CHOLINE UTILIZATION AT PERIPHERAL NERVE TERMINALS

by

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T. B. Tjandramaga, M.D.

ABSTRACT

Choline (Ch) uptake by sciatic nerves of the frog has been shown to conform with a two component mechanism of uptake found earlier for the giant axons of loligo.

At low concentration of Ch (10^{-5} M) there is evidence indicating uptake against a concentration gradient. The finding that Ch uptake is inhibited under anaerobic condition further suggests that the accumulation of Ch is an energy requiring process. Hemicholinium No. 3 (HC-3) showed a significant inhibitory effect on the Ch influx, which appears to be of the competitive type. This first component of uptake is compatible with a 'carrier' mediated process.

At greater concentrations (10^{-4} - 10^{-3} M), Ch enters the sciatic nerves at an apparent rate proportional to the concentration of Ch in the external medium, suggesting passive diffusion to be the second component of uptake.

Repetitive electrical stimulation using different frequencies (1, 5, 20 and 40 pulses/sec) has no obvious effect on the uptake of Ch.

No important migration of labelled Ch towards the axon terminals was found during a 24 hour incubation period under continuous stimulation (2/sec).

The dynamics of transmitter release at the neuromuscular junctions have been studied with the use of two different radioactive

tracers (^{14}C-Ch and ^{3}H-Ch) as precursors for acetylcholine (ACh) synthesis. The two tracers were loaded sequentially to replace the endogenous ACh stores. Each loading was followed by a washout procedure to remove the fraction of radioactive material not retained by the tissue. On the assumption that the retained tracer molecules have been transformed into labelled ACh carrying two different labels, studies were carried out to determine the release patterns of both tracers under different experimental conditions.

It was found that the newly introduced tracer is released in preference to the previously introduced one, providing evidence that newly synthesized transmitter is released preferentially. The preferential release of the second tracer cannot be demonstrated when the second tracer is introduced into the tissue in the presence of HC-3. From the release data the existence of a 'readily releasable' pool and a 'storage' pool of the transmitter is readily apparent. The existence of a surplus pool is suggested by experiments in which the second loading was carried out under resting conditions and in the presence of cholinesterase (neostigmine or eserine). ACh and electrical stimulation (40/sec) release the transmitter pool. Carbachol is more effective than ACh in releasing the surplus pool. Selective labelling of the surplus pool by loading under resting conditions was not successful. There is evidence of significant exchange and mixing of the newly synthesized transmitter with the transmitter pools even under resting conditions. KCl,in the presence of neostigmine, produced a release pattern similar to that produced by electrical stimulation. However, if eserine was the anticholinesterase used, the pattern of release with KCl was reversed.

The present studies in the neuromuscular junction of the frog provide some experimental evidence indicating that the overall model for transmitter dynamics known for the ganglion (Birks and MacIntosh, 1961) is applicable to the neuromuscular junction.

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SECTION I

INTRODUCTION

A. THE NEUROMUSCULAR JUNCTION: STRUCTURAL FEATURES

The term neuromuscular junction is used to describe the localized synaptic zone that relates motor nerve terminals to skeletal muscle fibres.

Classical light-microscopical studies reveal that the motor axon, quite close to the branching of the terminal, loses its myelin sheath and expands to make a series of discrete contacts with a specialized region of the muscle fibre, the motor end-plate. This is shown diagrammatically in Fig. 1A (Couteaux, 1958).

There have been several thorough electron-microscopic studies of neuromuscular junctions of amphibia, reptiles and mammals (Palade and Palay, 1954; Robertson, 1956, 1960; Reger, 1958; Anderson-Cedergren, 1959; Birks, Huxley and Katz, 1960), and it is now well established that all have the common structural features that are required for physiological explanations of chemical transmission at the neuromuscular synapses (Fig. 1B). The structural design of the neuromuscular junction is very efficient for the operation of a chemically transmitting synapse (see review by Eccles, 1964). Firstly, there is a synaptic cleft (of about 500 Å) width completely separating the surface membranes of the nerve terminal and the motor end-plate. Secondly, there are numerous vesicles (about 500 Å in diameter) in the motor ending, and they tend to collect in groups at some regions of the presynaptic membrane, particularly in proximity to the junctional folds. These synaptic vesicles are thought to be the storage sites of the transmitter (De Robertis and Bennett, 1955; Robertson, 1956). Mitochondria are concentrated within the nerve ending.

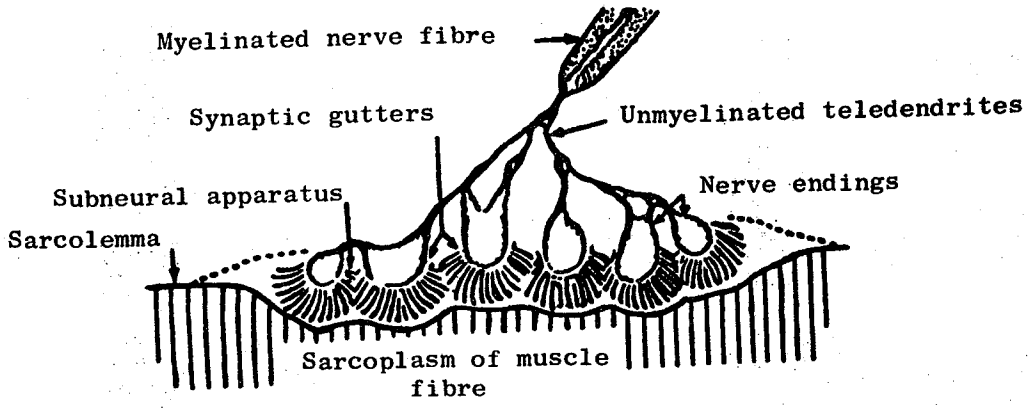


Fig. 1A. The light microscopic appearance of the neuromuscular junction - depicted diagrammatically.

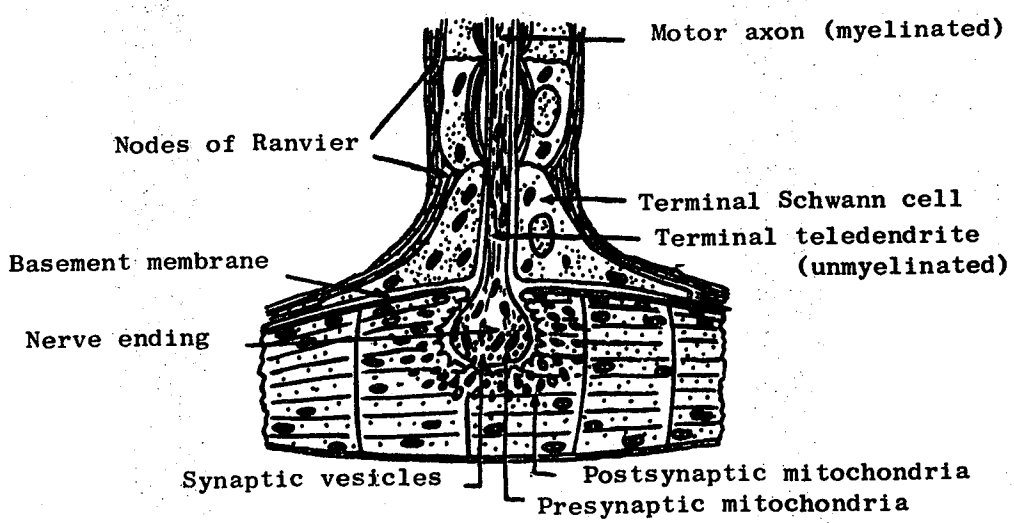


Fig. 1B. The principal structural features of the neuromuscular junction as revealed by electron microscopy.

B. CHEMICAL TRANSMISSION ACROSS THE NEUROMUSCULAR JUNCTION

Du Bois-Raymond (1877) in discussing the possible mechanisms of transmission from nerve to muscle at the neuromuscular junction suggested that it could be either electrical or chemical. In this way he initiated a controversy that would last for 60 years. Elliot (1904a, 1904b), who was impressed by the close correspondence between the effects of adrenaline on various organs and those produced by stimulation of their sympathetic nerve supply, suggested that sympathetic nerve impulses liberate adrenaline at the junctional regions of the smooth muscle fibres. Dixon (1906) suggested that vagal stimulation releases a muscarine-like substance from the dog's heart.

Dale (1914) noted that the actions of parasympathetic stimulation were mimicked very faithfully by acetylcholine (ACh). He also was able to differentiate between the nicotinic and muscarinic actions of ACh. The former action first stimulates then paralyzes ganglia, and is blocked by curare, whereas the muscarinic action is abolished by a small dose of atropine and unaffected by curare.

The experimental proof of the existence of chemical transmission was provided by the classical experiments of Loewi (1921). He showed that the fluid collected from a beating isolated frog's heart during vagal stimulation inhibited a second isolated frog's heart. A substance called the "Vagusstoff", was apparently liberated at the vagal nerve endings in the first heart. Since this substance had the same effect on the second heart as vagal stimulation, it could be concluded that the nerve acted on the heart by liberating this substance at its endings. As a result of the earlier pharmacological studies of Dale, the Vagusstoff

was identified as acetylcholine.

In the early 1930's a series of classical investigations (Feldberg and Gaddum, 1934; Feldberg and Vartianen, 1934; Brown, Dale and Feldberg, 1936; Dale et al., 1936) established that ACh was liberated at a wide variety of mammalian peripheral synapses. The release of ACh was demonstrated in the autonomic nervous system at all preganglionic endings, and at the terminals of postganglionic parasympathetic fibres (review by Hebb, 1963). An important aid to the experiments on the preganglionic sympathetic endings was the method described by Kibjakow (1933) for the perfusion of the superior cervical ganglion in situ in isolation from the rest of the body. It was thus possible to collect in a relatively small volume of solution any substance liberated from the presynaptic nerve endings. In 1933 a new terminology was suggested by Dale to describe nerve fibres in the autonomic nervous system. He proposed that fibres which act by the liberation of ACh should be called cholinergic and those which act by the liberation of the adrenaline-like substance ("sympathin") adrenergic.

The concept of chemical transmission across the neuromuscular junction was given a secure foundation in 1936 when the release of transmitter (ACh) at the neuromuscular junction was first demonstrated convincingly by Dale and his colleagues (Dale et al., 1936). Their evidence showed that ACh was released by stimulation of the motor nerve in amounts corresponding to those to be expected from previous experiments on ganglia; that ACh mimicked the effect of an asynchronous discharge of the motor nerve; and that certain pharmacological agents, in particular curare, modified the response to motor nerve stimulation in the same way

as they modified the action of ACh. Following this, the term cholinergic was also applied to the somatic motor fibres. The entire period of the development of the concept of chemical transmission and the identification of ACh as a transmitter compound was reviewed by Dale (1938).

C. IDENTIFICATION OF ACETYLCHOLINE AS THE CHOLINERGIC NERVE TRANSMITTER

Among a large series of choline esters prepared by Hunt and Traveau (1906), acetylcholine was later shown to be the most active biologically, on an average about 1000 times more active than choline (Dale, 1914). Dixon and Hamill (1909) applying to parasympathetic nerves the idea, previously suggested by Elliot (1904), of chemical transmission of nerve impulses, claimed to have shown that vagal stimulation liberated a muscarine-like substance from the dog's heart. However, this concept was not pursued further at the time.

During studies on ergot extracts, Dale found a substance which produced actions similar to those produced by muscarine and identified this substance as acetylcholine. He also found it to have on many organs actions which closely corresponded to those produced by the stimulation of their parasympathetic supply. Furthermore, in his publication (1914) he also made the important suggestion that the brevity of its action might be due to its destruction by a tissue esterase. Following Loewi's experiments (1921), the evidence that the cholinergic nerve transmitter might be ACh became more convincing, especially once its presence in animal tissues was definitively established by Dale and Dudley (1929). Using chemical methods these authors demonstrated its presence in the bovine and equine spleen. However, the amounts of ACh present in tissues were found to be so small that they were below the limits of accurate

chemical detection. In order to obtain quantitative information from small samples of tissue, or tissue fluids, another method of identification was necessary.

It became thus necessary to develop bioassay methods sensitive enough to measure the small amounts of acetylcholine present in biological samples.

Bioassay

Probably the first bioassay method available was based on the observation of Führer (1917) that the eserinized leech muscle is extremely sensitive to ACh, approximately one million times more than the uneserinized leech muscle. This method was used for the assay of ACh by Minz (1932) and later by Dale. In 1933 Chang and Gaddum published a paper on the properties of a number of preparations which are specially sensitive to ACh and showed how they can be used in different combinations to distinguish ACh from other bioactive substances, including other choline esters. There are numerous bioassay methods available, in between the ones most widely used we should mention: the negative inotropic action of acetylcholine on the heart of the frog, the hypotensive effect in the cat, and the contracting effect on the intestine of the guinea pig, or the rectus abdominis muscle of the frog. A detailed review of these bioassay procedures for identification of ACh has been published by Whittaker (1963).

In order to conclude from these bioassays that the actions observed are due to ACh, certain other conditions must be fulfilled. The action of the unknown must be potentiated by the drugs that inhibit the enzyme which hydrolyzes ACh, the unknown must be inactive after a 10 min

exposure to 1N alkali and, of course, it must display the same properties of ACh.

D. EARLY EXPERIMENTS DEMONSTRATING ACETYLCHOLINE SYNTHESIS

ACh synthesis in the ganglion was first demonstrated by Brown and Feldberg (1936, see also review by Feldberg, 1945). They found that, after a period of prolonged stimulation, the amount of ACh released by the preganglionic nerve of a perfused sympathetic ganglion was greater than the total amount that it originally contained. This can only be explained if the supply of preformed ACh had been supplemented as the stimulation progressed, by the synthesis of new ACh.

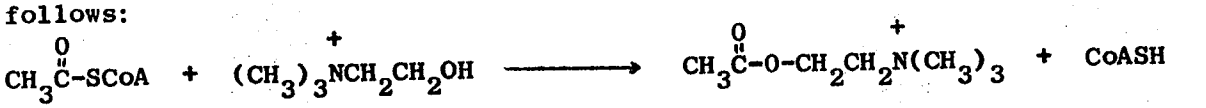
The first in vitro demonstration of ACh formation by nervous tissue, however, was obtained by Mann et al. (1938, 1939) on respiring slices of mammalian brain. They used a bioassay to measure the amounts of ACh formed. The amounts of ACh synthesized by the slices were small with a maximum yield of 50 ug/g tissue/hr (Mann et al., 1938, 1939). By these experiments, it was shown that isolated nervous tissue is capable of synthetizing ACh.

The next significant step was made by Stedman and Stedman (1937, 1939) who demonstrated the production of ACh by ground brain tissues suspended in chloroform-saline or ether-saline, thus showing that the synthetic mechanism survives disruption of the cell.

At this stage little was known about the conditions necessary to activate the synthetic mechanism, or what are its energy or substrate requirements. Evidence pertaining to these questions was provided by Nachmansohn and Machado (1943) who showed that,in the presence of choline, extracts of brain and nervous tissue will synthesize ACh. The synthesis

will continue even under anaerobic conditions and in the absence of glucose, as long as long as adenosine triphosphate (ATP) is supplied, which was found to accelerate greatly ACh synthesis. The enzymatic system was termed choline acetylase by Nachmansohn and Machado (1943). Further observations by these authors suggested that the enzyme concerned in the synthesis contains -SH groups. This was later confirmed by Reisberg (1954) who showed that choline acetylase activity is depressed by sulfhydryl inhibitors, such as p-chloromercuribenzoate and iodobenzoate, that react with thiol groups.

Feldberg and Mann (1946) reported that a heat stable, dialyzable substance, normally present in extracts prepared from acetone dried tissue, is required for optimal synthesis of ACh. Similar observations were made independently by Lipton (1946), Lipmann and Kaplan (1946) and Nachmansohn and Berman (1946). This substance was called the coenzyme of acetylation or coenzyme A (Lipmann, 1954). It is of general importance in biological acetylations and its constitution is now well established (Vovelli et al., 1951; Snell et al., 1950; Lynen and Reichert, 1951). Acetyl-CoA is essential for the formation of acetylcholine, and together with choline it is the substrate of choline acetylase, the enzyme that controls the rate of synthesis of ACh (Korkes et al., 1952). The reaction is as follows:



Acetyl coenzyme A — Choline — Acetylcholine — Coenzyme A

Acetyl-CoA is formed during the oxidative breakdown of fatty acids, amino acids and sugars. Pyruvate is often the immediate precursor of acetyl-CoA,

the reaction involved being as follows:

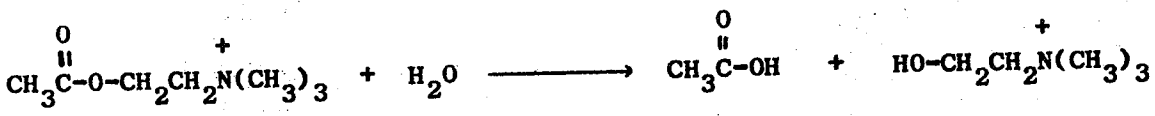
$$\text{Pyruvate} + \text{DPN} + \text{CoA} \longrightarrow \text{Acetyl-CoA} + CO_2 + DPNH_2$$

Thus oxidative metabolism of glucose via pyruvate is a principal source of acetyl-CoA and hence of ACh in the brain. In brain cell extracts, in which pyruvate oxidation does not proceed, acetate is a source of acetyl-CoA so long as ATP, CoA and the necessary activating enzyme (acetyl kinase) is present (Berg, 1956).

