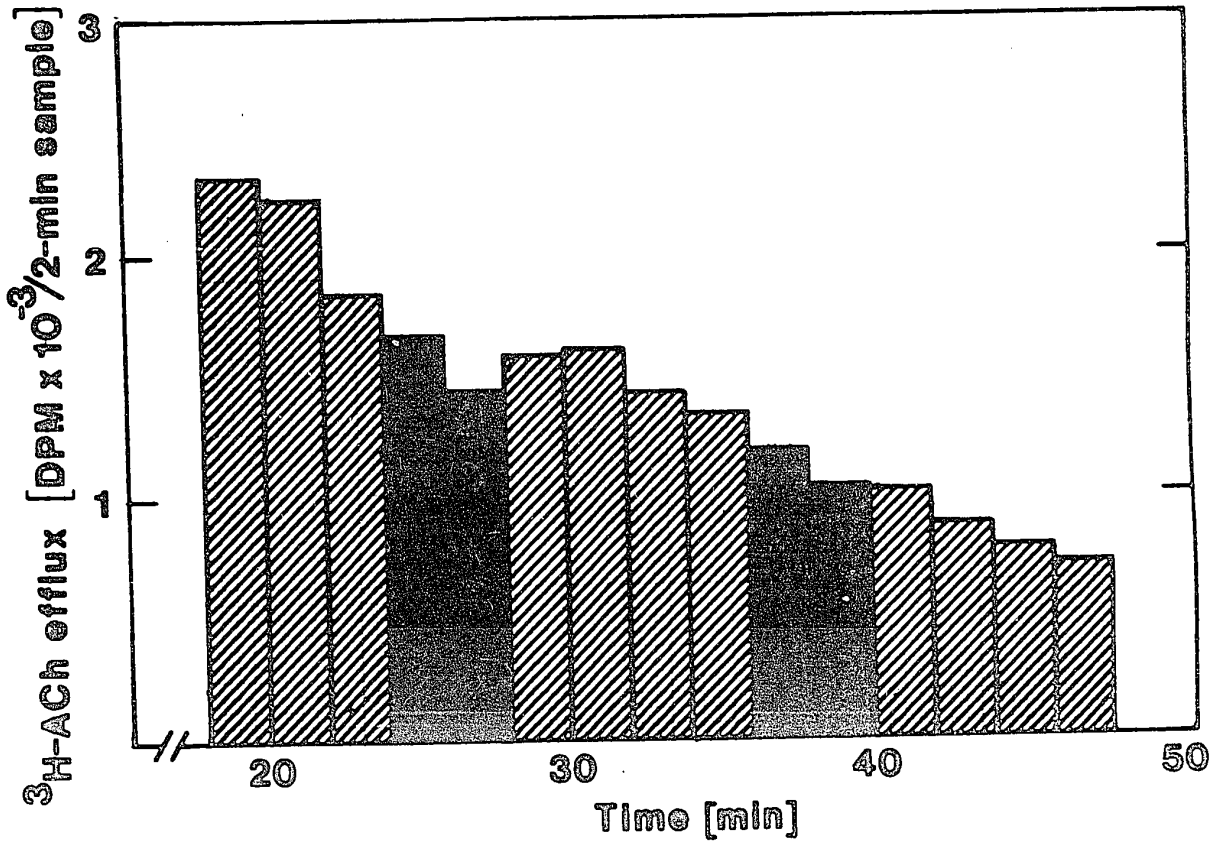


FIG. 8. The effect of antidromic nerve stimulation (20 Hz, during the collection periods indicated by the black columns) upon the efflux of radioactivity from a cat's superior cervical ganglion perfused with DFP-Krebs. The ganglion had previously accumulated ^{3}H-ACh during perfusion for 90 minutes with ^{3}H-ACh (2.8 x 10^{-6} M) in DFP-Krebs.

SECTION II

THE RECAPTURE OF ACETATE AND OF CHOLINE BY PREGANGLIONIC FIBRES

The experiments described in Section I demonstrate that exogenous ACh cannot substitute for precursor choline for the maintenance of transmitter stores. The experiments described in this section studied whether the metabolic products of neuronally released ACh, acetate and choline, can be recaptured for synthesis into ACh.

A. Recapture of Acetate by Ganglia

In 3 experiments, non-stimulated ganglia were perfused for 90 minutes with ^{14}C-acetate (2.8×10^{-6} M) and choline in DFP-Krebs. At the end of this time the ganglia were removed and extracted with TCA. The total radioactivity and the radioactivity of ACh were measured; the total ACh content was compared to total ACh content in the control contralateral ganglia. Table 13 summarizes the results of these experiments. The ganglia accumulated ^{14}C-radioactivity but little, if any, of this was as ^{14}C-ACh. However, ACh had been synthesized by these ganglia because their total ACh content had increased by 74%; this was due to the accumulation of surplus ACh since choline and DFP were present in the perfusion medium.

Two other experiments were designed to test if increased transmitter turnover would increase the labelling of ACh from ^{14}C-acetate. In these experiments, ganglia were perfused for 60 minutes with low glucose (1.98 mM)-Krebs containing ^{14}C-acetate (2.8×10^{-6} M), choline and DFP. The preganglionic nerve was stimulated (5 Hz) throughout the experiment and the effluent from the ganglia was collected in 5-minute samples which were used for bio-assay of the total ACh released by nerve stimulation. The results of one experiment

Table 13. ^{14}C-ACh and total ACh in ganglia perfused for 90 minutes with ^{14}C-acetate (2.8×10^{-6} M) in Krebs solution containing choline and DFP.

Experiment	Perfused Ganglion			Control Ganglion	
	Total Radioactivity (DPM)	^{14}C-ACh (DPM)	Total ACh Content (ng)	Total ACh Content (ng)	% Increase in ACh Contents
1	11,108	133	550	300	+83
2	8,958	0	385	220	+75
3	9,737	0	330	200	+65
Mean ± S.E.	9,890 ± 587	44 ± 44	422 ± 66	240 ± 31	+74 ± 5

are illustrated by Fig. 9 which shows that the release of ACh by nerve stimulation was maintained in the presence of the low glucose-Krebs; a total of 366 ng was released in 60 minutes. The release of ACh in the other experiment was also well maintained, although total ACh released was somewhat lower (210 ng). At the end of these experiments, the perfused ganglia were removed and extracted with TCA. ^{14}C-ACh was determined, and total ACh content was compared to total ACh content in the control ganglia. Table 14 summarizes the results of these analyses. The perfused ganglia were not depleted of ACh and thus transmitter released by nerve stimulation must have been replaced by ACh synthesis. The accumulation of extra ACh in these ganglia further demonstrates that the ganglia synthesized surplus ACh even in the low glucose medium. However, no significant amount of ^{14}C-ACh was detected in these ganglia; <1% of the radioactivity was on ^{14}C-ACh. These experiments demonstrate that exogenous acetate is not used for ACh synthesis during transmitter turnover. Thus it seems unlikely that acetate, derived from the hydrolysis of released ACh in vivo, can be recaptured by preganglionic fibres for ACh synthesis.

B. Recapture of Choline by Ganglia

In these experiments it was necessary to compare the amount (DPM) of radioactivity collected from a ganglion stimulated in the presence of neostigmine or HC-3 with the amount of radioactivity (DPM) collected from a ganglion stimulated in the absence of these drugs. This comparison can best be made if the ganglion's endogenous ACh is labelled (see Collier and MacIntosh, 1969). In the present experiments, the rate of nerve stimulation was close to physiological levels (5 Hz). Four experiments demonstrated the labelling of ganglionic ACh. Ganglia were perfused with ^{3}H-choline

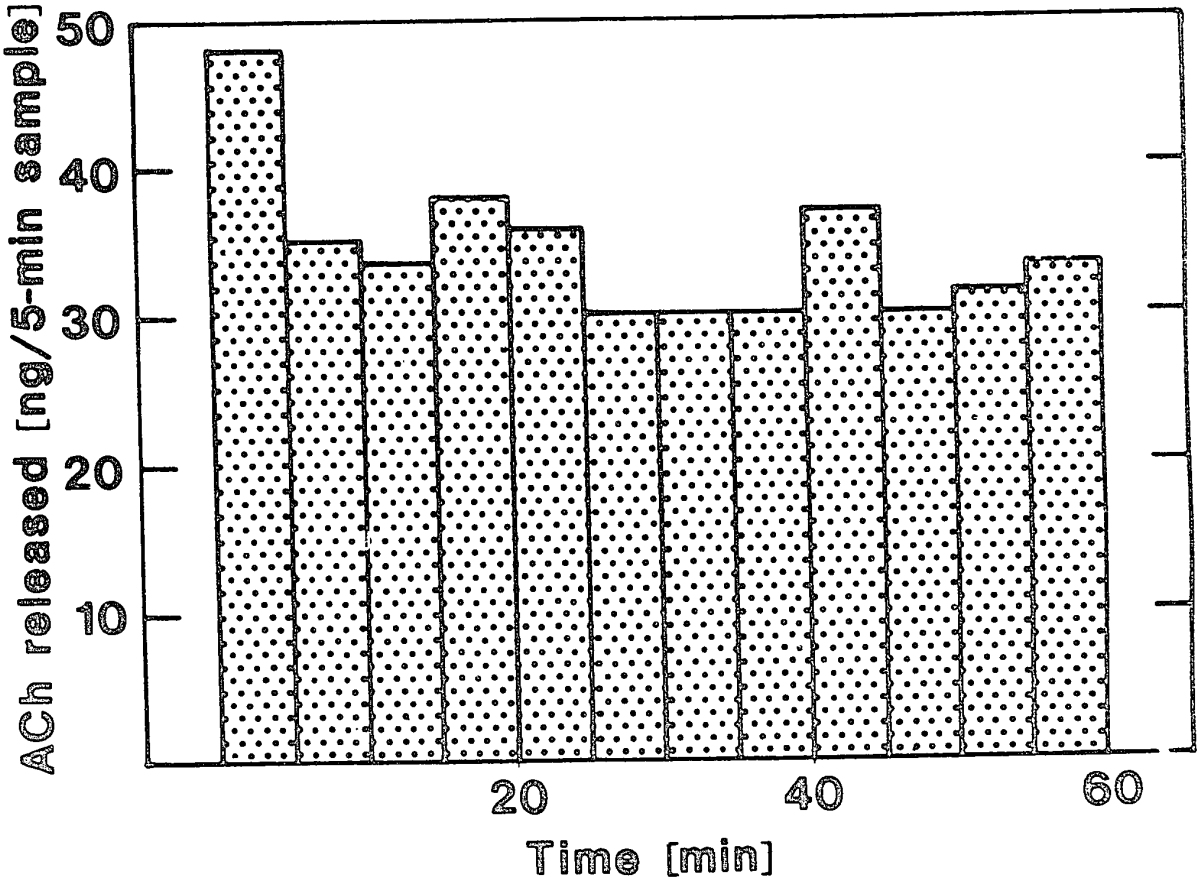


FIG. 9. The release of ACh by nerve stimulation (5 Hz) from a cat's superior cervical ganglion perfused with ^{14}C-acetate (2.8×10^{-6} M)-choline and DFP in low glucose Krebs.