E. HYDROLYSIS OF ACh

In 1914 Dale suggested that the rapid disappearance of ACh from blood and tissues is due to the action of an esterase. Further support for this idea was obtained by Führer (1917) who showed that in the presence of eserine ACh becomes far more effective in causing a contraction of leech muscle, while the action of choline, which also stimulates the muscle in a smaller degree, is not affected by eserine. This suggested that the potentiating action of eserine was due to an inhibitory effect on the hydrolysis of ACh. Subsequently, a similar conclusion was reached by Loewi et al. (1930) and Matthes (1930) who independently demonstrated the enzymatic nature of the tissue hydrolysis of ACh. Two years later, in 1932, Stedman et al. (1932) prepared from horse serum an eserine sensitive enzyme which hydrolyzed ACh. They named this enzyme, that they considered to be a specific esterase for acetylcholine, choline esterase (ChE). However, it splits butyrylcholine and proprionyl-choline at a higher rate than acetylcholine. Later, Nachmansohn and Rothenburg (1945) demonstrated that the cholinesterase in nerves, muscle tissue and erythrocytes, in contrast to the serum esterase, has a high affinity for ACh and acetyl-B-methylcholine, but splits butyrylcholine at a very low

rate. Enzymes of this type were called acetylcholinesterases by Augustinsson and Nachmansohn (1949). Following recommendation of the Enzyme Commission in 1964 the name acetylcholinesterase is used to denote the so-called true or specific enzyme which hydrolyzes ACh into choline and acetate according to the following reaction:



Acetylcholine Acetic Acid Choline

The use of histochemical techniques has been a great aid in mapping tissular and cellular distribution of the esterase, and in pinpointing the location of ACh in such tissues as skeletal muscle, where the overall ACh activity is low (Koelle, 1950; Couteaux and Taxi, 1952; Coers, 1953; Gerebtzoff et al., 1954; Couteaux, 1958). The skeletal neuromuscular junction has been intensively studied with respect to both the localization of acetylcholinesterase and also the pharmacological actions of the anticholinesterases. Prior to the development of histochemical methods, Marnay and Nachmansohn (1938) obtained indirect evidence that the acetylcholinesterase of skeletal muscle was concentrated in the terminal areas of motor nerves. This selective localization of acetylcholinesterase at the endplate region has been confirmed and clearly demonstrated by the ultramicroanalytical determinations of Giacobini and Holmstedt (1960). The highest concentration of acetylcholinesterase (which accounts for most of the cholinesterase at neuromuscular junctions (Denz, 1953)) appears postsynaptically, at the surface on the subsynaptic membrane of the neuromuscular junction and at the infoldings of the subneural apparatus. The axonal terminals contain relatively little enzyme

(Couteaux, 1958; Barnett, 1962). The amount of acetylcholinesterase present at the neuromuscular junction was estimated by Marnay and Nachmansohn (1938) to be capable of hydrolyzing some 10^9 molecules of ACh in ine millisecond. The hydrolysis of ACh is also catalyzed by various inorganic and organic agents, although the most important is the acetylcholinesterase of living tissues (see reviews by Davies and Green, 1958; Nachmansohn, 1959).

F. DISTRIBUTION OF ACh AND CHOLINE ACETYLASE

Between 1943 and 1951 Feldberg with various colleagues carried out a number of studies of the distribution of choline acetyl transferase in the mammalian nervous system (Feldberg, 1945; Feldberg and Mann, 1946; Feldberg and Vogt, 1948; Feldberg et al., 1951). A result of first importance which emerged from these studies is that the enzyme could be detected only in those parts of the peripheral and autonomic nervous systems which contained cholinergic neurones. It was absent from sensory or adrenergic nerves. The ability to form and store ACh in the peripheral nervous system appears to be confined to motor nerves innervating skeletal muscles, autonomic preganglionic trunks and parasympathetic postganglionic fibres (Hebb, 1963). The evidence that postganglionic adrenergic nerves do not contain acetylcholine or its synthetizing system has been derived mainly from experiments in which the superior cervical ganglion and the proximal part of its postganglionic fibres have been analyzed after chronic preganglionic denervation (Hebb, 1963). After such a procedure the normally high content of ACh and choline acetyltransferase in autonomic ganglia is drastically reduced, suggesting that most of the enzyme is located presynaptically (Buckley et al., 1967). The reduction of

choline acetyltransferase levels in degenerating somatic nerves occurs at about the same rate as in the superior cervical ganglion (Hebb et al., 1959).

Hebb et al. (1964) suggest that ACh and its synthesizing enzyme are present in large amounts at the motor nerve endings. They found that the end-plate zone of the rat hemidiaphragm contains 90% of the total ACh content of the whole muscle, while the nerve free part contains only 10%. Potter (1970) confirmed the localization in the neuromuscular junction of both choline acetyltransferase and ACh. He also showed that following denervation both enzymes levels were reduced to a small percentage of their normal value. It had previously been noted by Bhatnagar and Mac-Intosh (1960) that some ACh can persist in somatic muscle for long periods of time (up to 60 days). Only recently has it been shown by Miledi and Slater (1968) that the residual enzyme and ACh may be present in the neighbouring Schwann cells.

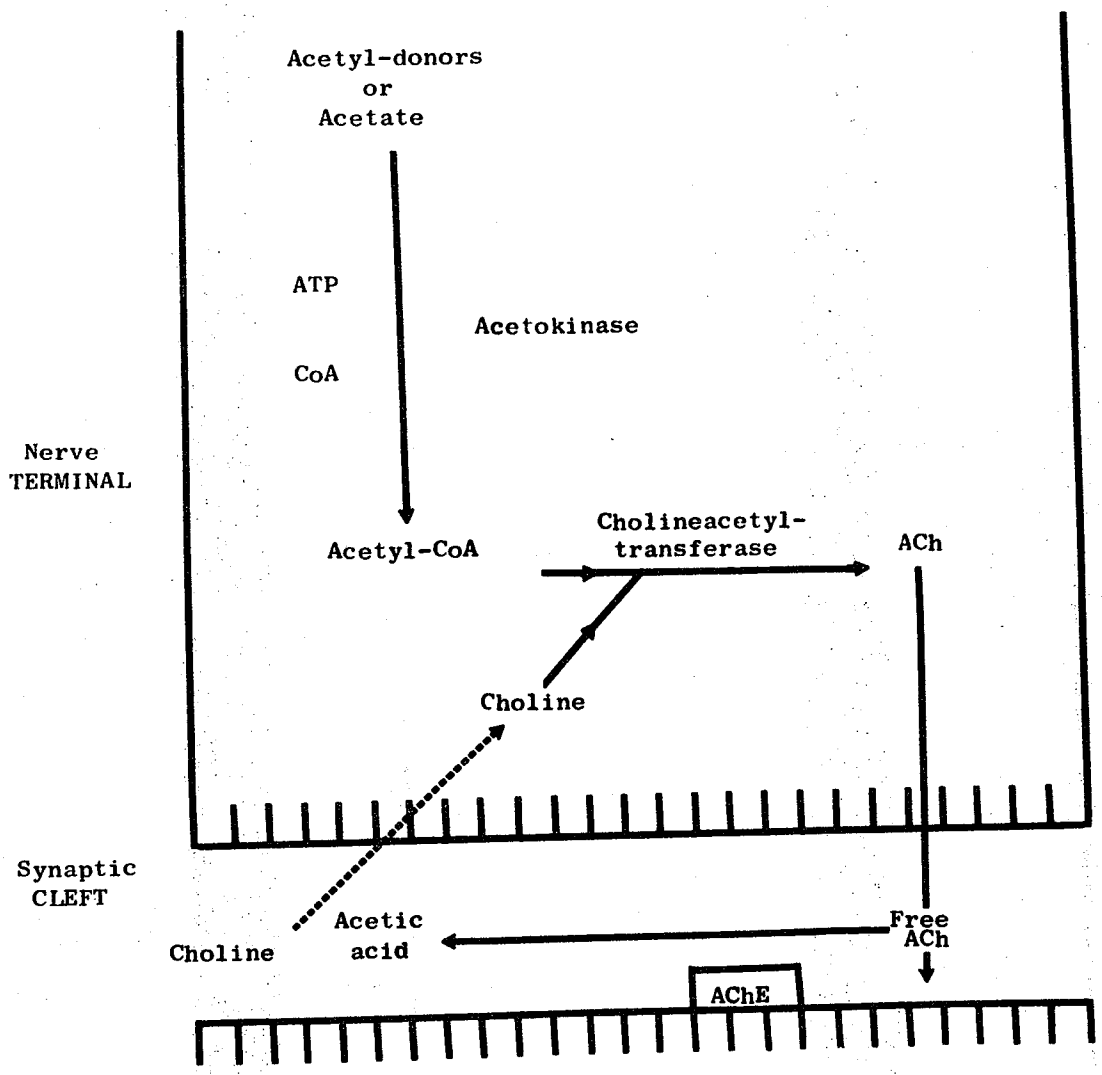


Fig. 2. A schematic summary of the metabolism of ACh at the nerve ending. As indicated, ACh is formed from acetyl-coenzyme A and choline under the influence of the enzyme cholineacetyl-transferase. Free ACh released is broken down to acetic acid and choline by the enzyme acetylcholinesterase, and the choline moiety is reabsorbed. The high energy compound acetyl-CoA is formed from ATP, coenzyme A, and acetate under the influence of the enzyme acetokinase.

G. RELEASE OF ACETYLCHOLINE BY MOTOR NERVE ENDINGS

Early experiments (Hess, 1923; Shimidzu, 1926) had shown that ACh (or an ACh-like substance) was released by nerve muscle preparations during nerve stimulation. These authors did not exclude the possibility that autonomic or sensory nerve fibres, or the muscle itself, were the source of the ACh-like substance. Dale, Feldberg and Vogt (1936) found that after sympathectomy, stimulation of the appropriate ventral roots, induced release of ACh from mammalian tongue and leg muscles perfused with eserinized Tyrode solution. Direct stimulation of the muscle was not effective, except insofar as intramuscular nerve branches were excited. Contraction of the muscle was not important since the release continued after paralysis of the muscle by curare.

An objection was subsequently raised against this study because the release of ACh was demonstrated under 'unphysiological' conditions, i.e. during perfusion with Tyrode which rapidly leads to edema and loss of function (Fleish et al., 1938). However, Emmelin and MacIntosh (1956) have since demonstrated that perfusing with blood or plasma, which maintain the muscle in sound condition, the release of ACh can still be demonstrated if sufficient concentrations of anticholinesterase are used. It has also been shown by these authors that these results are not dependent upon the action of one particular anticholinesterase, and if no anticholinesterase is used, choline, the product of ACh hydrolysis, can be recovered from the effluent in the expected amount.

The mean amount of ACh released per impulse per nerve ending can be calculated, provided one knows the number of nerve endings in the muscle. In the original publications of Dale et al. (1936) and Emmelin

and MacIntosh (1956) no attempt was made to calculate the mean release per nerve ending. Approximate calculations based on these experiments were published later by Dale (1938), Acheson (1948) and MacIntosh (1959). The amount estimated ranged from 0.5 to 7 x 10^{-18} mole. In later experiments with isolated diaphragm preparations (Brooks, 1954; Straughan, 1960; Krnjevic and Mitchell, 1960, 1961) relatively high yields of ACh have been obtained, between 2 and 10 x 10^{-18} mole per nerve ending per impulse. The higher values were associated with stimulation for short periods at low rates (2 to 5/sec). These authors have also detected a spontaneous release of small amounts of ACh in the absence of nerve stimulation.

Soon after Dale et al. established the role of ACh as the chemical transmitter, the electrical events associated with synaptic transmission at the neuromuscular junction were seriously investigated by several electrophysiologists (e.g. Schaefer and Haas, 1939; Eccles and O'Connor, 1939; Feng, 1941). One of their first findings was the existence of a local electrical potential change at the end-plate region. The electrical properties of this end-plate potential (e.p.p.) differ markedly from nerve action potentials. They are non-regenerative localized depolarizations. The end-plate potential can easily be recorded from the innervated zone of a curarized muscle using the microelectrode techniques pioneered by Ling and Gerard (1949). The e.p.p. is the earliest response of the muscle fibre to a nerve impulse. It is a local depolarization which starts about 1 msec after the arrival of the impulse to the axon terminals. Normally it rises quickly above the threshold of the muscle membrane, initiating a new impulse which propagates along the muscle fibre and causes its eventual contraction. The amplitude of the

e.p.p. can be progressively reduced by the application of increasing doses of curare. The so-called 'curare paralysis' occurs when the size of the end-plate potential has been depressed below the threshold of the muscle fibre. Neither the nerve nor the muscle impulse are reduced by a paralysing dose of curare, only the e.p.p. The e.p.p. can be increased in amplitude and very strikingly in duration, by the application of anti-cholinesterase agents, which are known to inhibit specifically the enzymatic hydrolysis of ACh.

The local electrical potential change at the synaptic area of the muscle fibre caused by the iontophoretic application of ACh is affected in the same way as the e.p.p. by curare and by anti-ChE. Based on this evidence Eccles et al. (1942) concluded that the end-plate potential is caused by ACh release from the motor nerve endings (see review by Katz, 1962).

Intracellular recording at resting neuromuscular junctions has revealed the existence of small spontaneous depolarizations with a fairly uniform amplitude, which are termed miniature end-plate potentials (m.e.p.p.) and are confined to the end-plate region (Fatt and Katz, 1952; Boyd and Martin, 1956a; Liley, 1956a). Except for their spontaneous occurrence and small amplitude, m.e.p.p. have all the characteristics of e.p.p. With iontophoretic application of ACh to the end-plate region, m.e.p.p.'s can be mimicked, and these artificially induced m.e.p.p.'s respond to the application of curare and anti-ChE in the same way in which spontaneous m.e.p.p.'s do. Del Castillo and Katz (1956a) concluded that the m.e.p.p.'s represent spontaneous impacts of ACh on the motor end-plate. Miniature end-plate potentials which were first observed in frogs by Fatt and Katz

(1952), later by Boyd and Martin (1956a) and Liley (1956a) in mammals, and by Ginsborg (1960) in birds, have now been found at the end-plates of all vertebrate species which have been examined, including humans (Elmquist et al., 1960; Dahlback et al., 1961). The frequency of the m.e.p.p.'s is of about 1/sec (Fatt and Katz, 1952) and is dependent, among other factors, on the membrane potential of the motor nerve terminal. Their frequency increases when the terminals are depolarized, either by electric current (del Castillo and Katz, 1954c; Liley, 1956c) or by increasing the external potassium ion concentration (Liley, 1956c; Furukawa et al., 1957; Takeuchi and Takeuchi, 1961). Liley (1956c) reported that a 2.7 fold increase in external potassium ion concentration (above 10 mM) resulted in a 55-fold increase in the frequency of m.e.p.p. Katz (1962) recalculated Liley's measurements and found that there was a linear relationship between the calculated membrane depolarization and the logarithm of the frequency of the m.e.p.p.'s. Hyperpolarization of the nerve endings decreases m.e.p.p. frequency (del Castillo and Katz, 1954). The fact that the frequency of the m.e.p.p.'s is controlled presynaptically, and only presynaptically, by the membrane potential of the nerve endings (del Castillo and Katz, 1954b; Liley, 1956b) is important evidence pointing to the nerve endings as the site of their origin. This is confirmed by other findings; e.g. the spontaneous discharge ceases when the nerve endings disintegrate after nerve section (Birks et al., 1960) and they are stopped by botulinum toxin (Brooks, 1956; Thesleff, 1960). Botulinum toxin has been shown to prevent the release of ACh from nerve endings (Burgen et al., 1949).

Since the general relation between depolarization of nerve

endings and increased rate of ACh release has been established, it is believed that the normal e.p.p. (that is the response of a single end-plate to a nerve impulse) is made up of a synchronized volley of a few hundred m.e.p.p.'s (del Castillo and Katz, 1954a; Boyd and Martin, 1956b; Martin, 1955). It is a momentary increase of the frequency, or statistical probability, of an event which occurs spontaneously at very low rate. In other words, the action potential of the nerve raises the probability of the unitary event of secretion (which corresponds to the m.e.p.p.) by several orders of magnitude, so that instead of a secretion of 1 unit of ACh per msec, three hundred units are secreted within a fraction of msec. Each such unit consists of a packet, called quantum, containing several thousand ACh molecules (see review by Katz, 1962). Thus it appears that the release of ACh as seen by its effect on the end-plate membrane always occurs in quanta or multiples of quanta. The size of the quantum, or the amount of ACh in a quantum, seem to be constant, and changes in the overall rate of release are determined by variations in the number of quanta released (del Castillo and Katz, 1956). Factors other than the membrane potential of the nerve endings are known to influence the spontaneous quantal release, e.g. the temperature, the osmotic pressure, and raising the magnesium or lowering the calcium ion concentration of the solution bathing the synaptic region (Fatt and Katz, 1952; Furspan, 1956; del Castillo and Katz, 1956; Liley, 1956; Boyd and Martin, 1956; Li, 1958).

The amount of ACh released by a nerve impulse depends very much on the ratio Ca/Mg in the medium, the number of quanta being low if the ratio is reduced and vice versa. A muscle can be paralysed by either

lack of Ca or excess Mg (del Castillo and Engbaek, 1954; del Castillo and Katz, 1954). The release of ACh is also apparently increased by stretch (Hutter and Trautwein, 1955) and in the presence of adrenaline (Krnjevic and Miledi, 1958). Experiments on the effects of low Ca and high Mg on the neuromuscular synaptic potentials, led to the formulation of the quantum hypothesis by del Castillo and Katz in 1954. They provided the essential evidence for the quantal nature of transmitter release by demonstrating that synaptic potentials can be fractioned into units, identical in all respects to the spontaneous or m.e.p.p. Del Castillo and Katz thought that the invariable unit of ACh-secretion and its quantal character may depend on the way in which the transmitter substance is stored and distributed within the nerve ending. It had been suggested earlier, on morphological grounds, that transmitter substances are packed in minute secretory granules (Feldberg, 1945), which in the electron microscope have the appearance of small vesicles (de Robertis and Bennet, 1954; Robertson, 1956). Del Castillo and Katz (1955, 1956) saw in these synaptic vesicles, whose existence in motor nerve terminals had been brought to their attention by Robertson (1956), a possible morphological counterpart of the quantal release. It has been postulated that synaptic vesicles move toward the presynaptic membrane and discharge their content, and evidence supporting this was obtained by Birks, Katz and Miledi (1960) who carried out parallel studies on the electron microscopical appearance and the spontaneous electrical activity of the junction. With respect to the manner of discharge, del Castillo and Katz believed that during a critical collision between a vesicle and the presynaptic membrane the colliding membranes are made sufficiently leaky to allow the vesicular

contents to escape into the synaptic cleft. This scheme has two merits; it would explain how the transmitter molecules, normally enclosed within their intracellular storage bags (the synaptic vesicles), are able to penetrate two successive membrane barriers simultaneously. Secondly, the scheme does not require any immediate change in the cytoplasm to occur on arrival of the nerve impulse. What is needed is a greatly increased reactivity of the presynaptic membrane, so that for a given rate of collisions a much larger proportion of vesicles will succeed in discharging their contents (Katz, 1962).

Investigation of the changes in ACh content resulting from variations in parameters of nerve stimulation, have led to the functional separation of transmitter stores in the nerve ending solely on the basis of release data.

The basic observation which has led to the concept of an 'immediately available store' is that, at many synapses, after one synaptic potential (in this case an e.p.p. elicited by nerve stimulation) further stimuli elicit potentials which are smaller in amplitude (because of a smaller quantal content). This effect lasts for periods up to 10 seconds (Liley and North, 1953; Lundberg and Quilisch, 1953a,b; Takeuchi, 1958; Curtis and Eccles, 1960; Eccles et al., 1961; Thies, 1965; Elmquist and Quastel, 1965b). At the neuromuscular junction it can be shown that the size of this depression of the second response, is directly correlated with the number of quanta released by the first impulse, and, if this number is drastically reduced (e.g. by increasing the magnesium ion content of the bathing medium) the depression disappears, and the second response has a larger quantal content than the first (del Castillo and

Katz, 1954b; Hubbard, 1959, 1963). Conversely, if the quantal content of the first e.p.p. is increased by, for example, increasing the calcium ion concentration of the bathing medium, this depression is potentiated in magnitude but not in duration (Lundberg and Quilisch, 1953b; Takeuchi, 1958; Thies, 1965). The simplest explanation of these findings is to assume that a store of immediately available preformed quanta of transmitter is emptied.

During repetitive stimulation of curarized neuromuscular junctions, the amplitude of the e.p.p. declines progressively ('early tetanic rundown') to a plateau. The level of this plateau is inversely dependent upon the frequency of stimulation (Liley and North, 1953; Hubbard, 1963; Elmquist and Quastel, 1965b). Early tetanic rundown is ascribed to a progressive depletion, by repeated stimulation, of the 'immediately available store'. The quantum content of the e.p.p.'s does not fall to zero, because the 'immediately available store' is sustained by a 'repletion' or 'mobilization' process. The constant level then obtained, would represent the rate at which ACh is made available for release by this process. A corollary of the concept of an immediately available store is the concept that an impulse releases only a fraction of this store (Liley and North, 1953).

In the sympathetic ganglia, the assumption that in the nerve endings there is a functional separation of transmitter stores, is based both on release data and on measurements of the ACh content.

The first quantitative study of the metabolism of ACh at active nerve endings was by Brown and Feldberg (1936), using perfused superior cervical ganglia of cats subjected to prolonged repetitive stimulation.

The ACh released from the activated nerve endings diffused into the perfusion fluid, which contained eserine (to prevent hydrolysis of ACh), and could be estimated in successive samples of the venous effluent. As control, they used the contralateral unstimulated ganglion. The contents of the stimulated ganglion and of the unstimulated control were extracted with trichloroacetic acid and the ACh of each was determined. In such experiments Brown and Feldberg found regularly that the rate of ACh release was high at the beginning of stimulation, but fell off progressively to reach (after 20-30 minutes) a much lower level, which was then maintained with little further decline. Measurements of the ACh content of the stimulated ganglion and the control, however, yielded about the same amount of ACh. This paradoxical association of a declining ACh output with a well-maintained store of ganglionic ACh was confirmed by other workers. Kahlson and MacIntosh (1939) reported similar findings. These authors suggested that stimulation depleted the ACh store, but that the deficit went unobserved because ACh was rapidly resynthesized in the short period between removal of the ganglion and its disintegration in the extracting medium. Perry (1953) advanced the hypothesis that only part of the extractable ACh of the ganglion is readily available for release by nerve impulses, and that while the ACh released by stimulation (under the usual conditions of perfusion with eserinized Locke's solution) is quickly replenished by synthesis, the newly formed ACh only becomes 'available' for release at a relatively slow rate. This rate is equal to the steady rate of ACh release following the initial progressive decline of output during prolonged stimulation. From the foregoing it appears that the amount of ACh released by a nerve impulse is regulated

by at least three factors: 1) the amount of transmitter in the nerve ending immediately available for release; 2) the fraction of this amount which is released by a nerve impulse; and 3) the extent to which 'repletion' of, or 'mobilization' into the 'immediately available store' is able to keep pace with release.

H. INHIBITION OF ACETYLCHOLINE SYNTHESIS BY HEMICHOLINIUM-3

The name hemicholinium was introduced by Schueler (1955) to denote a group of quaternary bases characterized chemically by the incorporation of a choline (or choline-like) moiety into a six-membered ring through hemi-acetal formation. These products are notable for their high toxicity. The most potent product investigated was designated hemicholinium No. 3 or HC-3 (Schueler, 1955).

Their most striking pharmacological action is respiratory paralysis; this is central in origin and late in onset (in the larger common laboratory animals). When the dose is not too large, this respiratory paralysis can be prevented by the administration of either eserine or choline. Schueler noted that hemicholinium intoxication presents several features grossly resembling some phases of poisoning by botulinus and suggested that it might be due to interference with some cholinergic mechanism. MacIntosh et al. (1956) thought that a substance having such effects might be a specific poison of ACh synthesis, and they were able to verify this idea with compound No. 3 (HC-3). They found that low concentrations of HC-3 (0.01 - 0.1 mM) inhibited the synthesis of ACh "in vivo" and "in vitro" (perfused superior cervical ganglion of the cat, minced brain of the mouse). This inhibition was reversed by adding choline. MacIntosh et al. (1956), therefore, suggested that HC-3

somehow inhibited the formation of ACh. However, choline acetylase extracted from acetone-dried powder of brain was hardly inhibited at all by HC-3, even when the concentration of HC-3 was one thousand times that of choline. This evidence indicates clearly that HC-3 inhibits the formation of ACh at some stage previous to the process of choline acetylation. HC-3 therefore does not appear to act in the manner of botulinum toxin, which has been shown to block release and to have no effect upon formation of ACh (Guyton and MacDonald, 1947; Burgen et al., 1949; Brooks, 1954, 1956). By way of interpretation, MacIntosh et al. postulated that HC-3 may compete with choline for transport to intraneuronal sites of acetylation, by a specific carrier system.

The work of MacIntosh et al. with minced mouse brain (1956) indicated that HC-3 would inhibit the synthesis of ACh only if the concentration of choline was much below that usually used for determining the activity of the synthesizing enzyme. This was confirmed by Gardiner (1957, 1961), who presented further evidence bearing upon the hypothesis of a carrier transport mechanism for choline into nerve cells. Gardiner (1957, 1961) studied the effects of HC-3 upon ACh formation in minced guinea pig brain, homogenate brain mitochondrial fractions, and activated homogenate preparations after ether treatment. HC-3 was found to be a potent inhibitor of ACh formation in minced brain, much less so in homogenates, and not at all in ether-treated homogenates. Treatment with ether is thought to disrupt the membrane which encloses intracellular particles. The observations of Gardiner (1961) suggest, therefore, that HC-3 inhibits not by a direct effect on choline acetyltransferase, but by an indirect effect which involves the integrity of the particles. It

was found further, that the degree of inhibition was dependent, among other things, upon the concentration of choline in the medium. As the concentration of choline was reduced below 50 uM, the inhibition produced by a given concentration of HC-3 increased. The results thus suggest that HC-3 inhibits ACh synthesis by competing with choline for access to the synthesizing enzym . Putting the two observations together, it is reasonable to conclude that HC-3 is able to block the passage of choline across the membrane enclosing the particles.

Earlier Hebb and Smallman in their studies on the intracellular distribution of choline-acetyl-transferase had already considered the possibility that the enzyme was enclosed within a barrier or membrane (1956). Later work (Hebb and Whittaker, 1958; Bellamy, 1959; Whittaker, 1959) supports this idea by showing that both, cholineacetyltransferase and acetylcholine, are associated with particles that have sedimentation characteristics similar to those of mitochondria, but which may be distinguished from them. Whittaker (1959) has shown that organic solvents will damage these particles to such an extent that they no longer retain ACh. It seems likely that this is the membrane through which the passage of choline is restricted and at which HC-3 competes with it. The postulate that HC-3 inhibits choline transport systems is further supported by the observations of MacIntosh et al. (1958) who showed that HC-3 competitively inhibits choline excretion from the avian kidney (a function that does not involve choline acetylase), by Hodgkin and Martin (1965) who demonstrated competitive inhibition of choline entry into squid axons by HC-3 and by Schuberth et al. (1966), who showed that HC-3 inhibits the active uptake of isotopically labelled choline into nerve

tissue in vitro.

The results obtained with the preparations discussed above support the conclusion that the effectiveness of HC-3 depends upon the structural integrity of tissues. Thus in the whole animal HC-3 is extremely toxic. In intact nervous tissue, i.e. in the superior cervical ganglia of the cat, perfusion with low concentrations of HC-3 (0.01 - 0.1 mM) inhibits the production of ACh. In minced brain, in which the nerve cells are still largely intact, HC-3 inhibits ACh synthesis, but less strongly. When the cells in turn are disrupted, HC-3 still inhibits the synthesis of ACh that proceeds in intracellular particles. When finally these particles are broken down by ether, HC-3 no longer inhibits ACh synthesis, nor does it inhibit the activity of cholineacetyltransferase extracted from acetone-dried powders of brain. It seems, then that HC-3 inhibits the formation of ACh by competing with choline, the precursor of ACh, for passage through membranes to reach the choline-acetylating systems (see discussion by Gardiner, 1961). The decreased ACh synthesis and content found in the HC-3 treated, stimulated, autonomic ganglia (MacIntosh et al.,1956), could thus be explained on the basis of decreased choline available for acetylation, in those cells which synthesize and release ACh as a transmitter. Later experiments by Birks and MacIntosh (1961) using perfused sympathetic ganglia have shown that HC-3 has no obvious effect on the output of ACh until the total quantity of ACh released amounts to about one third of the total initial content of the ganglia. Only subsequently does the rate of ACh release decline, as the stores become emptied. This decline in the release of ACh is related to a parallel decline in ACh content of the ganglion. A similar

depletion of the ACh store in the presence of HC-3, as estimated by measuring e.p.p.'s amplitude at neuromuscular junctions, has first been reported by Thies (1962) and later confirmed by Elmqvist (1964) and Elmqvist and Quastel (1965a). This suggests that HC-3 acts at the neuromuscular junction in the same way as in sympathetic ganglia, and that the reduction of ACh release occurs, in the presence of the drug, as a result of store depletion, provided the nerve terminals are stimulated sufficiently. The usual delay in muscular paralysis in hemicholinium poisoning, when the drug is given in doses that block ACh synthesis entirely, can easily be explained by the time required to deplete presynaptic stores of ACh. The failure to obtain neuromuscular block upon stimulation of a nerve-muscle preparation reported earlier (Schueler, 1955, 1960) reflected the very slow rate of stimulation that this author used. It has also been suggested that HC-3 might, by itself or after acetylation, compete with ACh for presynaptic storage sites, later being released by the nerve-endings as false transmitters (Burgen et al., 1956; Bowman and Rand, 1961; MacIntosh, 1961). However, the observations discussed above do not support this notion. The evidence reviewed above presents a strong case for a presynaptic site of action for HC-3. Such evidence does not, of course, rule out the possibility of action at the postsynaptic membrane.

I. ACETYLCHOLINE STORES IN THE NERVE ENDINGS

Sympathetic Ganglion:

One of the most remarkable features of sympathetic ganglia is the ability of the synthesizing mechanisms in the nerve terminals to maintain the concentration of ACh in the ganglia at or near normal levels

during intensive and prolonged activity (Birks and MacIntosh, 1961; Matthews, 1963). As long as the perfusion medium contains the ingredients (primarily choline and glucose) required to support the synthetic processes, the total amount of ACh present in the ganglion is unchanged by prolonged, intensive, stimulation of the preganglionic nerve. The striking synthetic capabilities of the cholinergic nerve endings is illustrated further by the fact that an amount of ACh equivalent to the total resting content (300 mug per ganglion) can be recovered from the perfusion fluid during 10 minutes of preganglionic stimulation at the rate of 20 cps.

By the use of HC-3, that inhibits ACh synthesis, and of drugs that prevent the enzymatic hydrolysis of the released ACh (e.g. eserine), it has been possible to develop some concepts about the intraganglionic distribution of ACh and its availability for synaptic activity (Birks and MacIntosh, 1961). Essentially the technique used by Birks and MacIntosh involved accurate measurements (using the cat's blood pressure method) of the ACh content and output of the perfused superior cervical ganglia. Studies were performed under resting conditions, and during prolonged activation by maximal preganglionic volleys over a wide range of frequencies, using perfusion fluids of altered composition. Ganglia perfused with media lacking choline and containing HC-3, are unable to maintain their normal content of ACh during intensive, prolonged stimulation. Under these conditions, the ganglia lose approximately 85% of their normal ACh content (or about 220 mug). From these facts, Birks and MacIntosh concluded that the storage forms of ACh can be classified as 1) the so-called "depot" ACh and 2) the "stationary" ACh. The "depot"

form represents the component available for release by the incoming nerve impulse; the "stationary" form, the component remaining (15% of the normal content or about 40 mug). This component is not available for release, since the "stationary" ACh represents that fraction of the total store that remains in a ganglion after ACh depletion by prolonged stimulation in the presence of HC-3. They further suggested that the "stationary" pool of ACh is probably confined to the preganglionic axons (as distinct from nerve terminals) that penetrate into the ganglion proper.

The analysis of the time course of ACh release during prolonged stimulation has shown that the "depot" ACh is composed of two subfractions, one of which is smaller and more readily liberated than the other. Birks and MacIntosh (1961) suggest that the two reservoirs of depot ACh are connected in series, and that ACh from the larger, more stable, subfraction may have to pass into the small, more readily releasable, subfraction before it can finally be liberated. This "series" hypothesis is similar to the one proposed by Perry (1953) to account for the paradox of a declining ACh output from Locke-perfused ganglia whose ACh stores were supposedly well maintained. Perry suggested that newly synthesized depot ACh could be made available (i.e. transformation of synthesized ACh into an available form) at the rate of about 4 mug/minute, which corresponds to the final steady efflux of ACh during prolonged stimulation. However, from the results of Birks and MacIntosh (1961) it has become clear that the rate at which the depot can be depleted, or replenished depends on the composition of the perfusion fluid, which affects the speed of synthesis.

Since "depot ACh" is present in nerve endings which have not been treated with cholinesterase inhibitors, it has been assumed that the depot ACh is in some way protected from the nerve ending's own acetylcholinesterase, possibly by inclusion in synaptic vesicles. It has been observed with several kinds of synapse (Edwards et al., 1958; Palay, 1958; Birks et al., 1958) that vesicles are not uniformly distributed within nerve endings, but show some tendency to be grouped close to the presynaptic membrane. Birks and MacIntosh (1961) suggest that the vesicles so located might be thought to contain the readily releasable fraction of the depot ACh, which occupies the strategic sites of the presynaptic membrane where ACh is ejected.