Table 14. ^{14}C-ACh in ganglia perfused for 60 minutes with ^{14}C-acetate (2.8×10^{-6} M) in low glucose Krebs solution containing choline and DFP; the preganglionic nerve was stimulated (5 Hz) throughout.

Expt.	Perfused Ganglion				Control Ganglion	
	Total Radioactivity (DPM)	^{14}C-ACh (DPM)	Total ACh Released (ng)	Total ACh Content (ng)	Total ACh Content (ng)	Increase in ACh Content (%)
1	8,316	0	210	500	288	+74
2	10,133	120	366	600	342	+75

(10^{-5} M, 100 mCi/m-mole) in Krebs solution for 45 minutes during constant electrical stimulation (5 Hz). At the end of this loading perfusion, the ganglia were perfused for an additional 16 minutes with unlabelled choline-Krebs; the nerve was not stimulated. The ganglia were removed and labelled choline, labelled ACh and total ACh determined. These values are summarized in Table 15. The radioactive ACh was equivalent to 90 ng of ^{3}H-ACh (calculated from the specific activity of ^{3}H-choline added to the perfusion medium); thus, about 25% of the ganglion's ACh was labelled by 45 minutes of stimulation at 5 Hz and this compares to 85% when perfusion is for 60 minutes during constant stimulation at 20 Hz (Collier and MacIntosh, 1969). The specific activity of ACh in the present experiments was 303 $\pm$ 44 DPM/ng (mean $\pm$ S.E.) so that the release of as little as 2-3 ng would be detected easily.

In the experiments which studied whether ganglia recapture choline from hydrolyzed ACh, both ganglia of the cat were perfused. The releasable ACh pool of both ganglia was loaded as described above and perfusion was continued in both for 16 minutes with choline-Krebs. At the end of this procedure, one of 3 paired experiments was done. Group (1): one ganglion was perfused with neostigmine-choline-Krebs and the contralateral ganglion was perfused with choline-Krebs. Group (2): one ganglion was perfused with HC-3-Krebs and the contralateral ganglion was perfused with choline-Krebs. Group (3): one ganglion was perfused with neostigmine-choline-Krebs and the contralateral ganglion was perfused with HC-3-Krebs. Within each group, the experiment to be done on the right or the left ganglion was altered with each experiment. In each paired experiment, nerve stimulation (5 Hz) was tested for its ability to release labelled ACh into one of the above perfusion media (released labelled ACh was detected as ACh in the presence of neostigmine,

Table 15. Labelled ACh and labelled choline in ganglia perfused for 45 minutes with ^{3}H-choline-Krebs during constant stimulation (5 Hz) and then perfused at rest for 16 minutes with choline-Krebs.

Experiment	^{3}H-Choline (DPM)	^{3}H-ACh (DPM)	Total ACh Content (ng)	Specific Activity (DPM/ng)
1	21,129	97,684	300	326
2	18,922	145,306	350	415
3	60,380	105,364	400	263
4	44,974	87,538	420	208
Mean $\pm$ S.E.	36,344 $\pm$ 9,944	108,973 $\pm$ 12,649	367 $\pm$ 26	303 $\pm$ 44

and was detected as choline in the absence of neostigmine), and the amount of radioactivity released by the two ganglia was compared. At the end of the experiment, a comparison was made between the amount of labelled ACh in the two ganglia. Neostigmine was chosen as the anti-ChE agent because in the presence of this drug surplus ACh does not accumulate unless perfusion is for longer than 45 minutes (Katz, 1970; Collier and Katz, 1971). The experimental results were examined as follows: Group (1): if choline from ACh breakdown is removed by uptake before it reaches the site of collection, the total radioactivity released in the presence of neostigmine would be greater than the total radioactivity released in the absence of neostigmine. If the recaptured choline is available for ACh synthesis, the difference in the amount of radioactivity released should be detected as extra radioactive ACh in ganglia perfused without neostigmine. Group (2): in the presence of HC-3 and the absence of an anti-ChE agent, neuronally-released ACh is hydrolyzed, but recapture of choline would be prevented by the blockage of the choline-uptake mechanism. Therefore, the release of radioactivity in the presence of HC-3-Krebs would be greater than the release of radioactivity in the presence of choline-Krebs. If recaptured choline is synthesized into ACh, the captured labelled choline should be detected as extra radioactive ACh in those preparations perfused with choline-Krebs. Group (3): the recapture of choline would be prevented by both neostigmine (prevention of ACh hydrolysis) and HC-3 (blockage of choline uptake). Therefore the release of radioactivity into neostigmine-choline-Krebs would be similar to the release of radioactivity into HC-3-Krebs. Similar amounts of labelled ACh would be expected in both ganglia at the end of the experiment. However, if neostigmine increases ACh release, the release of radioactivity

by stimulation into the neostigmine-containing medium would be greater than the release into HC-3-containing medium and this difference would be matched by a difference in the ^{3}H-ACh left in the ganglion at the end of the experiment.

The assumption made in these experiments is that paired ganglia behave in a similar manner under similar test circumstances. One preliminary experiment tested this assumption. The depot ACh pool of both ganglia of the cat were loaded as described earlier (stimulation at 5 Hz for 45 minutes) and perfusion was continued with Ch-Krebs for 16 minutes; effluent collected during this time was discarded. The effluent from the ganglia was then collected in 2-minute samples for a total of 40 minutes, and total radioactivity in each was determined; the preganglionic nerve was stimulated for 20 minutes (5 Hz) between the 10th and the 30th minute. Figure 10 illustrates the extra radioactivity released above the spontaneous efflux from the two ganglia during the 20-minute stimulation. There was no large difference in the pattern of release of radioactivity. The total extra radioactivity released by the left ganglion during stimulation was 12,402 DPM and that by the right ganglion was 14,103 DPM. At the end of the experiment, the left ganglion contained 49,941 DPM ^{3}H-ACh compared to 50,593 DPM in the right ganglion. The specific activity of ACh in the ganglion at the end of the experiment was calculated by dividing the total DPM of ACh by total ACh; this was 145 DPM/ng for the left ganglion and 142 DPM/ng for the right ganglion. The specific activity of ACh in the ganglion at the time of onset of nerve stimulation was calculated by adding the amount of ^{3}H-ACh remaining (DPM) in the ganglion at the end of the experiment to the amount of ^{3}H-ACh released (DPM) by nerve stimulation, and dividing this value by total ACh content (ng)

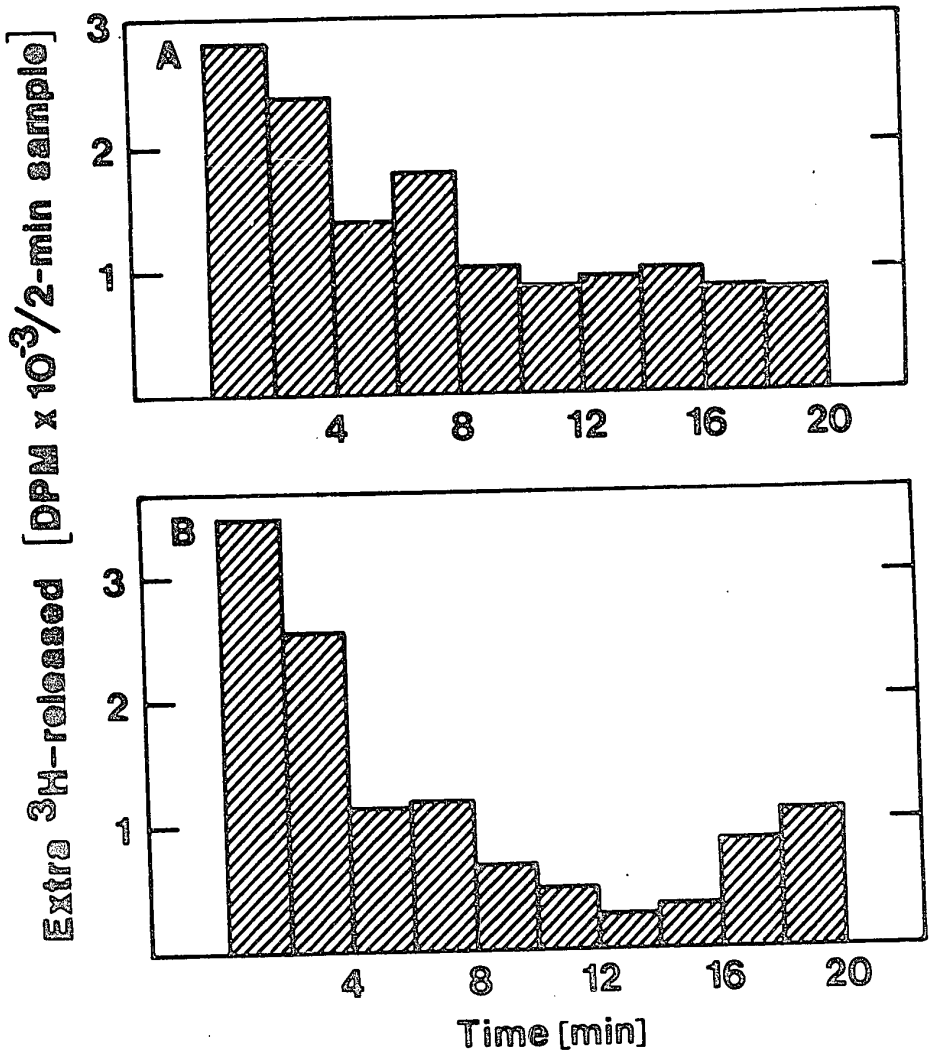


FIG. 10. The release of extra radioactivity above the spontaneous efflux from a paired experiment where the depot ACh pool of both ganglia had been labelled (see text); both ganglia stimulated (5 Hz) for 20 minutes during perfusion with choline-Krebs.

of the ganglion; this was 180 DPM/ng for the left ganglion and 182 DPM/ng for the right ganglion. These similar values demonstrate that the depot pools of both ganglia had been labelled to the same extent, and that the ganglia behaved similarly when subsequently stimulated.

a) Group 1 experiments: Comparison of release into neostigmine-choline-Krebs and into choline-Krebs. The releasable ACh of both ganglia of the cat was labelled by perfusion with ^{3}H-choline during 5 Hz stimulation and this was followed by a washout perfusion with unlabelled choline-Krebs for 16 minutes; effluent collected during this time was discarded. One ganglion was then perfused for 40 minutes with choline-Krebs and effluent was collected in 2-minute samples. The preganglionic nerve was stimulated (5 Hz) for 20 minutes between the 10th and the 30th minute, and total radioactivity in each sample was determined. The contralateral ganglion was perfused with choline-Krebs until 4 minutes before the start of nerve stimulation when perfusion was switched to neostigmine-choline-Krebs. The preganglionic nerve was stimulated (5 Hz) for 20 minutes between the 10th and the 30th minute, and at the end of stimulation, perfusion was returned to choline-Krebs; thus the ganglion was exposed to neostigmine throughout the period of nerve stimulation and total exposure time to neostigmine was 24 minutes. The total radioactivity of each 2-minute sample was determined. Figure 11 illustrates the extra radioactivity released above the spontaneous efflux from a pair of ganglia during the 20-minute stimulation period, where one ganglion had been stimulated in the presence of neostigmine-choline-Krebs and the other stimulated in the presence of choline-Krebs. The release of extra radioactivity was clearly greater into the neostigmine-choline-Krebs solution; 28,551 DPM were released into this medium compared to 8,272 DPM released into choline-Krebs. The

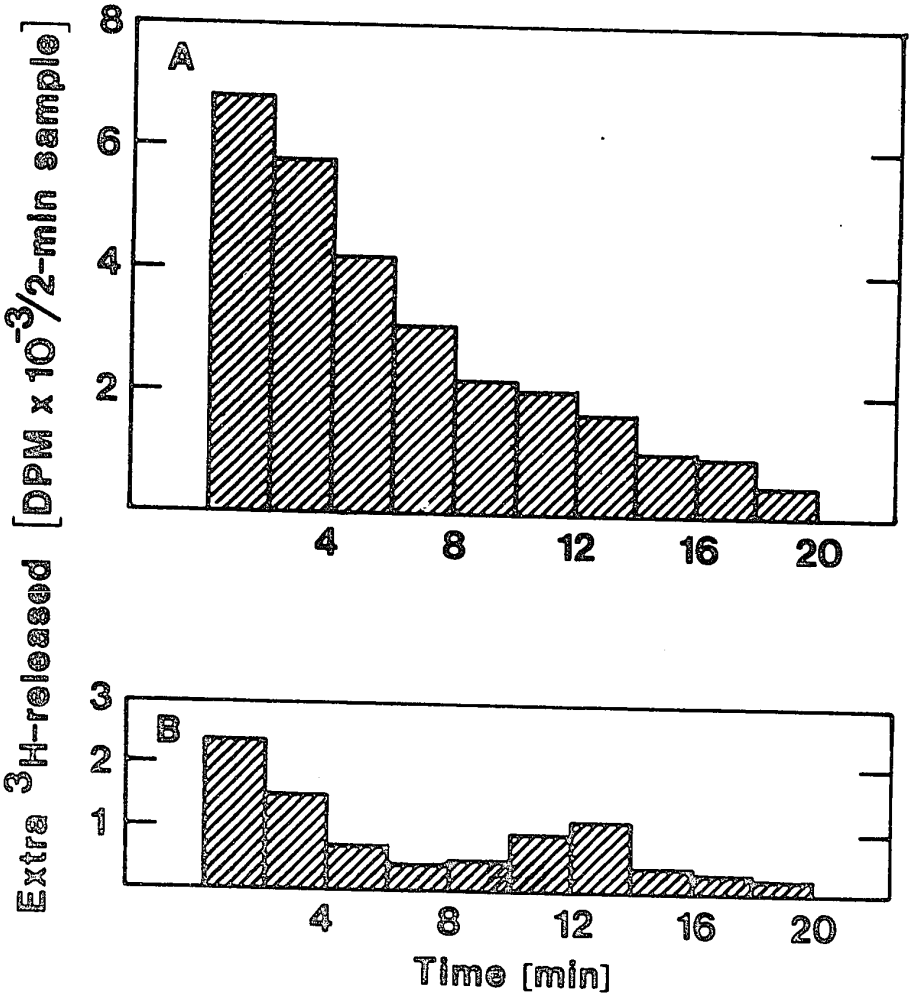


FIG. 11. The release of extra radioactivity above the spontaneous efflux from a paired experiment where the depot ACh pool of both ganglia had been labelled (see text); both ganglia stimulated (5 Hz) for 20 minutes during perfusion with (A) neostigmine-choline-Krebs or (B) choline-Krebs.

results of the other 2 experiments were similar (Table 16); the amount of radioactivity released into choline-Krebs was 37 ± 4% (mean ± S.E.) of that released into neostigmine-choline-Krebs, and this suggests that about 63% of the choline is recaptured under the conditions of these experiments. If the smaller release of radioactivity in the presence of choline-Krebs is due to the recapture by the ganglion of some of the labelled choline made available from the breakdown of the labelled ACh, then this extra radioactivity should appear in the final ganglion extract. If the recaptured choline was converted to ACh, then this extra radioactivity should appear as extra labelled ACh. The analyses of the ganglion extracts are summarized in Table 16. In each case most of the difference in the amount of radioactivity released was found to be as extra ACh in the ganglion stimulated in the presence of choline-Krebs; the extra radioactivity on ganglionic ACh represented 92% ± 7% (mean ± S.E.) of the radioactivity recaptured.

The total ACh content of the ganglia perfused with neostigmine was about the same as that of the ganglia perfused without neostigmine and this shows that surplus ACh had not accumulated in those preparations exposed to neostigmine-choline-Krebs for 24 minutes. The specific activity of ACh at the start of nerve stimulation was similar in each pair of ganglia, and this indicates that the labelling procedure with ^{3}H-choline had labelled both ganglia to the same extent. However, the specific activity of ganglionic ACh at the end of the experiment was consistently greater in those preparations which had been perfused with choline-Krebs. This is due to the extra radioactivity on ACh in these preparations formed from the recaptured labelled choline.

Table 16. Radioactivity released and radioactivity remaining in ganglia whose depot ACh pool had been labelled (see text); the ganglia were then stimulated (5 Hz) for 20 minutes during perfusion with neostigmine-choline-Krebs or choline-Krebs.

Experiment	^{3}H-Label Released (DPM)	Ganglion Content			*S.A. of ACh end of Expt. (DPM/ng)	*S.A. of ACh Start of Nerve Stim. (DPM/ng)
		^{3}H-ACh (DPM)	^{3}H-Choline (DPM)	Total ACh Content (ng)		
1. (a) Neostigmine-Choline	42,349	31,213	26,364	215	122	320
(b) Choline	16,922	58,366	22,450	225	259	334
Difference (a-b)	+25,427	-27,153	+3,914	-10	-137	-14
2. (a) Neostigmine-Choline	40,797	39,831	21,133	330	120	244
(b) Choline	16,822	59,788	18,870	300	199	248
Difference (a-b)	+23,975	-19,957	+2,263	+30	-79	-4
3. (a) Neostigmine-Choline	28,551	23,984	6,337	375	64	140
(b) Choline	8,272	41,549	9,248	320	129	155
Difference (a-b)	+20,279	-17,565	-2,911	+55	-65	-15

*S.A. = Specific Activity.

b) Group 2 experiments: Comparison of release into HC-3-Krebs and into choline-Krebs. The releasable ACh of both ganglia was labelled as described above, and perfusion was continued for 16 minutes with choline-Krebs; effluent collected during this time was discarded. In one ganglion, perfusion was continued for 40 minutes with choline-Krebs and the contralateral ganglion was perfused with HC-3 (1.7×10^{-5} M)-Krebs for 40 minutes. The preganglionic nerve was stimulated (5 Hz) between the 10th and the 30th minute and the effluent from the ganglia was collected in 2-minute samples for the determination of radioactivity. The ganglia were removed for extraction at the end of the experiment. Figure 12 illustrates the release of extra radioactivity above spontaneous efflux from one typical pair of ganglia during the 20-minute stimulation period. The release of extra radioactivity into HC-3-Krebs was clearly greater than release into HC-3-free medium. In this experiment, 29,346 DPM were released into HC-3-Krebs, and 8,977 DPM were released into choline-Krebs. The results of the other 2 experiments were similar (Table 17); the release of radioactivity into choline-Krebs was 47 $\pm$ 8% (mean $\pm$ S.E.) of the release of radioactivity into HC-3-Krebs. This suggests that in these experiments 53% of the labelled choline released into choline-Krebs escaped collection and this value is not too different from the value (63%) calculated from the experiments described as Group 1. The analyses of ganglion extracts from these experiments in Group 2 are summarized by Table 17. In all experiments, most of the difference in the radioactivity collected was accounted for by an increased amount of ^{3}H-ACh in the ganglion perfused with choline-Krebs; this extra DPM of ACh represented 88 $\pm$ 14% (mean $\pm$ S.E.) of the radioactivity recaptured. These experiments confirm that the labelled choline is recaptured largely