A third store of intracellular ACh has been described by Birks and MacIntosh (1961). This store can be demonstrated only in ganglia whose cholinesterase has been inactivated. This store has been called "surplus" ACh. Although it is formed rather slowly, it may rise to a level above that of the depot ACh. The fact that surplus ACh quickly disappears when the anticholinesterase is removed, or when the AChE enzyme is reactivated, suggests that it is located in a compartment where it can be destroyed in the presence of the active enzyme, most likely the cytoplasm of the nerve endings. It has been further demonstrated (Birks and MacIntosh, 1961) that the volley output from an eserinized ganglion remains constant while surplus ACh is accumulating. This leads to the conclusion that surplus ACh does not make an important contribution to the ACh released by nerve stimulation. It also provides an explanation for the paradox that during activation of a ganglion perfused with eserinized Locke solution, there is a progressive decline in the release

of ACh despite a well-maintained content of ACh in the ganglion (Brown and Feldberg, 1936; Kahlson and MacIntosh, 1939).

Further, Birks and MacIntosh (1961) have pointed out that the formation of surplus ACh, and also the steady release of ACh in minute quantity from the eserinized resting ganglion, are evidence that depot ACh undergoes a continuous slow turnover, even when no nerve impulses are arriving at the terminals. They propose that the continuous release of ACh during rest may represent a quantal discharge of depot ACh into the extracellular space, such as is known to occur at motor nerve endings in striated muscle, while the continuous formation of surplus ACh may represent a concurrent discharge of depot ACh into the presynaptic axoplasm.

The concepts developed by Birks and MacIntosh (1961) can be summarized in a hypothetical model as presented in Fig. 4.

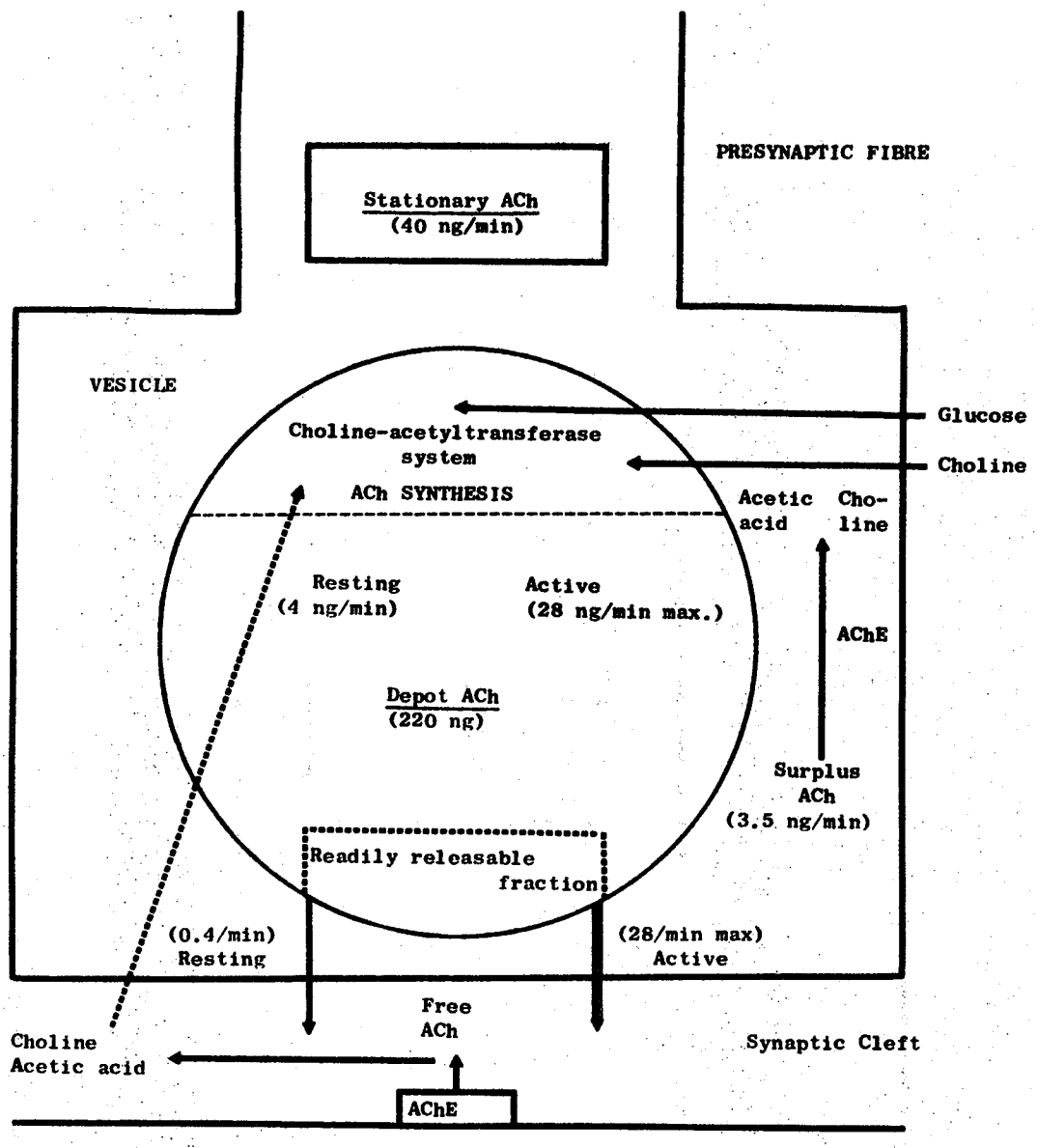


Fig. 3. Diagram of the acetylcholine transmitter system in presynaptic terminals of the cat superior cervical ganglion. Based on description by Birks and MacIntosh (1961).

The progress, made by Birks and MacIntosh (1961), in our understanding of the metabolism of ACh using conventional techniques of perfusion, bioassay, and pharmacological analysis, has been supplemented with information obtained with radioisotopic methods involving the use of labelled choline.

As has been mentioned earlier, an active superior cervical ganglion of the cat can, when perfused artificially, synthesize and release ACh over long periods of time (Brown and Feldberg, 1936; Kahlson and MacIntosh, 1939; Perry, 1953). For these processes to proceed optimally, circulating choline (Ch) must be supplied (Brown and Feldberg, 1936; Birks and MacIntosh, 1961). These authors have shown that physiological concentrations of Ch (about 10 uM; Bligh, 1952) are necessary to maintain normal tissue levels and high release rates of ACh during continuous nerve stimulation. Birks and MacIntosh (1961) estimated that synthesis of ACh within the ganglion could go up seven-fold during maximal preganglionic stimulation. Consequently, the demand for Ch should also be increased to ensure an adequate rate of synthesis of ACh in the active state. When the ganglion is perfused with choline-free Locke's solution, the amount of ACh released by preganglionic stimulation declines rapidly to a small quantity, indicating that a limited amount of Ch is available in the preganglionic nerve terminals. Friesen et al. (1965) confirmed that extracellular choline is important in maintaining ACh stores. These authors demonstrated that radioactive Ch added to the fluid perfusing a ganglion could be converted to ACh, and that the labelled ACh was released upon stimulation of the preganglionic nerve fibres. Paper chromatographic and paper electrophoretic methods were

used to characterize the ACh-like substance derived from the sympathetic ganglia (Friesen et al., 1965). The data obtained by these two procedures indicate that the choline ester present is ACh. In addition, the compound is indistinguishable from authentic ACh in all chemical tests performed. This work provides the first direct evidence that the cholinergic mediator released in the ganglion is ACh. This ability of the nerve ending to synthesize ACh has also been shown in other preparations using labelled Ch as the precursor: in the isolated rat hemidiaphragm (Saelens and Stoll, 1965; Potter, 1970); in cortex slices of the brain (Browning et al., 1966; Chakrin and Shideman, 1968); in the isolated cat heart (Wallach et al., 1967); and in the guinea-pig ileum (Mattila and Idanpaan-Heikkila, 1968).

Information on the incorporation of labelled Ch into the sympathetic ganglion was obtained from a study by Collier and Lang (1969). They found that the Ch taken up by the ganglion is incorporated at a measurable rate into phosphorylcholine (PCh) and into phospholipids as well as into ACh. The rate of formation of PCh and phospholipid from Ch however, was found to be low (2 ng/min each), and it is not affected by preganglionic stimulation or by exposure to HC-3 (procedures which are known to accelerate and retard respectively, the synthesis of ACh by intact ganglia). Free Ch liberated by PCh or phospholipid turnover seem unlikely to be an important source of Ch for ACh synthesis under physiological conditions (Collier and Lang, 1969).

Our knowledge of the ACh turnover during rest and activity in the perfused ganglion preparation has been advanced further by Collier and MacIntosh (1969) using a combination of bioassay procedures and

radiometric assay. Thus, assessment of transmitter turnover by bioassay is supplemented with measurements of the incorporation of labelled Ch into the transmitter depot and its subsequent release. They showed that at rest, with labelled Ch in the perfusion fluid, some of the ACh of the ganglion is slowly replaced with labelled ACh (about 20-25% of the ACh store in an hour). Fifteen to sixty minutes of preganglionic stimulation do not alter the total ACh content, but 80-85% of the total ganglionic ACh is replaced by labelled ACh. This percentage is probably an estimate of the size of the depot ACh available for release by nerve impulses. It agrees with previous estimates by Birks and MacIntosh (1961), who found that up to 85% of ganglionic ACh could be depleted by preganglionic stimulation when its replenishment was prevented by HC-3. It also agrees with estimates by Quastel (1962) and Birks (1963) who observed a similar loss of ACh in ganglia perfused with sodium-free media.

Though much of the labelled Ch from the perfusion medium taken up by the ganglia is rapidly converted to ACh and other esters (Collier and Lang, 1969), free Ch was found also to accumulate gradually, in amounts too large to be accounted for by passive diffusion into the aqueous phase of the ganglion (Collier and MacIntosh, 1969). This accumulation is more rapid in active ganglia and is reduced by HC-3. Collier and MacIntosh suggested that Ch may enter the nerve terminals by an active transport process which is blocked by HC-3, such as occurs in the giant axons of loligo (Hodgkin and Martin, 1965); or in erythrocytes (Martin, 1967). Although their experiments do not demonstrate that neural elements can accumulate Ch against an electrochemical gradient, their supposition is made more attractive in the light of Potter's (1968)

finding that isolated nerve terminals from brain can concentrate Ch from the surrounding medium. Collier and MacIntosh further found that the labelled-free Ch, unlike ACh, rapidly disappears when the ganglion is perfused with unlabelled Ch-Locke solution: Almost all (>90%) of the labelled Ch, but only a small fraction of the labelled ACh (synthesized by the ganglion under stimulation) is removed by the label-free washout. Therefore most of the label being retained was due to the synthesized labelled ACh. From their experiments using labelled ACh instead of Ch in the perfusion media (with eserine to preserve the ACh from its breakdown by AChE) it is clear that the ganglia strongly preferred Ch, the precursor of ACh, to the transmitter itself. Retention of labelled ACh by the ganglia in these experiments is only about 20% of the value obtained using labelled Ch.

The Ch present in plasma(Bligh, 1952) is, no doubt, the ultimate source from which ACh is manufactured by the ganglionic terminals. The immediate source, however, may be in part Ch derived from just-released ACh. Ch so formed should, at least for a moment, be available at the synaptic cleft, where it can compete with circulating Ch for capture by the nerve ending. Collier and MacIntosh (1969) obtained some evidence that the recapture of released Ch does contribute significantly to Ch turnover in ganglia. These authors subjected ganglia to the standard labelling procedure (60 min perfusion with labelled Ch with continuous preganglionic stimulation 20/sec), and then stimulated them in a medium containing physiological levels of Ch. They found that under these conditions stimulation discharged about twice as much label into the effluent when eserine was present than when it was absent. Furthermore, in the

presence of eserine the label was recovered as ACh, while in its absence it is recovered as Ch. It is unlikely that eserine acted here by promoting transmitter release per se, for raising the concentration of eserine does not have this effect (Emmelin and MacIntosh, 1956; Birks and MacIntosh, 1961; Matthews, 1966). Collier and MacIntosh (1969) concluded from these data that in the absence of eserine about half of the Ch formed from the just-released transmitter is recaptured.

The conclusion that ganglionic nerve terminals efficiently recapture the Ch formed from the ACh they release, but not the ACh itself, was first reached by Perry (1953) on the basis of his experiments on ACh and Ch release in perfused ganglia. More recently, analogous results have been obtained by Potter (1970) with the rat phrenic diaphragm, in this preparation about 35% of released ACh is shown to be re-uptaken as Ch.

Furthermore, Collier and MacIntosh (1969) found some indications that the newly synthesized ACh in the ganglion may be released preferentially. When a ganglion, in which the depot ACh had been almost completely labelled, was perfused with unlabelled Ch-Locke or plasma, it retained most of this label during a 20-min washout perfusion. At this point, a 10-min period of preganglionic stimulation released into the effluent, in the presence of eserine, about twice as much unlabelled than labelled ACh. Most of this unlabelled ACh must have been formed during the perfusion with unlabelled Ch-Locke; because when synthesis of ACh was prevented by HC-3, almost all the released ACh was labelled.

Two possible explanations were proposed by Collier and MacIntosh (1969). The first one postulates that during continuous activity,

the ACh store does not turnover as a uniform pool but that there is a preferential release of newly synthesized transmitter. In their experiments, ganglions were shown always to contain enough labelled ACh to account for more than two-thirds of the original transmitter depot (85% of the ACh content of the control ganglion). If we assume that this transmitter depot does not expand under the influence of eserine, we have to conclude that the ACh released by the ganglion is not a random sampling of the depot transmitter but that it includes a higher proportion of newly synthesized non-labelled transmitter. Therefore synthesized non-labelled transmitter does not mix rapidly with the preformed transmitter depot, but tends to remain transiently in a situation in which it is more likely to be released by nerve impulses. Such a preferential release of newly synthesized transmitter has been shown at an adrenergic junction by Kopin et al. (1968). The second explanation proposed by Collier and MacIntosh (1969) postulates that the effective size of the transmitter depot increases under the influence of eserine. It has been mentioned earlier that a ganglion perfused with Ch-Locke solution, containing eserine, accumulates ACh in some compartment not previously available for ACh storage. This excess ACh store, present only in eserinized preparations, called "surplus ACh" by Birks and MacIntosh (1961), does not seem immediately available for release. This has recently been confirmed by Collier and Katz (1970), who showed that preganglionic nerve stimulation (5/sec for 2 min) did not release surplus ACh, but perfusion with ACh (0.15 - 15 ug/ml), or injection of carbachol (0.5 - 2.5 ug) did. However, almost nothing is known about the rate of exchange of this surplus ACh with the original transmitter depot. It is therefore possible

that in the experiments of Collier and MacIntosh (1969) the labelled ACh loaded into the transmitter depot, may have subsequently become diluted by exchange with unlabelled surplus ACh which formed (after the loading period) in the presence of eserine. Such an exchange may explain, at least in part, why the specific activity of the released ACh is so much lower than that of the ACh depot at the end of the labelling procedure. It is likely that both of the processes described above participate in lowering the specific activity of the released ACh. Other experiments by Collier and MacIntosh (1969), in which the time course of the release of labelled and unlabelled ACh was studied, indicate that there may indeed be some preferential release of newly synthesized transmitter. These experiments, however, do not exclude exchange between the surplus and depot compartments as a factor contributing to the lowering of the specific activity of the released transmitter.

Neuromuscular Junction

Unlike the situation in the ganglia, ACh turnover at the neuromuscular synapses is much more difficult to investigate due to the fact that the motor nerve endings occupy such a small fractional volume of the muscle bulk in nerve-muscle preparations (Emmelin and MacIntosh, 1956; Straughan, 1960; Krnjevic and Mitchell, 1961). Therefore, the hypothetical models of how transmitter is manufactured, stored and made available for release in the motor nerve terminals, have to rely on the relevant data collected in the sympathetic ganglia.

The aim of our present studies is to investigate how much of the conceptual model based on the results from the ganglion (Birks and MacIntosh, 1961; Collier and MacIntosh, 1969), is applicable to the

peripheral nerve. Given the present commercial availability of labelled Ch with high specific activity, it has now become feasible to study the ACh turnover in the motor nerve-endings by following the utilization of radioactive Ch for ACh synthesis, and the subsequent turnover and release of the labelled ACh. A similar basic approach to the mammalian neuromuscular junction (using rat phrenic nerve-diaphragm preparations) has recently been reported by Potter (1970) who found that the results obtained with diaphragms are very similar to those previously observed with ganglia (Birks and MacIntosh, 1961; Collier and MacIntosh, 1969).

As a preliminary, the present experiments were begun to gain some insight into the uptake of radioactive choline by peripheral nerve, its utilization for ACh synthesis and its subsequent turnover and release at the neuromuscular junction.

The frog sciatic nerve-gastrocnemius muscle preparation was used because considerable information is available in the literature about this classical preparation; it is relatively easy technically and its long sciatic nerve trunk provides a large surface area for uptake studies and a good length for electrical stimulation. An added advantage of this preparation is its well-known stability over long periods of time.