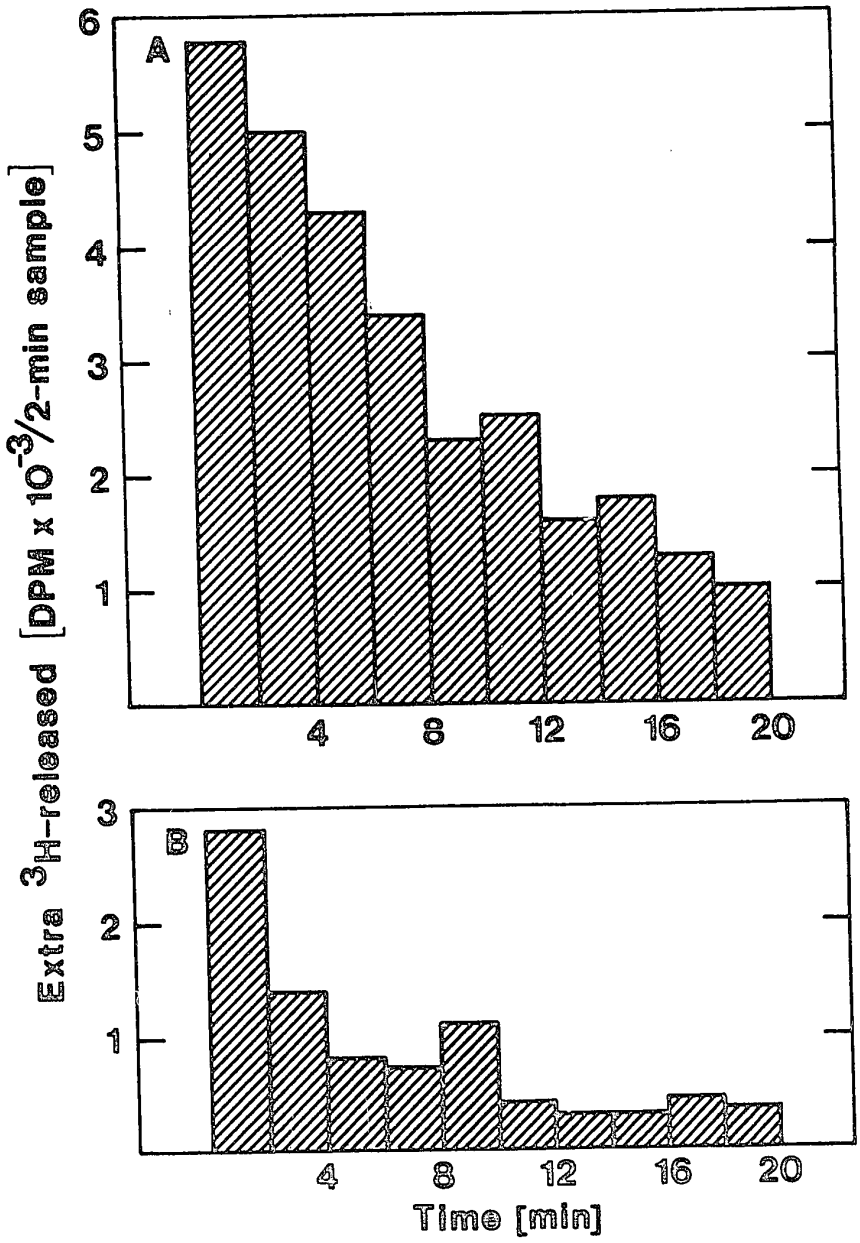


FIG. 12. The release of extra radioactivity above the spontaneous efflux from a paired experiment where the depot ACh pool of both ganglia had been labelled (see text); both ganglia stimulated (5 Hz) for 20 minutes during perfusion with (A) HC-3-Krebs or (B) choline-Krebs.

Table 17. Radioactivity released and radioactivity remaining in ganglia whose depot ACh pool had been labelled (see text); the ganglia were then stimulated (5 Hz) for 20 minutes during perfusion with HC-3-Krebs or choline-Krebs.

Experiment	^{3}H-Label Released (DPM)	Ganglion Content				*S.A. of ACh Start of Nerve Stim. (DPM/ng)
		^{3}H-ACh (DPM)	^{3}H-Choline (DPM)	Total ACh Content (ng)	*S.A. of ACh end of Expt. (DPM/ng)	
1. (a) HC-3	19,097	28,051	18,222	253	110	112
(b) Choline	10,222	38,227	17,174	420	91	115
Difference (a-b)	+8,875	-10,176	+1,048	-167	+19	-3
2. (a) HC-3	26,921	30,622	21,661	130	235	230
(b) Choline	15,170	39,085	25,830	250	156	217
Difference (a-b)	+11,751	-8,463	-4,169	-120	+79	+13
3. (a) HC-3	29,346	12,995	23,436	67	184	174
(b) Choline	8,977	28,540	30,524	240	118	156
Difference (a-b)	+20,369	-15,545	-7,088	-173	+66	+18

*S.A. = Specific Activity

for ACh synthesis.

In these experiments (Table 17) there was a partial depletion (53 ± 10%; mean ± S.E.) of the total ACh content in the ganglia stimulated in the presence of HC-3. Thus despite the greater amount of ^{3}H-ACh in the ganglia that had been perfused with choline-Krebs, the specific activity of ganglionic ACh at the end of the experiment was higher in the ganglia perfused with HC-3-Krebs. To calculate the specific activity of ganglionic ACh at the onset of stimulation, it was assumed that both ganglia at that time contained an amount of ACh equal to that measured at the end of the experiment in the ganglion perfused without HC-3 (ganglia perfused with choline-containing medium are not depleted of ACh (see Birks and MacIntosh, 1961)). At the start of stimulation both ganglia contained ACh of similar specific activity and this indicates that both had been loaded to a similar extent.

c) Group 3 experiments: Comparison of release into neostigmine-choline-Krebs and into HC-3-Krebs. The releasable ACh of both ganglia was labelled as described earlier and perfusion was continued for 16 minutes with choline-Krebs; effluent collected during this time was discarded. One ganglion was then perfused for 40 minutes with HC-3-Krebs and effluent was collected in 2-minute samples. The preganglionic nerve was stimulated (5 Hz) for 20 minutes between the 10th and the 30th minute, and total radioactivity in each sample was determined. The contralateral ganglion was perfused with choline-Krebs until 4 minutes before the start of nerve stimulation when perfusion was switched to neostigmine-choline-Krebs. The preganglionic nerve was stimulated (5 Hz) for 20 minutes between the 10th and the 30th minute, and at the end of stimulation, perfusion was returned to choline-Krebs. The ganglia were removed for extraction at the end of the experiment. Figure 13

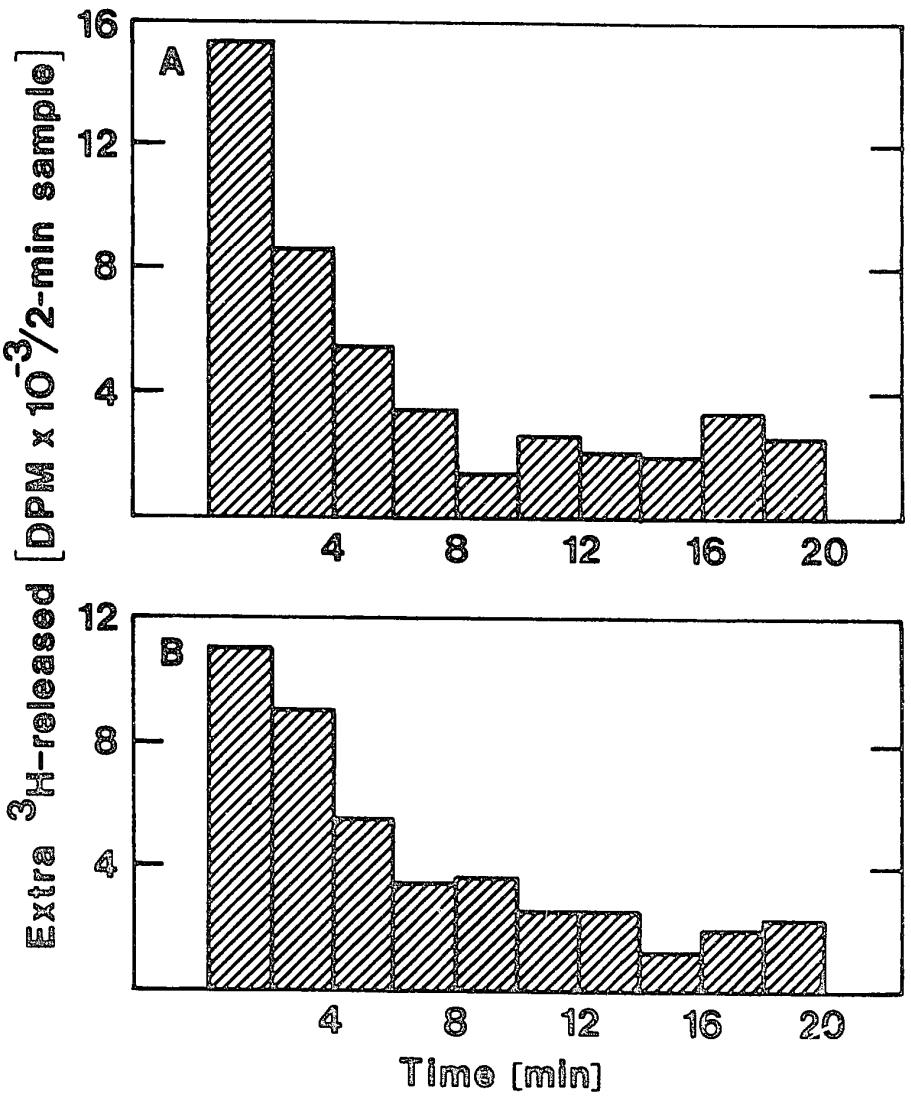


FIG. 13. The release of extra radioactivity above the spontaneous efflux from a paired experiment where the depot ACh pool of both ganglia had been labelled (see text); both ganglia stimulated (5 Hz) for 20 minutes during perfusion with (A) neostigmine-choline-Krebs or (B) HC-3-Krebs.

illustrates the release of extra radioactivity above the spontaneous efflux from one typical pair of ganglia during the 20-minute stimulation period. There was no large difference in the amount of extra radioactivity released into either medium. In this experiment, 48,005 DPM were released into neostigmine-choline-Krebs, and 44,100 DPM were released into HC-3-Krebs. The results of the other 2 experiments were similar (Table 18); the release of radioactivity into neostigmine-choline-Krebs was 88 $\pm$ 10% (mean $\pm$ S.E.) of the radioactivity released in HC-3-Krebs. This suggests that in these experiments most of the radioactivity released into either medium was collected in the ganglion effluent. The analyses of ganglion extracts in these Group 3 experiments are summarized by Table 18. There was only a small difference in the amount of labelled ACh left in the two ganglia although preparations stimulated in the presence of neostigmine-choline-Krebs consistently contained more ^{3}H-ACh.

In these experiments (Table 18) there was a partial depletion (42 $\pm$ 11%, mean $\pm$ S.E.) of total ACh content in the ganglia stimulated in the presence of HC-3, and therefore the specific activity of ganglionic ACh was higher in these preparations than in the ganglia stimulated in the presence of neostigmine-choline-Krebs. Both ganglia contained ACh of similar specific activity at the onset of nerve stimulation (calculated as described above) and this indicates that both had been loaded to a similar extent.

The results of Groups 1, 2 and 3 experiments are all consistent with the hypothesis that choline, derived from neuronally released ACh, can effectively compete with circulating choline for ACh synthesis; approximately 50-60% of the choline appears to be recaptured for ACh synthesis. This

Table 18. Radioactivity released and radioactivity remaining in ganglia whose depot ACh pool had been labelled (see text); the ganglia were then stimulated (5 Hz) for 20 minutes during perfusion with neostigmine-choline-Krebs or HC-3-Krebs.

Experiment	^{3}H-Label Released (DPM)	Ganglion Content			*S.A. of ACh end of Expt. (DPM/ng)	*S.A. of ACh Start of Nerve Stim. (DPM/ng)
		^{3}H-ACh (DPM)	^{3}H-Choline (DPM)	Total ACh Content (ng)		
1. (a) Neostigmine-Choline	24,841	51,849	30,374	400	130	192
(b) HC-3	32,323	46,744	38,415	228	205	198
Difference (a-b)	-7,482	+5,105	-8,041	+172	-75	-6
2. (a) Neostigmine-Choline	48,005	34,772	21,731	300	115	275
(b) HC-3	44,100	29,220	20,780	120	244	244
Difference (a-b)	+3,905	+5,552	+951	+180	-129	+31
3. (a) Neostigmine-Choline	9,122	52,309	36,156	280	187	219
(b) HC-3	11,585	46,018	45,396	220	209	206
Difference (a-b)	-2,463	+6,291	-9,240	+60	-22	+13

*S.A. = Specific Activity

conclusion is supported by the analysis of the results of this section that is illustrated by Fig. 14. The ratio of the specific activity of ACh in the ganglion at the end of the experiment to the specific activity of ganglionic ACh at the start of nerve stimulation is plotted for the 6 ganglia stimulated in each of the 3 perfusion media; a ratio of 1.0 indicates no change in the specific activity of ACh. When perfusion was with HC-3-Krebs, the specific activity of ganglionic ACh was the same at the end of the experiment as it was at the onset of stimulation and this indicates that released ACh came from a homogeneous pool of ACh when synthesis was prevented. When perfusion was with neostigmine-choline-Krebs, the specific activity of ganglionic ACh at the end of the experiment was 55% of its specific activity at the start of stimulation; thus newly synthesized transmitter, formed from unlabelled choline, had diluted the ganglion's labelled ACh. When perfusion was with choline-Krebs (the only one of the 3 media which allowed the recapture of labelled choline), the specific activity of ganglionic ACh at the end of the experiment was 78% of its specific activity at the start of stimulation, or about half-way between the situation when ACh synthesis was prevented and when ACh synthesis was from unlabelled choline. Thus it is clear that labelled choline was recaptured for ACh synthesis.

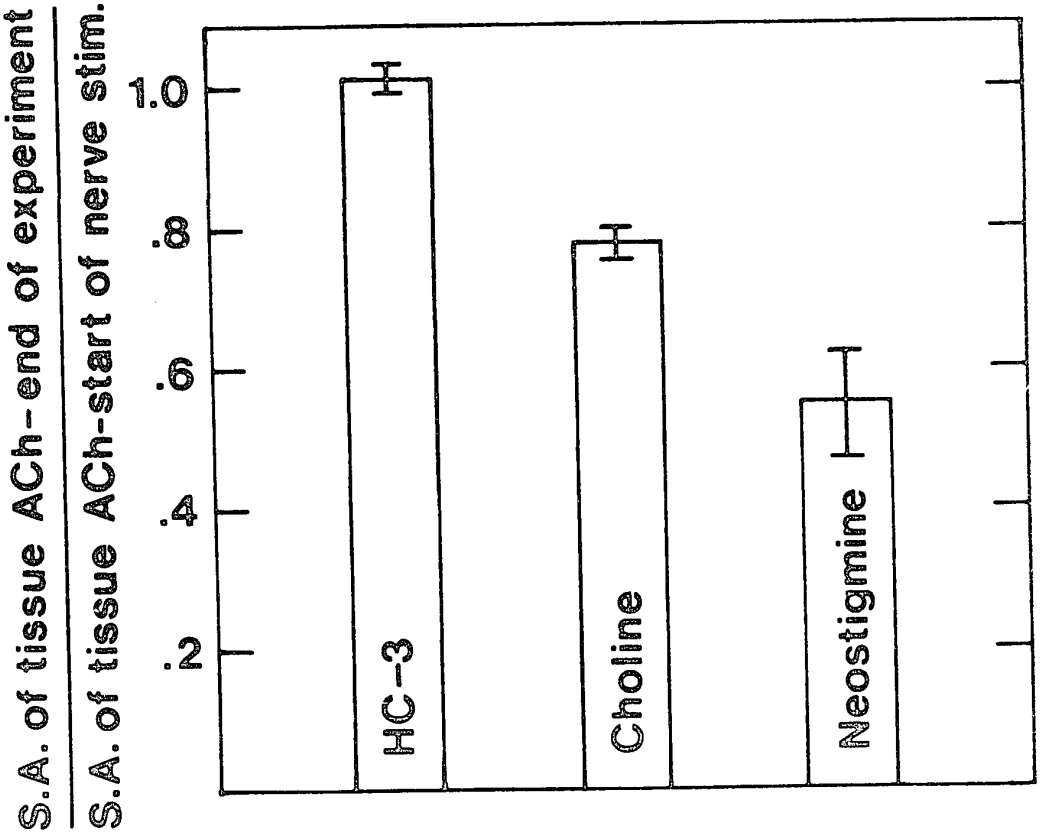


FIG. 14. The ratio of the specific activity of ganglionic ACh at the end of the experiment to the specific activity of ganglionic ACh at the onset of nerve stimulation in ganglia perfused with HC-3-Krebs, choline-Krebs or neostigmine-choline-Krebs, as indicated. Each column represents the mean ± S.E. of 6 experiments.

IV. DISCUSSION

A. Can Neuronally Released Acetylcholine be Recaptured by the Superior Cervical Ganglion and Re-Used for Transmission?

The use of radiotracer procedures has demonstrated that many non-cholinergic transmitters can be taken up by neurones - e.g., noradrenaline (Whitby, Axelrod and Weil-Malherbe, 1961; Iversen and Whitby, 1962; Iversen, 1963), γ-aminobutyric acid (GABA, Iversen and Neal, 1968), glycine (Neal and Pickles, 1969) and 5-hydroxytryptamine (Chase, Breese, Carpenter, Schanberg and Kopin, 1968). Once accumulated, these substances are available for release by nerve stimulation (Hertting and Axelrod, 1961; Gillespie and Kirpekar, 1965; Baldessarini and Kopin, 1967; Chase et al., 1968; Mitchell and Srinivasan, 1969; Srinivasan, Neal and Mitchell, 1969) or by high K^+ (Baldessarini and Kopin, 1967; Chase, Breese and Kopin, 1967; Machiyama, Balázs and Richter, 1967; Srinivasan et al., 1969). These findings suggest that the uptake and re-use of released transmitter may represent an important mode of termination of action of transmitter at many non-cholinergic synapses. The present experiments tested uptake and re-use of transmitter ACh at the cholinergic synapse in the superior cervical ganglion of the cat.