SECTION II

METHODS

A. ANIMALS

All experiments were performed with grass frogs (Rana pipiens) of both sexes during a period of time which included the four seasons (from August to July). Frogs weighed approximately 250-350 g and were kept in running tapwater at about 10°C.

B. TISSUE PREPARATIONS

Frogs were sacrificed by decapitation at the cervical level. A fine wire was then passed down the whole length of the vertebral canal, destroying the spinal cord. Such preparations could have had no sensations of pain and did not twitch during dissection. A circular skin incision was made round the abdomen of the frog at about the level of the lower ribs. The skin was then cut between the legs and this cut was continued both at the back and in front until it met the transverse circular incision. The skin was carefully stripped off from the lower part of the trunk, on each side, and from each leg at a time, as one would slip off a rubber glove. The abdomen was then opened by a longitudinal cut followed by a transverse incision. The animals were eviscerated, taking care to avoid rupturing of the intestine and spilling of the intestinal content into the abdominal cavity. The spinal column was severed just above the level at which the nerves which form the sciatic plexus emerge (7th, 8th and 9th spinal nerves). After this spinal transsection the sciatic nerves were dissected along their whole length, up to the level of the knee joint, where they insert into the gastrocnemius muscles. Side-nerve branches were trimmed to within about a few mm of the major trunk. The nerves were cleaned of adherent loose connective tissue using needle point forceps and fine scissors, while the perineural sheath was left

intact. Two types of tissue preparations were used: 1) single sciatic nerve, and 2) sciatic nerve-gastrocnemius muscle. The only difference between these preparations was the inclusion, in the latter, of the innervated gastrocnemius muscle. In the single sciatic nerve preparation, the gastrocnemius muscle was separated from the sciatic nerve at the level of the knee.

For experiments using the nerve-muscle preparation the attached gastrocnemius muscle was carefully dissected to preserve intact the branch of the sciatic nerve to this muscle. A fine cotton thread, tied to a fine sewing needle, was then passed through the tendon Achillis at its distal end and tied firmly several times round the tendon well clear of the muscle. The tendon was cut distal to the ligature. The muscle was separated from the tibio-fibular bone as far as the knee joint. The tibio-fibular bone was cut just below the knee and the femur and thigh muscles were cut above the knee so as to preserve the knee joint. The knee joint was used to tie another piece of cotton thread, to be coupled to a twitch recording system. During the whole procedure care was taken to keep the tissue constantly moist with oxygenated frog Ringer solution, and also to avoid stretching of the nerve and the muscle. The whole dissection was completed at room temperature. After the dissection, the tissue preparations (single nerve or nerve-muscle preparations) were allowed to rest in oxygenated Ringer solution while being gassed with 95% O_2-5% CO_2 in an Erlenmeyer flask for 1-2 hours, until the experiments were started.

C. <u>APPARATUS EMPLOYED FOR INCUBATION OF THE TISSUE</u>

For experiments measuring the uptake of radioactive choline

into the sciatic nerves under resting conditions, small (10 ml) Erlenmeyer flasks were used to incubate the tissues in a Dubnoff metabolic shaker.

For other experiments appropriate lucite chambers were used to incubate the tissues. To determine the effect of nerve stimulation on the uptake of labelled choline by the sciatic nerves, a two-compartment nerve chamber was employed (Fig. 4). The nerve chamber and its supporting frame were made from lucite. The chamber consisted of two main compartments each with the following dimensions: 5 cm x 0.5 cm x 0.5 cm deep, separated by a small well (0.5 x 0.5 x 0.5 cm). Each one of the two walls of the smaller central well had a small interconnecting groove in the upper edge. The two bigger compartments contained silver wire electrodes for stimulation and recording. A sciatic nerve trunk could be positioned with the proximal end in one compartment resting on the stimulating electrodes, passing through the grooves in the walls of the middle chamber, and resting the distal end in the second compartment, on the recording electrodes. The incubation medium could then be added or removed using an appropriate syringe with a hypodermic needle. During the whole experiment a lucite cover with stopcock grease on the edges could be placed to prevent evaporation of the medium and drying of the preparation.

To investigate the longitudinal migration of radioactive choline along the sciatic nerve, a special moist chamber was employed (Fig. 5). This chamber had two wells connected by an inverted T-shaped canal (2 mm in diameter), which accommodated a 6 to 7 cm segment of the sciatic nerves. The nerve could be led from one well, over a bipolar electrode for stimulating, through the centre of the T-system and out to the other well and recording electrode. During the actual experiment,

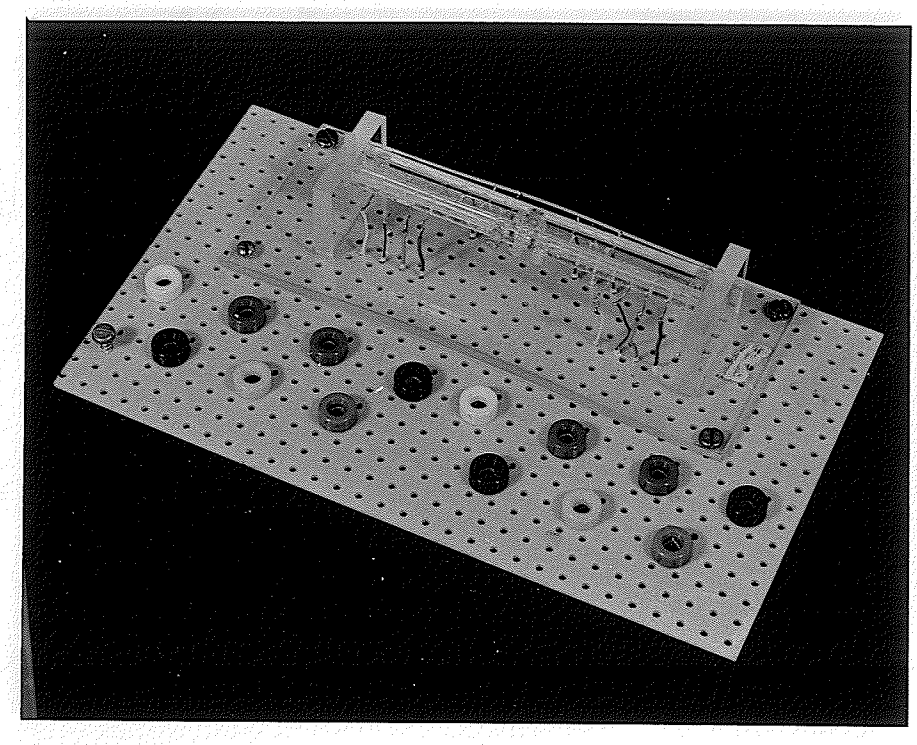


Fig. 4. Two-compartment nerve chamber.

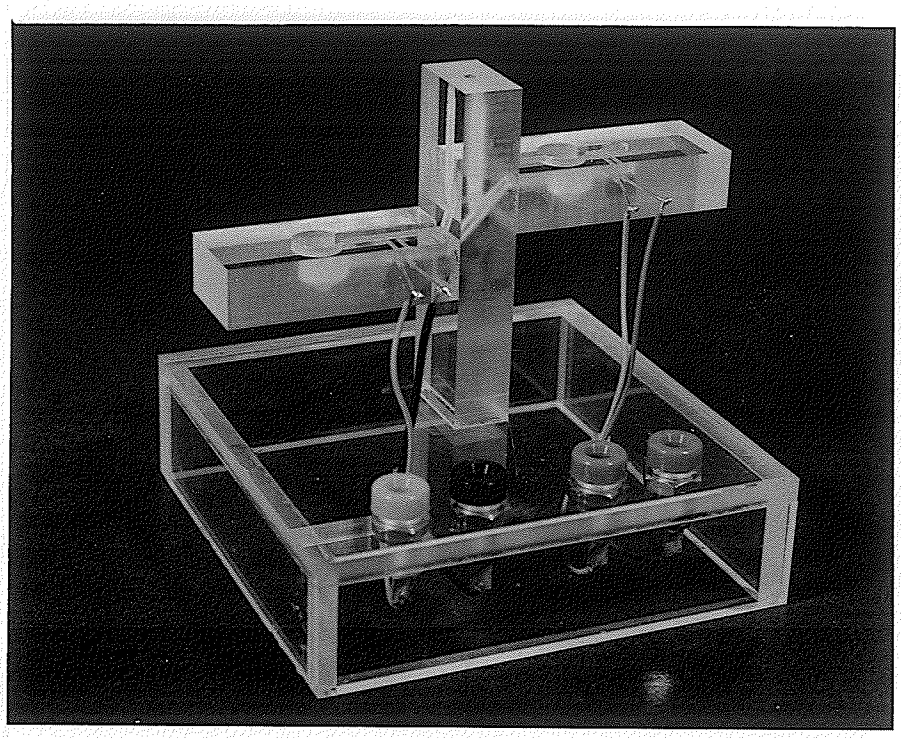


Fig. 5. Moist chamber.

each well and the surround region was kept moist with frog Ringer solution but the center portion of the T-system was filled with vaseline from the top; this effectively sealed the individual wells. During the experiments the wells were covered with a lucite slide and sealed with vaseline.

For experiments using the nerve-muscle preparation, a double nerve-muscle chamber was constructed to accommodate two such preparations (Fig. 6). This consisted of one common circular compartment in the middle (2 cm diameter; 0.5 cm deep) which was connected to two nerve chambers, 5 cm in length and each consisting of four wells which contained silver wire electrodes for stimulating and recording purposes. The common circular compartment with the two joining nerve chambers was constructed so as to be able to accommodate one pair of sciatic nerves coming from the same frog with the spinal cord and spinal column at their points of emergence remaining intact. It also served as a common reservoir for the incubation media of both nerves. The distal ends of the nerve chambers in turn were each connected to two horizontal muscle chambers. The chambers lay perpendicular to the nerve chambers and had each a 7 ml volume capacity. A small groove (2 mm wide) fitting the diameter of the sciatic branch going to the gastrocnemius muscle joined the nerve and the muscle chamber. The nerve chambers also contained silver wire electrodes for stimulation and recording purposes. During the actual experiment this interconnecting groove between the nerve chamber and the muscle chamber with the nerve running through it could be sealed using vaseline so as to prevent the nerve medium from mixing with the muscle medium. A stainless steel hook at one side of the muscle chamber was used to secure one end of the muscle (usually the Achilles tendon). At

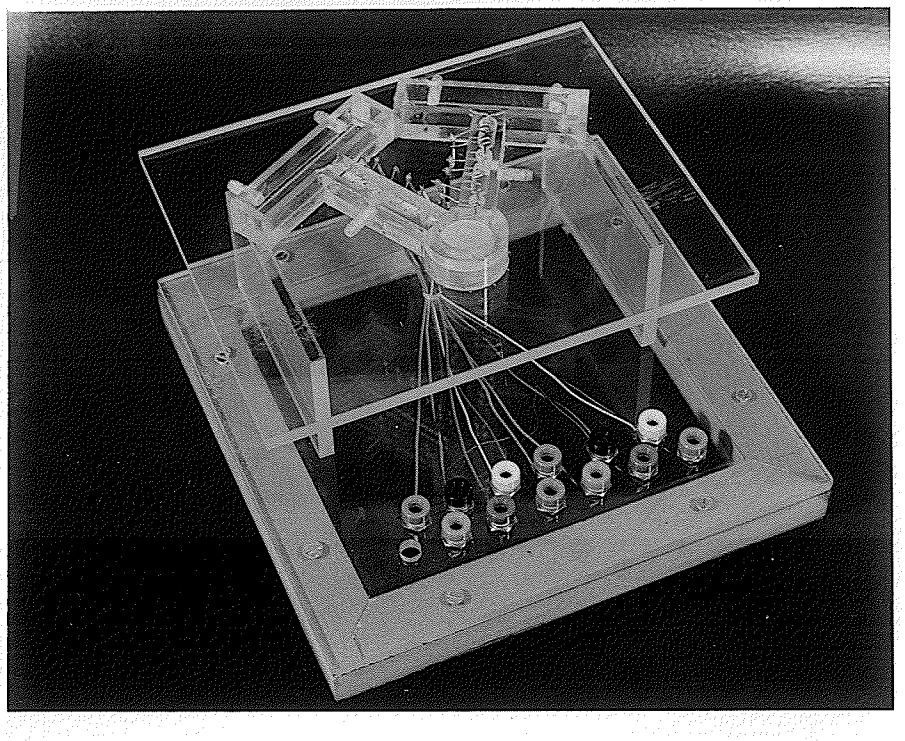


Fig. 6. Double nerve-muscle chamber.

the other side of the chamber, there was a pulley which rotated around a stainless steel axle. This enabled the cotton thread coming from the knee joint of the dissected nerve-muscle preparation (see Tissue Preparation) to be placed over the pulley for attachment to an isometric force transducer used to record muscle twitches. At the side of the muscle bath opposite the communication with the nerve chamber, two lucite nipples were placed, through which filling and emptying of the muscle chamber with the appropriate solutions could be effected. A system of rubber tubing was used to connect the chamber inlet nipples with bottles containing the various solutions serving as reservoirs. With the use of pinchcock clamps the inflow of fluid from the reservoir bottles containing the appropriate solutions could be regulated. A hypodermic needle inserted through the inlet nipple was used for bubbling the solution with oxygen and CO_2. This continuous bubbling also ensured during the experiment proper mixing of the bathing fluid. The outlet nipple was connected with a filter pump of the aspirator type, using rubber tubing and pinchcock clamps to regulate the force of aspiration used to empty the fluid in the muscle chamber.

D. SOLUTIONS

The 'standard' buffer solution used for all the experiments was "frog Ringer solution" which contained the following amounts of electrolytes: Na^+, 112; K^+, 1.9; Ca^{++}, 1.1; Cl^-, 116; HCO_3^-, 2.4; $H2PO_4^-$, 0.1 mEq/l (modified from Boyle and Conway, 1941). Glucose was added to make 10 mM. The solution was prepared first by dissolving all the ingredients except $CaCl_2$, which was added only after the initial solution had been bubbled with 95% O_2-5% CO_2 for 15 minutes. This solution was further equilibrated

with the same gas mixture for another 15 minutes. The pH of the solution was then corrected by adding NaOH to give a final pH value of 7.2.

Before the experiments the Ringer solution was bubbled with 95% O_2-5% CO_2 for 15 minutes. The acidity was controlled with indicator paper (B.D.H.). Labelled choline and other salt solutions were added as necessary for the experiment. No correction was attempted for the small osmotic error introduced by the addition of relatively small amounts of other substances.

For the experiments testing the effects of anaerobic condition, the Ringer solution was made up with pre-heated, glass-distilled demineralized water, and the solution was gassed with 95% N_2-5% CO_2.

The particular combinations of isotopes and other compounds present in the incubation media will be indicated with each experiment together with the incubation conditions.

E. CHEMICALS AND THEIR SOURCE OF SUPPLY

Radioactive choline-methyl-^{14}C chloride (10 mc/mM), choline-methyl-^{3}H chloride (100 mc/mM) D-mannitol-1-^{3}H (N)(100 mc/mM) were purchased from the New England Nuclear Corp. All the radiochemicals were stored at -15°C. Sources for the rest of the chemicals were the following.

Choline chloride	J.T. Baker Chemical Co.
Acetylcholine chloride	Calbiochem
Hemicholinium No. 3 (HC-3)	Aldrich Chemical Co.
Eserine salicylate (Physostigmine salicylate)	Sigma Chemical Co.
Neostigmine bromide	Sigma Chemical Co.
Carbachol	Calbiochem

All reagents used were analytical grade.

F. STIMULATION AND RECORDING

Sciatic nerves were stimulated in the nerve chamber via a pair of silver-wire electrodes. Supramaximal rectangular pulses of 0.2 msec duration were obtained from constant-voltage stimulator (Grass, SD-8) coupled to the electrodes.

Nerve action potentials corresponding to those from type "A" myelinated fibres were monitored visually to provide an index of the functional state of the nerve. This was most useful in experiments where cholinesterase inhibitors (eserine and neostigmine) were present, since contraction of the muscle was completely abolished early in the course of such experiments.

In the experiments with the sciatic nerve-gastrocnemius muscle preparation recording of the muscle twitch was effected by means of an isometric force transducer (Grass, FT.03) which was connected to the preamplifier of a polygraph (Grass model 5).

G. EXPERIMENTAL PROCEDURE

1. Choline uptake experiments

a. Resting uptake

Single sciatic nerves were prepared as described earlier (see Tissue Preparation). Pairs of sciatic nerves coming from the same frogs were used, one serving as control and the other as the treated preparation. The same length of nerve segments were taken for the experiments (usually around 6-7 cm).

To determine choline uptake into the sciatic nerves under resting conditions each nerve was incubated for a fixed period of time

(usually 60 min) at room temperature (20°C) in small (10 ml) Erlenmeyer flasks, under an atmosphere of 95% O_2-5% CO_2 (except in anaerobic experiments, where 95% N_2-5% CO_2 was used instead). During the whole incubation period the incubation vessels were kept in a Dubnoff shaker. For each nerve, 2 ml of frog Ringer solution containing (^{14}C) choline chloride as a tracer (10 mc/mM) with or without unlabelled choline chloride were used. Tracer amounts of (^{3}H) mannitol (100 mc/mM) were also added to measure the apparent extracellular space.