A previous study by Collier and MacIntosh (1969) showed that ACh uptake by the superior cervical ganglion exposed to labelled ACh represents only a few percent of the ganglion's endogenous store of ACh. These results were interpreted as evidence against the recapture of released transmitter by preganglionic fibres of the ganglion. Perry (1953) had earlier reached similar conclusions from less direct experiments. The conclusions of Perry (1953) and of Collier and MacIntosh (1969) are not unequivocal since eserine was used to protect the ACh from hydrolysis. ACh uptake by brain slices

in the presence of this anti-ChE is inhibited (Polak and Meeuws, 1966; Schuberth and Sundwall, 1967; Liang and Quastel, 1969b; Polak, 1969); ACh uptake by brain can be demonstrated only when an organophosphorus anti-ChE agent is used (Polak and Meeuws, 1966; Schuberth and Sundwall, 1967; Heilbronn, 1969; Liang and Quastel, 1969a; Polak, 1969). The present experiments therefore used DFP as the anti-ChE because DFP does not inhibit ACh uptake by brain (Polak, 1969). A second problem in the experiments of Collier and MacIntosh (1969) was that the ACh used in the uptake studies was labelled on the choline moiety and any slight breakdown of the labelled transmitter during perfusion would result in radioactive free choline, which could then be taken up by the ganglion and acetylated to labelled ACh. This artifact was avoided in the present experiments by using acetyl-1-^{14}C-choline; breakdown of the ACh would yield ^{14}C-acetate, which as shown by the present experiments, was not a substrate for ACh synthesis under the conditions of the experiments that measured ACh accumulation.

Although the present experiments demonstrated that ACh was accumulated by ganglia, only 14 ng of ACh was taken up and this represents <6% of a ganglion's ACh content. This accumulation of ACh by ganglia can only be demonstrated by the use of radioactive ACh, for a 6% change in ACh content cannot be detected with certainty by bio-assay methods. The exogenous ACh that accumulated in ganglia in the present experiments appeared not to mix with releasable transmitter stores for ganglia whose transmitter turnover was accelerated by constant preganglionic nerve stimulation accumulated no more ^{14}C-ACh than did ganglia whose preganglionic nerves were not stimulated. In these experiments, stimulation was at 20 Hz for 90 minutes and this would

result in an accelerated turnover of ACh compared to ganglia which were perfused in the absence of nerve stimulation. Furthermore, because these ganglia were perfused with a medium that was choline deficient, the ganglia that were stimulated were partially depleted of their total ACh content. The ganglia that were not stimulated did not lose ACh, but synthesized and stored some surplus ACh; the finding that exogenous ACh accumulation was similar in the stimulated and the non-stimulated preparations shows that ACh cannot substitute for precursor choline to maintain ACh stores. There was, however, some synthesis of ACh in the stimulated ganglia, and it is clear from the work of others that some ACh synthesis in response to nerve stimulation can occur in the absence of added choline (Brown and Feldberg, 1936b; Kahlson and MacIntosh, 1939; Perry, 1953; Birks and MacIntosh, 1961; Matthews, 1963), although the source of this choline for ACh synthesis is unclear. In the stimulated ganglia, it is likely that the ganglia's endogenous supply of choline provided some of the choline for synthesis of releasable ACh. For optimal synthesis, an extracellular source of choline must be provided. However, in the ganglia that were not stimulated, the endogenous supply of choline probably provided the source of choline for surplus ACh synthesis. The amount of surplus ACh accumulated in the non-stimulated ganglia in the absence of extracellular choline is much less than the amount of surplus ACh which can accumulate in preparations provided with a source of choline.

The present experiments demonstrated that the exogenous ACh which accumulated in ganglia was not available for release by nerve stimulation or by high K^+, although in all experiments that tested the release of

accumulated ACh, stimulation released unlabelled ACh. Similar results for brain have been reported. In vitro, labelled ACh that accumulates in brain slices is not available for release by K^+ (Katz et al., in preparation); labelled ACh accumulated by brain in situ is not available for release by direct cortical stimulation (Salehmoghaddam, Collier and Harfield, 1970). The lack of release of accumulated ACh in all these experiments suggests that re-use of exogenous ACh is not significant in ganglia or in brain.

Raised K^+ and exogenous ACh can release surplus ACh from ganglia (Collier and Katz, 1970; Katz, 1970; Collier and Katz, 1971); this surplus ACh is not released by nerve impulses and cannot mix with releasable ACh (Katz, 1970; Collier and Katz, 1971). The absence of extra radioactivity in the effluent of ganglia which had accumulated labelled ACh and were then exposed to high K^+ medium or to transient perfusion with unlabelled ACh in the present experiments, suggests that the accumulated ACh is not stored in the compartment in which surplus ACh accumulates.

Previous studies of the localization of AChE in the superior cervival ganglion of the cat have demonstrated the presence of the enzyme on and in preganglionic fibres (Koelle, 1951; Koelle, 1957; Koelle and Koelle, 1959). This would make the recapture of neuronally released ACh unlikely, unless special mechanisms existed to circumvent ACh hydrolysis. Koelle (1971) has recently reported, however, that AChE is located postganglionically, and if this is so, ACh recapture could be more easily accomplished. The present findings that labelled ACh is not entering the preganglionic surplus or depot ACh pools make it unlikely that ACh can be recaptured by pregang-

lionic fibres even when AChE is inhibited. ACh re-uptake is likely not of physiological importance and thus cholinergic synapses appear to be different from most non-cholinergic synapses (see Iversen, 1967, 1971).

B. Acetylcholine Uptake and Surplus ACh Release

Surplus ACh accumulates in ganglia exposed to an anti-ChE agent and provided with a source of choline, and is thought to be cytoplasmic (Birks and MacIntosh, 1961); it is available for release by ACh injected close to the ganglion or by ACh perfused through the ganglion (Collier and Katz, 1970; Katz, 1970). Since surplus ACh does not accumulate in the absence of an anti-ChE (Birks and MacIntosh, 1961), its presence and possible release in ganglia may be of importance only in the presence of an anti-ChE. However, since part of the bound ACh in brain is thought to exist free in the cytoplasm, and may correspond to surplus ACh in ganglia, its release by exogenous ACh could be of importance. Katz (1970) argued that the release of ACh by ACh in ganglia could be by exchange; this has also been suggested as the mode of release of ACh from ganglia by carbachol (McKinstry and Koelle, 1967). The argument made earlier that the uptake of exogenous ACh in the present experiments was not into the surplus ACh compartment of ganglia is not consistent with this idea, but it might be argued that exogenous ACh can accumulate into a surplus pool only if surplus ACh is present; the accumulation of the full complement of surplus ACh was avoided in these experiments (discussed in Section A). Therefore the experiments which directly compared ACh uptake in the presence and in the absence of surplus ACh were a fairer test of exchange. Those preparations which had accumulated

surplus ACh did not take up greater amounts of labelled ACh, although surplus ACh had presumably been released during exposure to ^{14}C-ACh, and some surplus ACh remained in the ganglion at the end of the experiment. Thus the presence of surplus ACh did not enhance ACh accumulation; and this suggests that ACh uptake is not related to surplus ACh release. Other experiments which directly compared the amount (ng) of exogenous ACh taken up to the amount (ng) of surplus ACh released by ACh confirmed that exogenous ACh does not exchange with surplus ACh; the release of surplus ACh was 35-70 times greater than the uptake of exogenous ACh.

These results leave unanswered the question of how exogenous ACh releases surplus ACh, and suggests the necessity for further experiments on the presynaptic actions of ACh. ACh can depolarize non-myelinated nerves (Armett and Ritchie, 1960) and it can induce antidromic firing at certain nerve terminals (Masland and Wigton, 1940; Riker, Roberts, Standaert and Fujimori, 1957; Randić and Straughan, 1964). The release of surplus ACh by ACh may be the result of presynaptic depolarization, but this seems unlikely because the concentration necessary for demonstrating the release of surplus ACh is about 200 times less that necessary to depolarize presynaptic nerve terminals in frog and rat sympathetic ganglia (Koketsu and Nishi, 1968). The possibility cannot wholly be eliminated that the release of surplus ACh by ACh is secondary to K^+ release as the result of ACh's depolarizing action on the postsynaptic membrane (see Katz, 1962), but the lack of release of depot ACh by exogenous ACh (Collier, Vickerson and Varma, 1969; Collier and Katz, 1970) argues against this.

C. Site of Acetylcholine Accumulation and Termination of Action of Transmitter

The experiments that showed that perfused ACh could not displace ^{3}H-ACh accumulated by ganglia suggest that the labelled ACh was not binding to some extracellular site; ACh stimulated the ganglion, but did not increase the efflux of radioactivity from the ganglion which had previously accumulated labelled ACh. Ganglia which had been perfused with ^{3}H-ACh for 90 minutes and then with ACh-free medium for 50 minutes lost more than 80% of their ^{3}H-ACh. Accumulated ACh is thus not very firmly held by the ganglion. Labelled ACh accumulated by brain slices also diffuses out into the ACh-free medium (Heilbronn, 1970). Hexamethonium, atropine and HC-3 competitively inhibit ACh uptake by brain (Polak and Meeuws, 1966; Schuberth and Sundwall, 1967; Liang and Quastel, 1969b; Polak, 1969), but these drugs were without measurable effect in blocking uptake of ACh in ganglia. This suggests that the uptake of ACh in brain might be by a different mechanism than the uptake of ACh by ganglia and further demonstrates that ACh is not accumulating on nicotinic or muscarinic receptors; the lack of inhibition of ACh uptake by HC-3 further demonstrates that ACh accumulation is not related to the choline uptake mechanism.

The present experiments demonstrated that the amount of exogenous ACh accumulated by ganglia that were chronically (7 days) denervated was similar to the amount accumulated by acutely denervated ganglia. One week after denervation, the superior cervical ganglia had lost approximately 85% of their stored ACh (see also MacIntosh, 1938; Feldberg, 1943; Banister and Scrase, 1950; Friesen et al., 1967); chronically decentralized ganglia can

no longer synthesize depot or surplus ACh (Feldberg, 1943; Banister and Scrase, 1950; Katz, 1970; Collier and Katz, 1971). Since preganglionic nerve terminals form only about 1% of the perfused ganglion's volume (MacIntosh, 1963), the destruction of preganglionic elements would not be expected to result in a dramatic reduction in exogenous ACh uptake if ACh is entering both pre- and postganglionic fibres non-selectively. It can be argued, therefore, that there is no preferential uptake of ACh into presynaptic fibres; ACh may be accumulating to a significant extent in adrenergic cell bodies or glial cells. Recent experiments by Henn and Hamberger (1971) have shown that glial cells can accumulate and concentrate noradrenaline, serotonin, dopamine and GABA. If ACh was accumulating in cell bodies it was not released by antidromic nerve stimulation, or by ganglion stimulating concentrations of ACh or K^+. The non-specific uptake of ACh demonstrated by the present experiments is consistent with studies which showed similar ACh accumulation by end plate and non end plate regions of skeletal muscle (Adamič, 1970; Potter, 1970), as well as Polak's report (1969) that ^{3}H-ACh accumulated by brain was diffusely distributed throughout the cytoplasm of all cells as judged by radioautography. Other experiments have shown that there is no difference in the amount of ACh accumulated by incubated cerebral cortex slices or sliced cerebellum, or by synaptosomes prepared from cerebral cortex or cerebellum (Salehmoghaddam, 1971; Katz et al., in preparation). Cerebral cortex contains five to ten times more ACh (MacIntosh, 1941) and choline acetyltransferase activity (Hebb and Silver, 1956) than does cerebellum, and ACh release measured from cerebral cortex is about ten times greater than ACh release from cerebellum (Mitchell, 1961; Phillis and Chong, 1965). These values suggest that

cerebral cortex contains appreciably more cholinergic nerve endings than does cerebellum.

The non-specific uptake of ACh into cholinergic and non-cholinergic tissue makes the use of labelled ACh a poor experimental tool for studying cholinergic mechanisms. For example, Ritchie and Goldberg (1970) incubated isolated synaptosomes with ^{14}C-ACh or ^{14}C-choline and measured the amount of labelled ACh in the cytoplasmic and vesicular fractions prepared from lysed synaptosomes. Synaptic vesicles contained 15% of the labelled ACh present in the cytoplasm when the synaptosomes were incubated with ^{14}C-choline, but only 1.5% of the labelled ACh present in the cytoplasm after incubation with ^{14}C-ACh was associated with vesicles; these results were interpreted as indicating a separate vesicular and cytoplasmic ACh synthesis. If relatively few cerebral cortical nerve endings are cholinergic (Krnjević and Phillis, 1963; Krnjević and Silver, 1965; Mitchell, 1966), and if incubating synaptosomes with labelled ACh labelled the cytoplasm in all synaptosomes (cholinergic and non-cholinergic), then the conclusions of Ritchie and Goldberg (1970) should be reconsidered.

Termination of action of transmitter ACh is thought to be by hydrolysis of AChE or by diffusion from the synaptic cleft and ultimate destruction by blood ChE. The uptake of ACh by nervous tissue suggests the possibility of this uptake being another mode of termination of action of released ACh. Accumulated ACh would then diffuse slowly out into the surrounding plasma for destruction by blood ChE. If this ACh uptake is of importance in cholinergic synapses, then, in the presence of drugs which block ACh uptake, greater amounts of ACh that have been released from endogenous stores should

be collected from cholinergic tissue. The experimental test of this hypothesis has produced somewhat confusing results. Atropine, which blocks ACh uptake by brain (Polak and Meeuws, 1966; Schuberth and Sundwall, 1967; Liang and Quastel, 1969b; Polak, 1969), increases the release of ACh from brain in vivo (MacIntosh and Oborin, 1953; Mitchell, 1963; Szerb, 1964; Polak, 1965; Collier and Mitchell, 1966) and in vitro (Polak, and Meeuws, 1966; Molenaar and Polak, 1970; Bourdois, Mitchell and Szerb, 1971). Certain investigators have suggested that the increased release of ACh in the presence of atropine may be due to the drug's ability to block ACh uptake sites (Giarman and Pepeu, 1964; Szerb, 1964; Polak, 1965; Celesia and Jasper, 1966), but this now seems to be unlikely (Polak and Meeuws, 1966; Dudar and Szerb, 1969; Molenaar and Polak, 1970; Bourdois et al., 1971; Polak, 1971). The release of ACh from incubated cerebral cortex is not enhanced in the presence of hexamethonium (Katz et al., in preparation), a drug which blocks ACh uptake by brain (Liang and Quastel, 1969b). HC-3 and hexamethonium have been reported to increase the initial output of ACh in the effluent of perfused ganglia (Matthews, 1966), but these drugs did not block ACh uptake by ganglia in the present experiments. The relevance of ACh uptake to termination of action of transmitter remains to be determined and the physiological significance of ACh uptake by ganglia, if any, remains unclear.

D. The Recapture of the Metabolic Products of Acetylcholine

ACh released from the superior cervical ganglion of the cat cannot be detected in the absence of an anti-ChE drug (Brown and Feldberg, 1936b) due

to the hydrolysis of ACh to acetate and choline by AChE. Whether these metabolic products of ACh degradation can be recaptured for ACh synthesis in ganglia was not known with certainty.

a) The recapture of acetate. In the present experiments, surplus ACh accumulated in ganglia that were perfused with ^{14}C-acetate and choline in DFP-Krebs, because total ACh content increased. Although the ganglia accumulated ^{14}C-acetate, little, if any, of the radioactivity was synthesized to ACh. These findings are similar to experiments by others, that tested acetate uptake by brain; acetate was accumulated, but was a poor precursor for ACh synthesis (Cheng, Nakamura and Waelsch, 1967; Browning and Schulman, 1968; Heilbronn, 1970; Nakamura, Cheng and Naruse, 1970; Sollenberg and Sörbo, 1970). Experiments on incubated brain suggest that acetyl groups for ACh synthesis are derived from glucose added to the incubation medium through metabolism via the Embden-Myerhoff pathway. How intramitochondrially synthesized acetyl CoA reaches the site of ACh synthesis in the cytoplasm is unclear, since the mitochondrial membrane is impermeable to acetyl CoA (Lowenstein, 1964; Tuček, 1967).

The experiments of Kahlson and MacIntosh (1939) on the superior cervical ganglion of the cat demonstrated that in the absence of glucose, ACh release is poorly maintained and synthesis is impaired. Although low doses of glucose injected to the ganglion restore transmission, acetate has no such effect. Similar conclusions had earlier been reached by Quastel et al. (1936) who studied ACh metabolism by brain. Quastel et al. (1936) and Kahlson and MacIntosh (1939) concluded that glucose is essential for ACh synthesis; this implies that since acetate cannot improve synthesis in the

absence of glucose, acetate cannot serve as a source of active acetate for ACh synthesis. These conclusions must, however, be accepted with caution. The impairment of ACh release in the experiments of Kahlson and MacIntosh (1939) may have been partly due to conduction failure in presynaptic fibres in the absence of glucose (Larrabee and Bronk, 1952; Krnjević and Miledi, 1958; Larrabee, 1958; Larrabee and Klingman, 1962). However, the depletion of ACh in these experiments in the absence of glucose (and choline) was the same as the depletion of ACh when glucose was present (but not choline, see Birks and MacIntosh, 1961). The low glucose Krebs solution was not employed in the present experiments to examine whether exogenous acetate can effectively compete with glucose as the source of acetyl groups, but rather to examine if, in the presence of low, but adequate levels of glucose to maintain synthesis and release of ACh, the uptake of acetate and its synthesis to ACh was enhanced. In low glucose medium containing ^{14}C-acetate, choline and DFP, the release of ACh from the ganglion during nerve stimulation was maintained and total ACh content increased due to the formation of surplus ACh. This is in contrast to the findings of Kahlson and MacIntosh (1939) which showed that ACh release is not well maintained and total ACh content is depleted in stimulated ganglia perfused in the absence of glucose. Thus adequate metabolism was well maintained in the present experiments which used low glucose-Krebs. Even with the maintained release of ACh and the concomitant formation of surplus ACh in the present experiments, exogenous acetate did not serve as a source of acetyl groups. These findings therefore suggest that the recapture of

acetate from neuronally released ACh is unlikely to be important for ACh synthesis. The acetyl moiety for ACh synthesis must be derived from the metabolism of precursors.

b) The recapture of choline. In the absence of choline added to the perfusion medium, ACh synthesis and release is maintained, but at a low level, although more ACh is released than initially contained in the ganglion (Brown and Feldberg, 1936b; Kahlson and MacIntosh, 1939; Perry, 1953; Birks and MacIntosh, 1961; Matthews, 1963). Some of the endogenous choline used in the absence of added choline may be coming from the ganglion's preganglionic store of choline, and some from phospholipid-choline. Brain tissue can also synthesize ACh in the absence of added choline (Quastel et al., 1936) and in this tissue the release of bound choline for ACh synthesis seems to be important (Browning, 1971; Collier et al., 1972).

Superior cervical ganglia accumulate labelled choline from a perfusion medium and readily incorporate this choline into ACh (Friesen, Kemp and Woodbury, 1965; Collier, 1969; Collier and Lang, 1969; Collier and MacIntosh, 1969; Collier and Katz, 1970; Katz, 1970; Collier and Katz, 1971). In addition to the supply of choline made available to ganglia from the circulating plasma, Perry (1953) and later Collier and MacIntosh (1969) suggested that choline obtained from the hydrolysis of neuronally released ACh can be recaptured for ACh synthesis. Their experiments showed that the release of ACh was greater from test ganglia perfused in the presence of eserine than was the release of choline from ganglia perfused in the absence of eserine when stimulation was for at least 10 minutes. Emmelin and MacIntosh (1956) demonstrated that the superior cervical ganglion stimulated

for only a short period of time releases as much choline when eserine is absent, as ACh when eserine is present. This might be interpreted as evidence against the recapture and re-use of choline, but these results are difficult to interpret.