At the end of the incubation period each sciatic nerve was separated from its incubation medium. Further handling of tissue and its incubation medium is described in "Preparation of Samples for Measurement of Radioactivity".

b. Choline uptake during stimulation

For choline uptake experiments involving electrical stimulation of the sciatic nerves the incubation was performed in the two-compartment lucite nerve chambers. The sciatic nerves were mounted on two such chambers (see Apparatus Employed for Incubation). Each chamber was then filled with 2 ml incubation medium of the appropriate composition. One nerve of each pair of sciatic nerves was stimulated while the other served as resting control. Supramaximal stimuli of 0.2 msec duration were delivered at the appropriate rate, to the stimulated preparation, during the indicated period of incubation. Subsequent procedures after the end of the incubation were the same as those described for incubation under resting conditions.

2. Migration of choline in sciatic nerve axons

To determine whether migration of labelled Ch (taken up in the

proximal segment of the sciatic nerve) distalwards, is fast enough to be an important factor in the supply of Ch to the motor nerve endings, a series of exploratory experiments were carried out using the moist chamber previously described. A 6 to 7 cm segment of sciatic nerve was placed in one well, over a bipolar electrode, led through the centre of the T-system and out to the other well. 0.5 ml of Ringer solution containing ^{14}C-Ch (1 μc) was added to the well associated with the stimulating electrode. The distal well was filled with the same volume of label-free Ringer solution. Prior to stimulation of the nerve, the wells were covered with lucite covers and sealed with vaseline. The nerves were then continuously stimulated at a frequency of 2/sec for 24 hours. Radioactivity measurements in the Ringer solution in the distal well were made at the start and at the conclusion of the experiment.

3. Choline utilization at the neuromuscular junction

Utilization of the proximally supplied labelled Ch for transmitter synthesis at the neuromuscular junction was estimated using the sciatic nerve-gastrocnemius muscle preparation. A pair of such preparations coming from the same frog were mounted on the double nerve-muscle chamber described earlier (see Apparatus Employed for Incubation). The grooves at the junction of the nerve- and muscle-chambers were sealed with vaseline to prevent the incubation media of the nerves and muscles from mixing. ^{14}C-Ch (10^{-4} M) in 2 ml Ringer solution was used to incubate the nerves in the nerve chambers, whereas the muscle baths were filled with 6 ml label-free Ringer solution bubbled in 95% O_2-5% CO_2. Samples (100 μl each) were collected from the muscle baths at appropriate times to monitor the amount of radioactivity present. No correction was

attempted for the relatively small amount of fluid taken for radioactivity determinations. Test stimuli were delivered through the sciatic nerves and the changes in the levels of radioactivity in the muscle baths were measured. However, after a series of exploratory experiments using different incubation periods (up to 6 hours), and test stimuli with a variety of frequencies, it was apparent that contamination of the muscle bath (by radioactivity coming from the nerve bath) was a problem which was difficult to control with the method of sealing used. This apparent method of supplying labelled Ch through the proximal part of the sciatics was not pursued.

In all of the following experiments the nerve-muscle preparations were exposed to labelled Ch in the muscle baths (instead of in the nerve bath) to give a Ch supply to the motor nerve endings directly. These studies were all conducted at room temperature (20°C). In each experiment incubation began with Ch-free Ringer solution. The preparations were first submitted to a preconditioning period of 30 minutes during which the functional state of the nerve-muscle preparation was tested by delivering low frequency electrical stimulation (0.2/sec during 10 minutes) followed by rest. The preparations were then incubated for 60 min with 2 uc (0.2 mM) of ^{14}C-Ch in 6 ml of Ringer solution containing 2×10^{-5} M neostigmine placed in the muscle bath portion of the nerve-muscle chamber. During this incubation the sciatic nerve trunk was stimulated continuously at a frequency of 20/sec. Nerve stimulation was stopped at the end of this loading procedure. Neostigmine was included in the bath to prevent the re-uptake, by the nerve ending, of the choline originated by the hydrolisis of the ACh liberated by the nerve. Collier and MacIntosh (1969)

have shown that the uptake of ACh as such is small.

After incubation with the tracer, the preparations were subjected to 8 successive washings with label-free neostigmine-Ringer solution, to remove most of the free or loosely bound tracer (presumably most of the free Ch) and to establish a washout curve of the tracer until a low steady efflux of it was obtained. It is assumed that this steady efflux represents the residual flux of labelled Ch together with the resting release of labelled ACh. The washout was done by rapidly emptying and refilling the muscle bath at 5 minute intervals with 6 ml of fresh Ringer solution(containing either neostigmine or eserine). At the end of the loading period and of each washing, samples of the muscle bath (100 ul each time) were collected for radioactivity determination.

4. Preferential release experiments

In the experiments designed to test the hypothesis (Collier and MacIntosh, 1969) that newly synthesized transmitter is preferentially released, a second loading procedure was carried out. This second loading was in all respects the same as the first one, except that the radioactive tracer used was ^{3}H-Ch 4 uc (0.04 uM) instead of ^{14}C-Ch. This was followed by a similar washout procedure for 8 periods of 5 min as described earlier. The ^{3}H-labelled transmitter, supposedly formed during the second loading procedure may be considered as being the newly synthesized transmitter. The ^{14}C-labelled transmitter could consequently be thought of as the 'old' transmitter.

After the second washout procedure the incubation medium was changed to choline free neostigmine-Ringer solution, and this was the medium for the whole 'test period'. During the first part of the test

period (15 to 20 minutes before the test stimulation), 3 to 4 samples (100 ul each) were collected at 5 minute intervals, for the measurement of baseline radioactivity in the samples. The baseline radioactivity was taken to equal the mean value of the radioactivity readings obtained during that 15 to 20 min period. The test stimuli were then delivered for 30 minutes, during which 6 other samples were collected (also at 5 min intervals) to measure the release of labelled ACh during the course of stimulation. In the first series of experiments the radioactivity level of the incubation medium was followed for an additional 15 minutes to have some idea about the time necessary for the radioactivity to level off after stimulation was arrested. After the last sampling the muscle bath was emptied and the neostigmine-Ringer solution was replaced for another test period. In this series of experiments the preparations were subjected to two 30 min test periods of electrical stimulation using a frequency of 40 pulses per second.

5. Surplus pool experiments

In another series of experiments the second labelling procedure (with ^{3}H-Ch) was conducted under resting conditions, in an attempt to label the surplus ACh pool. Two different anticholinesterases (neostigmine and eserine) were used in two sets of experiments. After the two successive loading periods with the two different radioisotopes and the following washout procedures, the preparations were subjected to thirty-minute periods of stimulation using electrical stimulation or the exogenous application of ACh, carbachol, or KCl.

In a third group of experiments a modification was introduced in the composition of the bathing fluid. The first loading was performed

in the absence of anticholinesterase, and after the second loading period unlabelled-Ch was constantly present (10^{-5} M). This procedure was adopted to be able to compare our results with those of Collier and Katz (1970).

H. RADIOACTIVITY DETERMINATIONS

1. Preparation of Samples for Measurement of Radioactivity

In the choline uptake experiments, after the incubation period, each tissue was soaked briefly (about 1 sec) into ice-cold Ringer solution, in order to remove adhering radioactive material. It was then gently blotted on Whatman filter paper and immediately weighed, using a precision balance, (Federal Pacific Electric Co.). After weighing, the whole tissue was introduced into a scintillation vial containing 0.3 ml Nuclear Chicago Solubilizer (N.C.S.). The vial was then closed tightly with a plastic cap. The vials containing the tissues and N.C.S. solubilizer were then placed in a Dubnoff shaker and agitated at 70°C for 30 minutes. At the end of this this time the tissues were completely dissolved. The vials were then left to cool for a brief period in melting ice. After cooling, 10 ml scintillation fluid were added to each vial, and the sample was ready for radioactivity measurement.

Samples of incubation media were prepared by pipetting 0.1 or 0.3 ml of each into scintillation vials and adding to each vial 10 ml of scintillation fluid.

2. Scintillation Solutions

Scintillation fluids used were mixtures with relative high efficiency and good water miscibility of the following compositions:

(1) For the solubilized tissue samples:

6 g 2,5-diphenyloxazole (PPO)/litre of toluene.

(2) For the medium samples:

5.65 g PPO, 0.1 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 250 ml ethylglycolmonomethyl-ether (EGME)/litre of toluene.

Radioactivity determinations were performed with the use of a Phillips liquid scintillation spectrometer. A monolabel or a double label technique was used, as appropriate. Channel ratios and external standardization were found to be constant for all vials, so that no correction for quenching was considered necessary.

3. Calculation of Choline Distribution

The number of counts per minute (CPM) obtained by double-label liquid scintillation spectrometry of the samples was used to calculate the distribution of the ^{14}C-labelled-choline and of the extracellular marker ^{3}H-mannitol. This involved the following steps:

Medium ^{14}C content: The final radioactivity of the medium after incubation with the tissue was used for calculation. CPM/sample obtained as raw data from the scintillation counter was corrected for background ^{14}C counts (by subtraction). This value was multiplied by the dilution factor to get the number of CPM/ml of medium for ^{14}C.

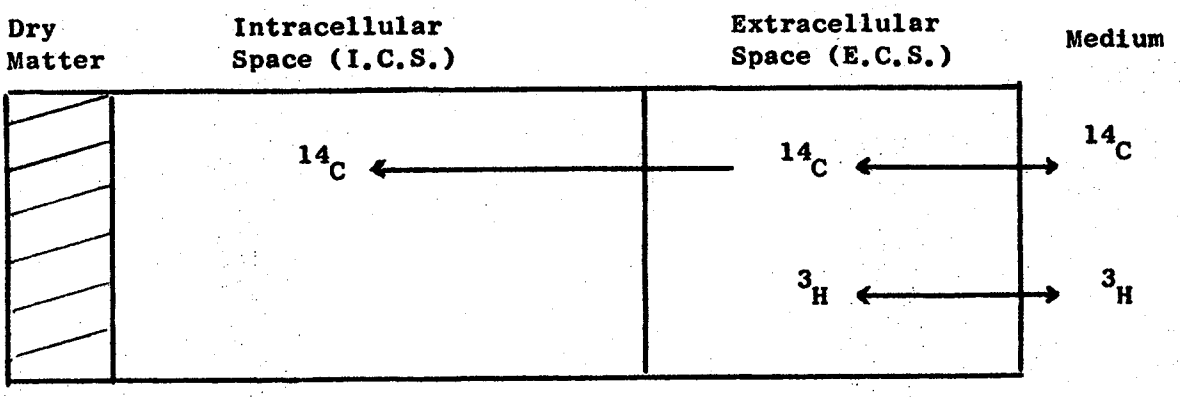
Medium ^{3}H content: ^{3}H background counts were also subtracted from the value of CPM/sample. In addition, however, a certain percentage of ^{14}C overflow (around 13% of the CPM value for ^{14}C in the same sample, which represents the energy value of ^{14}C being counted in the ^{3}H channel) was also subtracted as a correction factor. To obtain the CPM/ml of

medium for ^{3}H this corrected value was multiplied by the dilution factor.

Tissue content of the ^{14}C-labelled Choline:

CPM/sample of wet tissue was corrected for background radioactivity in the same way as described for the medium.

The tissue sample represents the total weighed tissue after the blotting procedure. This tissue is still in wet condition and can conceptually be visualized as consisting of three compartments, the dry matter, the intracellular space and the extracellular space. The tissue in the incubation medium together with the distribution of the two radioisotopes (^{14}C-choline and ^{3}H-mannitol) can be represented diagrammatically as follows:



As indicated in the diagram the total water content of the tissue is composed of the intracellular water and the extracellular water. After equilibration, the extracellular space has the same ionic composition as the incubation medium. The ^{3}H-mannitol present in the medium, which is used as extracellular space marker, only fills the extracellular water space of the tissue. Its distribution in the tissue water is thus an estimate of the extracellular space. Substances penetrating into the cell (into the I.C.S.) occupy a larger space than the E.C.S. of the tissue water.

The apparent extracellular space, taken to equal the mannitol space, was measured in each tissue sample. This value was determined individually for each nerve because it depends somewhat on the amount of adhering medium and, therefore, on the handling of the tissue. It is, thus, an operational parameter reflecting accurately the amount of extracellular water in a particular tissue sample rather than the extracellular space in vivo.

The value of the total tissue water (I.C.S. and E.C.S.) was determined separately for the sciatic nerves by finding the difference in weight of a series of sciatic nerves before and after drying to constant weight, this was done in vacuo in a dessicator at 80°C overnight. The weights were then used for calculation using the following equation:

$$\frac{\text{Wet weight - dry weight}}{\text{Wet weight}} \times 100\%$$

This gave an average value for total water content of:

$$\frac{75.5 \pm 0.6\%}{\text{(mean } \pm \text{ S.E. for 8 determinations)}}$$

For calculations of the per cent penetration of choline, this average tissue water content was corrected for the apparent extracellular space mentioned earlier, which is taken to equal the mannitol space measured in each tissue sample. For measuring the extracellular space in the sciatic nerves, Inulin-^{3}H was first used in preliminary studies, but the values thus obtained had a very large variability. Mannitol-^{3}H, on the other hand, gave more constant results, so that only mannitol-^{3}H was further used as extracellular marker in each uptake experiment. As an example of one series of such measurements the extracellular space of sciatic nerves measured with mannitol-^{3}H gave an average value of

$$\frac{48.2 \pm 3.4\%}{\text{(mean } \pm \text{ S.E. for 6 determinations)}}$$

Further calculation of the Tissue ^{14}C takes the following form:

$$\frac{\text{CPM of tissue sample (corrected for background)}}{\text{water weight of tissue in grams x 0.75 = wet weight of tissue}}$$

represents the value of CPM/ml of tissue water.

The fraction of these two spaces occupied by a substance is usually referred to as the virtual space of that substance. Thus, if the whole tissue water were completely equilibrated with the medium, the virtual space is 100%.

The ratio $\frac{\text{CPM/ml tissue water}}{\text{CPM/ml medium}}$ for ^{14}C = (a), representing the fraction of total choline space.

Tissue content of the ^{3}H-mannitol (the extracellular marker):

This was calculated in a similar way. The only difference being the correction for ^{14}C overflow as was discussed earlier.

The ratio $\frac{\text{CPM/ml tissue water}}{\text{CPM/ml medium}}$ for ^{3}H represents the fraction of the extracellular space = (b).

The results of choline uptake experiments are expressed as per cent penetration of choline into the intracellular water space, i.e. the concentration of choline in the intracellular water is expressed as a percentage of the final concentration of choline in the medium. This term can also be interpreted as the hypothetical volume of intracellular water equilibrated with choline from the medium. Results calculated in this way include a correction for the dry weight of the tissue and for the apparent extracellular space, taken to equal the mannitol space.

The percent penetration can then be calculated according to the following formula:

$$\% \text{ Penetration of choline} = \frac{(a - b)}{1 - b} \times 100$$

or

$$= \frac{\text{Total choline space - mannitol space}}{(1 - \text{mannitol space})} \times 100$$

Knowing the concentration of choline in the medium one can thus calculate the amount of choline influx into the intracellular space of the tissue.

I. MEASUREMENT OF INCREASE IN RADIOACTIVITY LEVEL DURING STIMULATION

In the "preferential release" experiments and the "surplus pool" experiments the content of radioactivity of each sample from the incubation medium of the nerve-muscle preparation was determined by double-label liquid scintillation spectrometry as described earlier. To be able to compare the time course of release of the two radioactive tracers during stimulation, the values of both tracers in CPM/ml, had to be normalized to overcome the great differences between their energies of radiation and also the difference in the specific activities of the two tracers used. To do this the results of the radioactivity contents for both the ^{14}C and the ^{3}H tracers in the incubation medium were expressed as the per cent increase in radioactivity compared to the corresponding mean values of baseline radioactivity for the two tracers during the first 15 to 20 min. of the test period. Both of these baseline values were taken to be equal to 100%. In this way the relative behaviour of the two tracers in reference to their corresponding mean baseline values can be followed during the whole time course of stimulation.

On the assumption that the residual flux from the tissue of labelled Ch at the start of the test period is minimal, the mean base-line radioactivity of the 15 to 20 min period before the test stimulation would reflect the spontaneous release of ACh while the per cent increase in radioactivity during stimulation would represent the increment due to the release by stimulation of labelled ACh.

SECTION III

RESULTS

I. SINGLE SCIATIC NERVE

A. Uptake of Choline

In these experiments the per cent penetration as defined earlier (Methods p. 60) was taken to be a measure of Ch transport across the cell membrane.

1. Resting uptake

Figure 7 shows the results obtained when sciatic nerves were incubated under resting conditions for 60 min at room temperature in varying concentrations of Ch. Penetration values higher than 100% were obtained at the lowest concentration tested (10^{-5} M). As the choline concentration in the medium was increased, the per cent penetration decreased progressively. In the presence of HC-3 (5×10^{-4} M) the per cent penetration for the 10^{-5} M concentration of Ch was reduced far below the 100% value (45.4 ± 3.4%) with a mean value for the differences in per cent penetration between the control and the treated paired sciatic nerves of 76%. The mean of the differences in per cent penetration between untreated and HC-3 treated nerves was reduced as the concentration of Ch used was increased. No decrease in per cent penetration with this concentration of HC-3 could be detected when the concentration of Ch used was 10^{-2} M or higher.

The significance of the difference between the means of the control and treated nerves was determined by Student's t-test applied to the paired data of per cent penetrations in the absence and presence of HC-3.

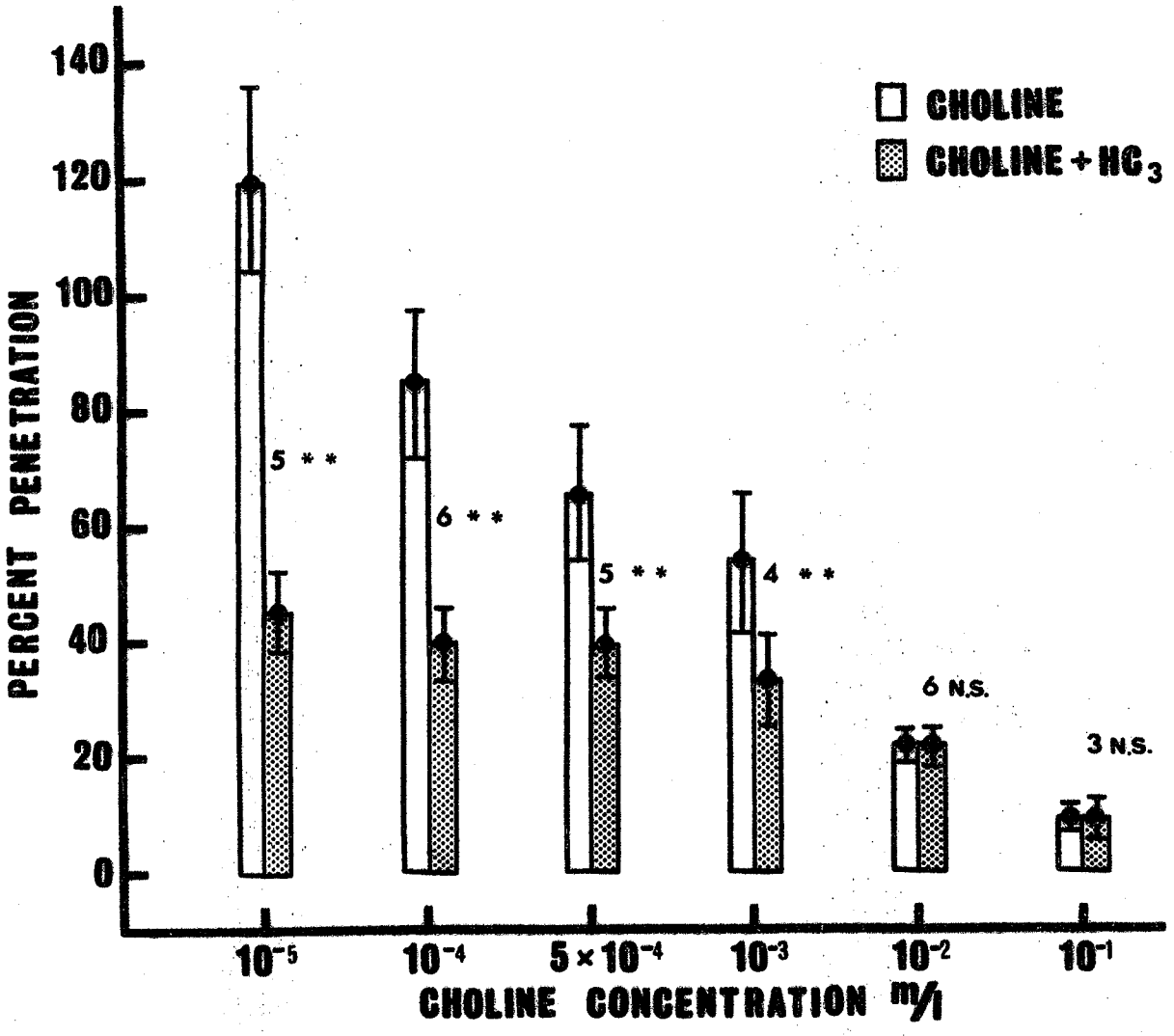
The penetration value derived from the intracellular concentration of C^{14} choline after 60 min of incubation, is a measure of the net

Fig. 7. Uptake of choline by isolated sciatic nerves in the absence and presence of hemicholinium-3 (HC-3: 5×10^{-4} M). The sciatic nerves were incubated for 60 minutes in different concentrations of choline, as described in Methods. The blank columns represent the control and the stippled columns represent the treated nerves. Vertical lines represent the standard error of the mean calculated from the total number of pairs of experiments indicated by the figures next to the columns. Incubation condition: resting at room temperature (20°C) in 2 ml Ringer solution. ^{14}C-choline (10 mc/mM) was present at concentrations giving between 40000 and 60000 counts per min/ml, together with traces of ^{3}H-mannitol as extracellular marker giving about three times as many counts. Significance levels:

$P < 0.01$ **

$P < 0.05$ *

N.S. non-significant


140
120
100
80
60
40
20
0
PERCENT PENETRATION
CHOLINE
CHOLINE + HC3
5 * *
6 * *
5 * *
4 * *
6 N.S.
3 N.S.
10-5
10-4
5 x 10-4
10-3
10-2
10-1
CHOLINE CONCENTRATION m/l

influx of choline. Therefore the effect of different concentrations of choline in the external medium upon its influx into the nerve can be calculated from the penetration data in terms of the net Ch influx and expressed as n-mole/ml per hour. The Ch influx pattern presented (Fig. 8) is consistent with a two-component uptake mechanism of Ch into the sciatic nerve, similar to that described for the giant axons of Loligo (Hodgkin and Martin, 1965). The first component of the uptake mechanism is a carrier mediated transport, which can be demonstrated only at low concentrations of Ch (10^{-5} M), where Ch is found inside the nerve at concentrations higher than in the medium. This first component could account for the differences between the measured influx in the absence and presence of HC-3, a drug which is known to have a highly specific inhibitory effect on the uptake of Ch (Gardiner, 1961; Hodgkin and Martin, 1965; Potter, 1967). The second component is a passive transport (diffusion) which allows Ch to enter the axons at a rate proportional to the external concentration.

Figure 8 represents the influx of Ch resulting from different concentrations in the external medium. It can be seen from this figure that with HC-3 (5×10^{-4} M) the influx of choline is reduced. However, the influx of choline still increases linearly with increasing concentrations of Ch in the medium.

Using the same data, the inhibitory effect of HC-3 on the transport of Ch can further be demonstrated by the double reciprocal plots (Lineweaver-Burk, 1934), of the rate of influx versus Ch concentration in the medium in the absence and presence of HC-3 (Fig. 9). Two straight lines were obtained, which intersect at the same point on the

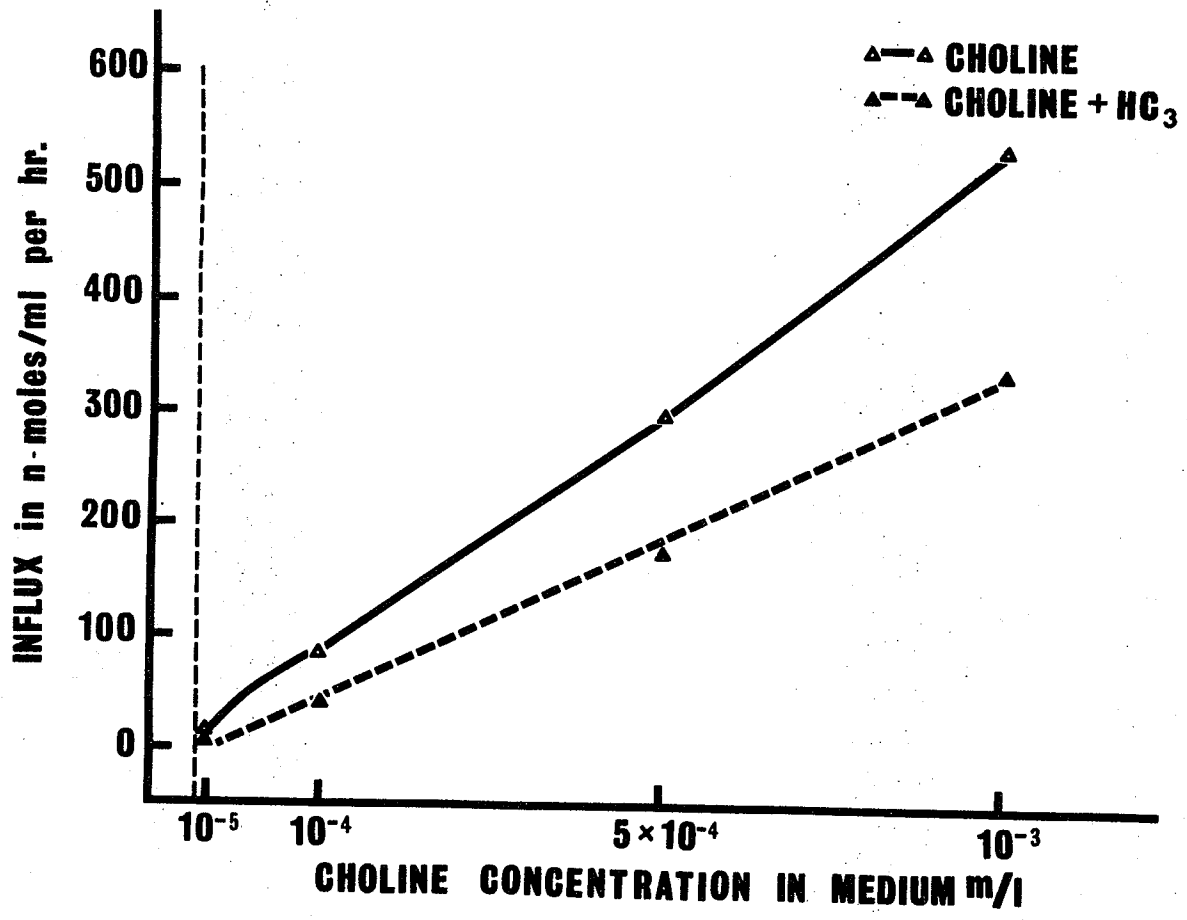
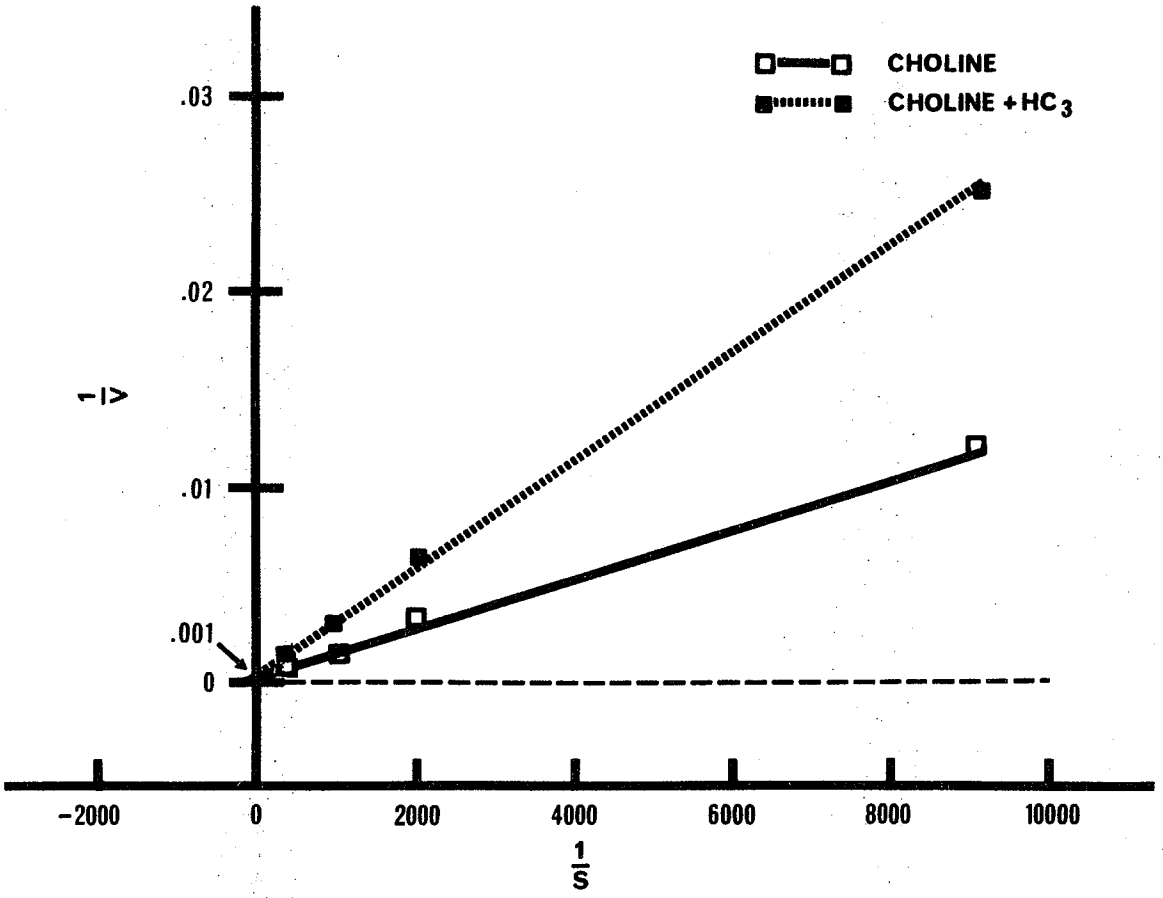


Fig. 8. Effects of different choline concentrations on the influx of choline in the absence and presence of HC-3 (5×10^{-4} M). Data were calculated from the mean values of per cent penetrations of choline in Fig. 7. Values were obtained by transforming the mean values of per cent penetration into the absolute amounts of Ch influx per ml intracellular water per hour. The best fit curve and line were estimated by eye.

Fig. 9. Lineweaver-Burk plot of choline uptake in the absence and presence of hemicholinium-3 (5×10^{-4} M) in the incubation medium. S is the concentration of choline in the medium (M/l) and V is the choline flux into the sciatic nerves (n-mole/ml intracellular water per hour). The data are from the same experiments presented in Fig. 7 and 8. The lines fitted to the control (□) and to the solid squares intercept the ordinate at the same point (0.001). These lines intercept the abcissa at $-1/Km$ and at $-1/Km\ (1+ {}^{i}/Ki)$.

Km is the apparent dissociation constant for choline (6.2×10^{-4} M). i is the concentration of HC-3 (5×10^{-4} M/l) and K_i its apparent dissociation constant. The intercepts of the two lines with the ordinate is $1/V_{max}$ where V_{max} is the apparent maximum flux of choline (in this case 833 n-mole/ml intracellular water per hour).

The lines of best fit for the two series of data points were estimated by eye. The data shown were derived from the mean values of choline influx presented in Fig. 8.


CHOLINE
CHOLINE + HC3
.03
.02
.01
.001
0
-I>
-2000
0
2000
4000
6000
8000
10000
1/S

ordinate (b: 0.001) indicating that the inhibition is most likely of competitive type (Schuberth and Sundwall, 1967).

Due to the wide range of Ch concentrations used (10^{-5} to 10^{-3} M) the y-intercept obtained in this Figure is close to zero. A negative K_i value was derived (for explanation see DISCUSSION).

2. Anaerobic incubation

The uptake of choline by sciatic nerves incubated anaerobically was compared with that in aerobic conditions in a medium containing 10^{-4} M of choline. As shown in Table 1, the uptake of choline during anaerobic incubation (incubation in an atmosphere of 95% nitrogen and 5% carbon dioxide) was consistently decreased.

TABLE 1

EFFECT OF ANAEROBIC CONDITION ON CHOLINE UPTAKE IN SCIATIC NERVES. INCUBATION WITH 10^{-4} M Ch, 60 MIN, RESTING CONDITION, AT ROOM TEMP. (20°C)

Sciatic Nerve Pair No.	Aerobic condition (95% O_2 - 5% CO_2)	Anaerobic condition (95% N_2 - 5% CO_2)
	% Penetration	
1	128.2	93.3
2	159.0	143.4
3	73.5	51.5
4	134.4	76.3
5	129.6	99.4
6	80.5	78.0
Mean ± S.E. M.	117.5 ± 13.6	90.3 ± 12.6

Difference in % Penetration: 27.2

t : 3.5186 ** ($P < 0.01$)

3. Influence of nerve stimulation on choline uptake

The values of the differences in per cent penetration of choline into stimulated and unstimulated sciatic nerves are shown in Fig. 10. These are results from experiments in which the control and treated nerves were mounted on double-compartment nerve chambers (see METHODS) for incubation. While the treated nerves were being stimulated, the electrode wires of the control nerves were left unconnected. Nerve stimulation with different frequencies (1, 5, 20 and 40 pulses/sec) during 60 min each, tested on 4 pairs of nerves, did not result in any significant change in Ch uptake by the sciatic nerves. Analysis of variance of the differences gave an F ratio of 0.470 which is non-significant (S.E. of the mean: 20.7%). It should be noted however that with frequencies of stimulation lower than 20 pulses/sec, there was a progressive decrease (not significant) in the mean uptake. At higher frequencies (40 per second) there was an increase in the mean Ch uptake (also not significant).