The present experiments overcame some of the problems associated with the work of Perry (1953) and of Collier and MacIntosh (1969) by demonstrating that the greater release of ACh in the presence of an anti-ChE agent (as compared to the release of choline in an anti-ChE-free medium) is not due to a direct effect of the drug on release and by further demonstrating more clearly that recaptured choline is synthesized into ACh. In the present experiments, the release of radioactivity from ganglia whose depot ACh had been labelled was consistently less in preparations stimulated during perfusion with drug-free Krebs solution (i.e., no neostigmine or HC-3) than it was during perfusion with Krebs solution containing neostigmine or HC-3; neostigmine would block choline recapture by preventing the hydrolysis of neuronally released ACh and HC-3 would block the recapture of choline by competing with choline for carrier uptake sites. The difference in the amount of labelled material released in these experiments was balanced by extra radioactivity appearing on ACh in preparations stimulated during perfusion with drug-free medium, in which condition the recapture of choline was possible. Clearly the choline that was recaptured entered preganglionic fibres and was used for ACh synthesis. These experiments used neostigmine as the anti-ChE in order to prevent surplus ACh from forming in ganglia during the 24-minute exposure to this agent while protecting released ACh from hydrolysis (Katz, 1970; Collier and Katz, 1971).

In the experiments by Collier and MacIntosh (1969), eserine was used, but the release of ACh into eserine or neostigmine is the same (Emmelin and MacIntosh, 1956; Katz, 1970; Collier and Katz, 1971). The release of radioactivity and the amount of radioactive ACh remaining in ganglia at the end of the experiment was similar in ganglia perfused with medium containing neostigmine and in ganglia perfused with medium containing HC-3. This suggests that the greater release of radioactivity in the presence of neostigmine is not due to a direct effect of neostigmine on ACh release. Although the release of ACh in the presence of HC-3 may be slightly greater during the first few minutes of nerve stimulation (Matthews, 1966; but see MacIntosh et al., 1956 and Birks and MacIntosh, 1961), the release of preformed ACh is not affected by HC-3 during longer periods of stimulation (Collier and MacIntosh, 1969).

That labelled choline was recaptured for ACh synthesis was also shown by comparing the specific activity of labelled ACh in the ganglion at the start of nerve stimulation and at the end of the experiment. The specific activity of labelled ACh in ganglia did not change when perfusion was with HC-3-Krebs. When neostigmine-choline-Krebs was used, the specific activity fell by 45% due to the synthesis of unlabelled ACh from the choline added to the perfusion medium, but when perfusion was with choline-Krebs, the specific activity fell by only 25% and thus in this condition newly synthesized ACh must have come, in part, from choline made available from the released labelled ACh.

In adrenergic synapses, the recapture of released noradrenaline is probably important in the termination of action of transmitter and in its

re-use. However, in preparations stimulated at high frequencies (e.g. 20-30 Hz), the overflow of released transmitter away from the synaptic area is considerably greater than when stimulation is at low rates (e.g. 5-10 Hz; Brown and Gillespie, 1957; Brown, 1965; Hughes, 1970; Kirpekar and Wakade, 1970; Langer, 1970). This may indicate that at a high rate of stimulation the relative importance of recapture of released transmitter by presynaptic fibres is diminished (Häggendal and Malmfors, 1969), although the reason for this is not clear. If the same were true for the recapture of choline by the superior cervical ganglion, a greater percentage recapture of choline from released ACh might be expected at lower rates of stimulation, when less ACh is released, than at higher rates of stimulation, when more ACh is released. The present experiments measured the recapture of choline from ACh in ganglia stimulated at 5 Hz; the earlier experiments of Collier and MacIntosh (1969) used unphysiologically high rates of stimulation (20 Hz). Both the experiments of Collier and MacIntosh (1969) and the present experiments, however, showed that approximately 50% of the choline is recaptured. This would suggest that, at least in the superior cervical ganglion, a constant fraction of the choline from ACh is recaptured at both low and high frequencies of stimulation.

Collier and MacIntosh (1969) argued that since ganglia that were perfused with medium containing added choline in a concentration of 10^{-5} M recaptured 50% of the choline from released ACh, then the concentration of choline produced from the released ACh must also have been about 10^{-5} M. This would suggest that the two "pools" of choline mixed, and that the ganglion sampled choline from a homogeneous "pool". The concentration of

choline produced from released ACh in the present experiments which stimulated ganglia at 5 Hz must have been less than the concentration of choline from ACh in the experiments of Collier and MacIntosh (1969) where stimulation was at 20 Hz, yet an equal percentage of the choline appears to have been recaptured in both sets of experiments. This may indicate that the "pools" of choline do not mix, but rather that a steady fraction of choline produced by ACh hydrolysis is taken up; this choline may be the most important source of precursor for ACh synthesis and choline recapture may be linked to transmitter release. However, since ganglionic ACh content is not depleted when ganglia are stimulated in the presence of both an anti-ChE agent (and therefore no recapture of choline) and added choline (Birks and MacIntosh, 1961), it would seem that in the absence of the recapture of choline, the ganglion can readily make use of the added choline.

The present experiments indicate that recapture of choline is efficient throughout the period of stimulation since the release of choline in the absence of an uptake blocking drug (direct or indirect) was always less than the release of ACh in the presence of the drug. This was not expected, since earlier work by others (Perry, 1953; Emmelin and MacIntosh, 1956) showed that in the first few minutes of stimulation, the amount of choline released in the absence of an anti-ChE was about the same as the amount of ACh released in the presence of eserine. The reason for this discrepancy between the present results and those of others is unclear.

The recapture of choline from released ACh may be important at many cholinergic synapses. The present experiments, like those of Collier and

MacIntosh (1969), show that the superior cervical ganglion of the cat recaptures about 50% of this choline. Using electrophysiological procedures, Bennett and McLachlan (1972b) have recently shown that recaptured choline is important for ACh synthesis in the guinea-pig ganglion. Potter (1970) demonstrated that the labelling of endogenous ACh from exogenous ^{14}C-choline in the stimulated rat phrenic nerve-diaphragm preparation is twice as fast when neostigmine is present (and choline from ACh not available) than when neostigmine is absent; he concluded that about half of the choline from hydrolyzed ACh is recaptured by the phrenic nerve endings.

V. SUMMARY

1. The recapture of ACh and its metabolic products, acetate and choline, was studied in the superior cervical ganglion of the cat using radiotracer techniques and standard procedures of ganglion perfusion.

2. The ratio of ^{3}H:^{14}C in ganglia perfused with acetyl-1-^{14}C-choline, DFP and ^{3}H-inulin was 3.2 times smaller than the isotope ratio in the perfusion medium. This suggests that more ACh was accumulated than can be accounted for by extracellular trapping. However, the amount of ^{14}C-ACh accumulated was <6% of the ganglion's endogenous ACh.

3. Accumulated labelled ACh was not displaced by unlabelled ACh, and accumulation of exogenous ACh was not blocked by atropine, hexamethonium or HC-3. These results suggest that accumulated ACh was not bound to nicotinic or muscarinic receptors, and that uptake of ACh is not related to the choline uptake mechanism. Eighty percent of the labelled ACh that had been accumulated was lost by spontaneous efflux within 50 minutes of perfusion with ACh-free medium.

4. There was no significant difference in the amount of ^{14}C-ACh accumulated by ganglia perfused at rest or during nerve stimulation. In these experiments, choline had been deliberately omitted from the perfusion medium and the stimulated ganglia therefore lost 29% of their endogenous ACh. This suggests that exogenous ACh cannot substitute for ACh synthesis from precursor choline to maintain ACh stores; recapture of neuronally released ACh by preganglionic fibres is therefore unlikely.

5. Nerve stimulation and transient exposure to a high K^+ (46 mM) medium did not release accumulated exogenous ACh, but did release unlabelled ACh. These results (and the failure of ACh to release accumulated ACh) suggest that the ACh taken up by ganglia did not mix with releasable or surplus ACh stores. This was confirmed by experiments which showed equal accumulation of ^{14}C-ACh by acutely (30 minutes) and chronically (7 days) decentralized ganglia.

6. The amount of labelled ACh accumulated was the same in preparations which had or had not previously accumulated surplus ACh. Double-isotope experiments showed that exogenous ACh released surplus ACh but the amount released was 35-70 times the amount of ACh taken up. These results suggest that the release of surplus ACh by ACh is not by exchange.

7. Ganglia perfused at rest with ^{14}C-acetate accumulated radioactivity, but little, if any, radioactivity was as ^{14}C-ACh. Nerve stimulation increased transmitter turnover, but did not enhance the labelling of ACh from ^{14}C-acetate. These results suggest that the recapture and re-use of acetate from hydrolyzed released ACh is unlikely.

8. In paired experiments, the depot ACh pool of both ganglia of the cat was labelled by perfusing with ^{3}H-choline-Krebs during nerve stimulation; the release of radioactivity by nerve stimulation was then measured. Release of radioactivity from ganglia perfused with choline-Krebs was 37% of the release from ganglia perfused with neostigmine-choline-Krebs and 47% of the release from ganglia perfused with HC-3-Krebs. The difference between release of radioactivity in these experiments was balanced by extra radio-

active ACh in ganglia perfused with choline-Krebs. There was no difference in release of radioactivity nor in radioactive ACh in ganglia when one ganglion was perfused with neostigmine-choline-Krebs and the other was perfused with HC-3-Krebs. The specific activity of labelled ACh in ganglia perfused during nerve stimulation with HC-3-Krebs did not change; in ganglia perfused with neostigmine-choline-Krebs the specific activity at the end of the experiment fell to 55% of its specific activity at the start of nerve stimulation but in ganglia perfused with choline-Krebs, the specific activity only fell to 78%. These results suggest that approximately 50% of the choline made available from ACh is recaptured for ACh synthesis.

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