B. Migration of labelled choline along sciatic nerve axons

We found in a series of 5 experiments in which sciatic nerves were incubated for 24 hours under constant stimulation of 2 pulses/sec (METHODS), that the radioactivity level in the distal well (with respect to the stimulating point, which was bathed with ^{14}C-choline) rose only up to about 10 times the background activity. This suggests that no significant migration of labelled Ch occurred through the axon. At least, it is obvious that Ch flux through the axon can not be an important source of Ch to sustain ACh synthesis during nerve activity.

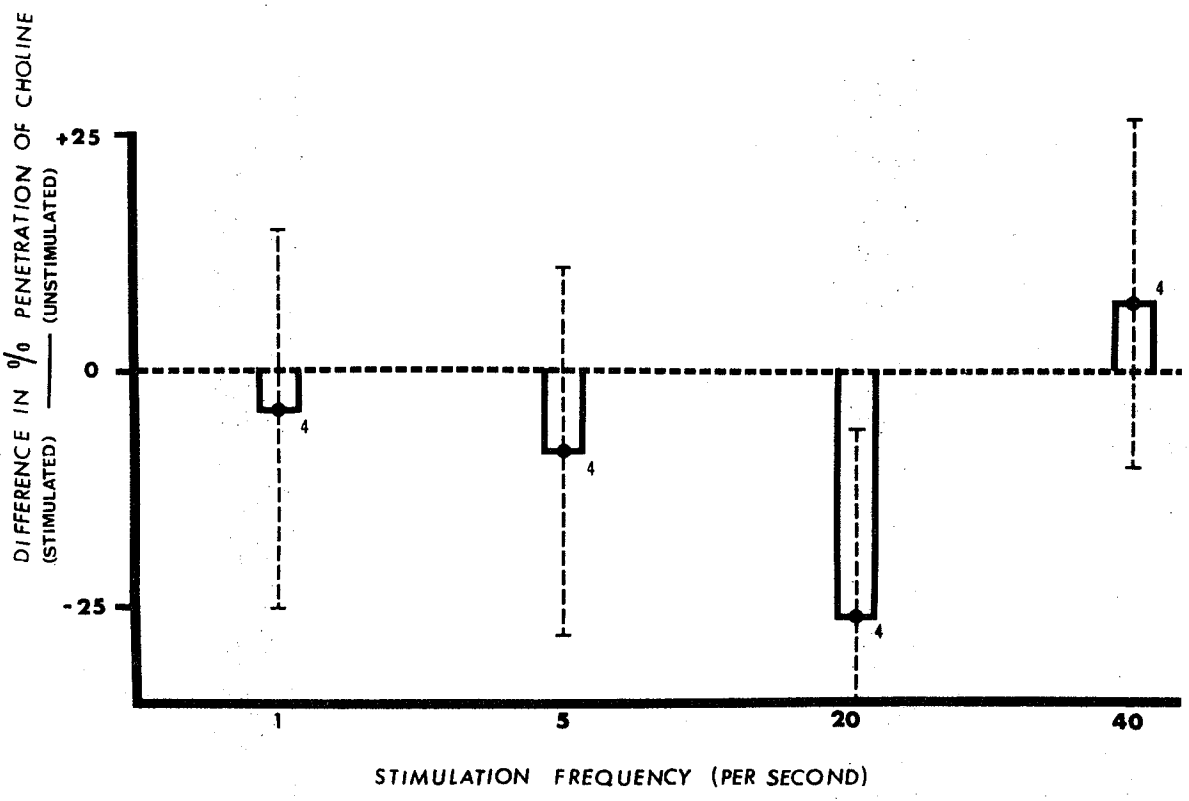


Fig. 10. Effects of electrical stimulation (1, 5, 20, 40/sec) on choline uptake.

Figures next to the columns indicate number of pairs of sciatic nerves tested.

Incubation: in 2 ml Ringer solution containing 10^{-4} M choline at room temperature (20°C).

II. SCIATIC NERVE-GASTROCNEMIUS MUSCLE

Preferential release experiments

Six nerve-muscle preparations were loaded successively with two radioactive tracers, first with ^{14}C-choline and the second time with ^{3}H-choline, using the standard procedure for loading described in METHODS. Each of these loading periods was followed by a washout procedure. As the ^{3}H tracer in this experimental procedure is the more recently introduced tracer, it represents the newly synthesized transmitter. Such doubly loaded nerve-muscle preparations were subjected to two test periods and the radioactivity level of the muscle baths was regularly monitored. It was found that the ^{3}H-tracer released during the two test periods was consistently higher than the corresponding ^{14}C-tracer (Fig. 11).

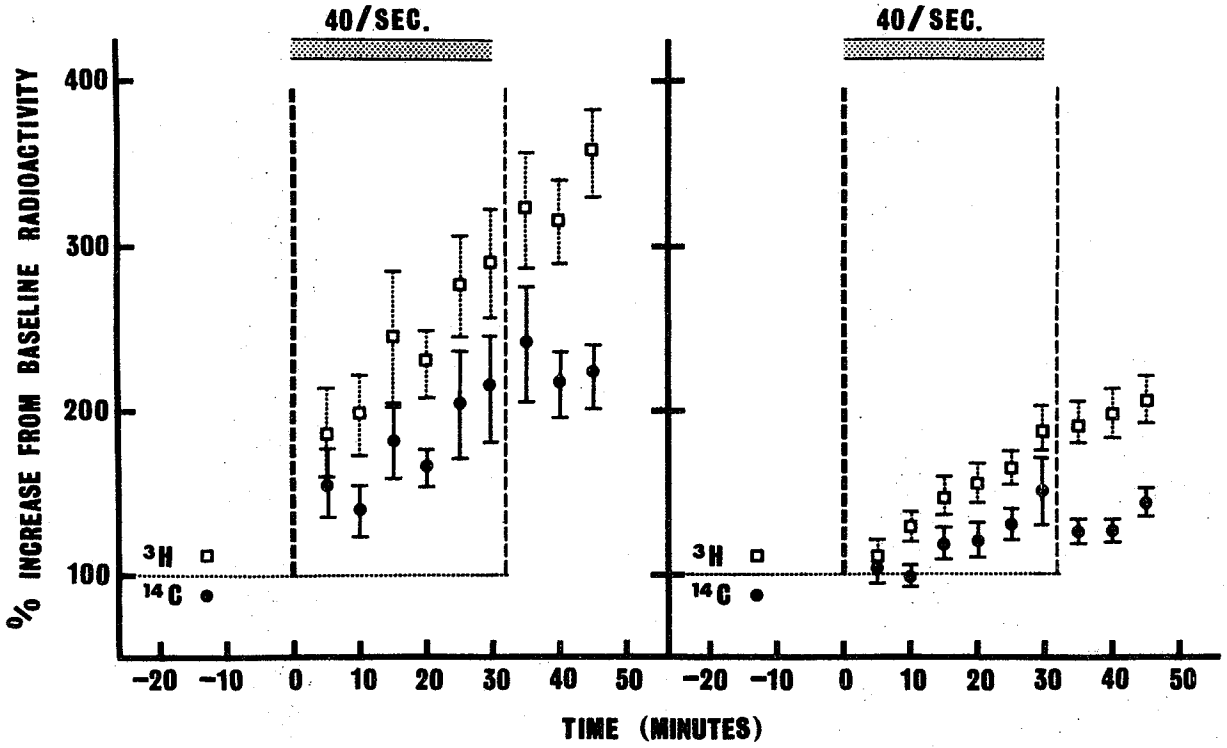
Further analysis of the data using analysis of variance plus orthogonal comparisons (as will be described later) revealed the linearity of the increase in radioactivity levels for both tracers during the whole time course of stimulation.

Statistical analysis of the double tracer release data

An analysis of variance with orthogonal contrasts for single degree of freedom comparisons (Steel and Torrie, 1960) was used to determine the nature of the response curve of the two tracers released by stimulation. The values of release in per cent increase of radioactivity from baseline, obtained at six equally spaced intervals of time (see METHODS), were subjected to this analysis.

Table 2 is an example of this statistical analysis. It illustrates the analysis of the results from the 30-min period of stimu-

Fig. 11. The time course of release of ^{14}C-labelled ACh (●) and ^{3}H-labelled ACh (□) during two stimulation periods (40/sec. 30 min) from the sciatic nerve-gastrocnemius muscle preparation which had been loaded with ^{14}C-Ch and ^{3}H-Ch successively during two standard loading procedures followed by two washout procedures. Black dots represent the mean values of per cent increase from baseline radioactivity for ^{14}C-tracer, and open squares for the ^{3}H-tracer. Baseline value for ^{14}C was the mean value of ^{14}C radioactivity level during the first 15 minutes before the start of stimulation. The mean value of ^{3}H level during that same period of each panel was also taken as the baseline radioactivity level for ^{3}H. The vertical lines represent the standard errors of the means (of 6 experiments) derived from values in each individual interval.


40/SEC.
40/SEC.
% INCREASE FROM BASELINE RADIOACTIVITY
400
300
200
100
^3H
^{14}C
^3H
^{14}C
−20 −10 0 10 20 30 40 50
−20 −10 0 10 20 30 40 50
TIME (MINUTES)

lation shown in the first panel of Fig. 11. The results obtained by such analysis are displayed in Table 2.

TABLE 2

Analysis of variance of release data

SOURCE OF VARIATION	Degrees of freedom	Sum of Squares	Mean Square	F Ratio
BLOCKS	5	176764	35353	20.808
TREATMENTS:	11 :	148852	13532	7.964
Comparisons:				
1. T1 vs T2	1	68450	68450	40.288**
2. Linear	1	65897	65897	38.785**
3. Curvature (quadratic function)	1	576	576	0.339 N.S.
4. Slopes	1	2853	2853	1.679 N.S.
Remainder	7	11076		
ERROR	55	93442	1699	
TOTAL	71	419059		

Highly significant ** $P < 0.01$

Significant * $P < 0.05$

Not significant (N.S.) $P \geq 0.05$ statistically

As indicated in this table, the source of variation for treatments was partitioned into several components listed under "Comparisons".

This included the following tests using coefficients for orthogonal comparisons:

1. Differences between tracers ($Tracer_1$ versus $Tracer_2$).
2. Linear relationship with time.
3. Curved relationship with time (quadratic or second degree function).
4. Difference in linear relationship with time (slopes).

More complex relationships grouped into Remainder were not investigated.

Highly significant F ratios ($P < 0.01$) were obtained for comparisons 1 and 2. This means that the amount of tracer 1 is significantly different ($P < 0.05$) from the amount of tracer 2 (in this case, the increase in ^{3}H was greater than the increase in ^{14}C, as shown in the first panel of Fig. 16) and that the response curves of the two tracers are linear. A non-significant F ratio ($P \geqslant 0.05$) for quadratic response means that a parabola would not fit the data better, which is in effect a confirmation of the significant finding for linearity. In other words, the increase for each additional increment of both tracers during the time course of stimulation is constant. The non-significant finding for the fourth comparison, testing for difference in slopes, means (in addition to the findings in the other three comparisons) that the two response curves (per cent increase of radioactivity vs. time) for the two tracers are parallel. A similar statistical analysis of the data from panel 2 of Fig. 11 revealed the same significant values of F ratios for the first two comparisons and a non-significance for quadratic function. The fourth comparison, however, resulted in a significant F ratio implying

the existence of a difference in slope between the two response curves in that panel. Based on the results obtained by this statistical analysis with the four types of comparisons, it is possible to represent the release data of the double tracer experiments by simple graphs. The two response curves of the first panel of Fig. 11 for example, can essentially be represented by two parallel lines. The one above would represent ^{3}H tracer which was released in greater quantity. In the second panel the two lines have different slopes, as indicated by a statistically significant F ratio for the fourth comparison. The summary of these release data will be presented later (in Fig. 16).

Addition of HC-3 (10^{-2} M) to the muscle bath during the second loading period (with ^{3}H-Ch) resulted in a decreased response curve for ^{3}H tracer (Fig. 12 C and D and Fig. 16, second row panels C and D). This overlapping of the two curves implies that the increase in the release of both ^{14}C and ^{3}H tracers during stimulation is proportionately the same. Thus the ratio of ^{3}H over ^{14}C during stimulation remains the same as the baseline ratio. In panel A, row I, of that same figure, however, although the ratio of ^{3}H over ^{14}C during the last 25 min of stimulation remains constant, (the two response curves remain parallel), this ratio is different from the baseline ratio. This means that ^{3}H release increased proportionately more than ^{14}C release under stimulation.

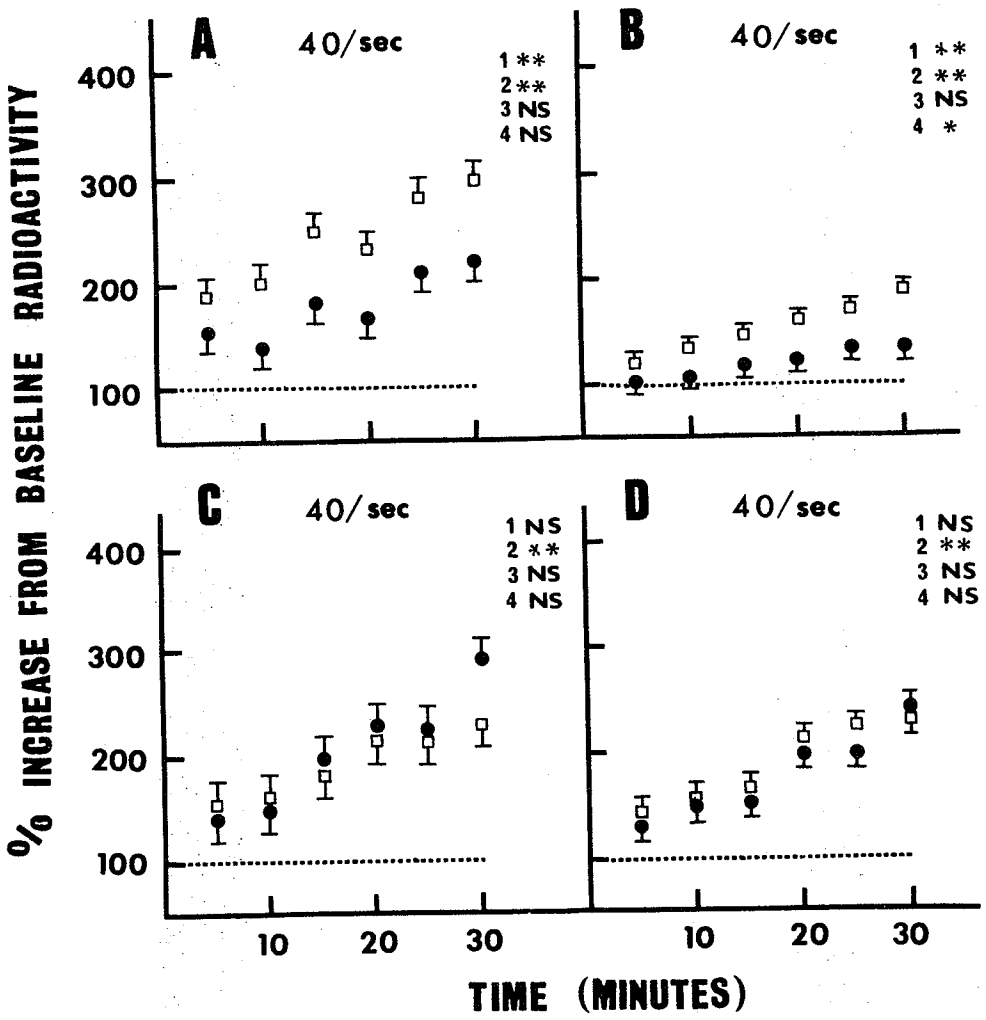
Surplus Pool Experiments

Either neostigmine or eserine (2×10^{-5} M) was used as the acetylcholinesterase (AChE) inhibitors in this series of experiments. In these experiments, during the second loading procedure (with ^{3}H choline) the nerve was not stimulated to provide optimal conditions for the

Fig. 12. PREFERENTIAL RELEASE EXPERIMENTS

^{14}C (●) and ^{3}H (□) release by electrical stimulation (40/sec, 30 min). Results shown in panels A and B correspond to the results in Fig. 11 (6 experiments). Standard error values were obtained from the error terms of the analysis of variance. C and D show the release data obtained when loading of the second tracer (^{3}H) was in the presence of HC-3 (10^{-2} M) (4 experiments). The figures correspond to the numbers of comparison indicated in the analysis of variance table. The significant values for the tests involved are indicated by asterisks.

PREFERENTIAL RELEASE



formation of excess ACh (surplus pool). After completing the loading procedures, each preparation was subjected to four successive test-periods using either electrical impulses (40/sec), exogenous ACh (2×10^{-5} M), carbachol (1×10^{-4} M), or KCl (40 mM) to stimulate release of the tracers. The results from experiments with neostigmine are shown in Fig. 13, and from those with eserine in Fig. 14. The effects of the ACh releasers (electrical impulses 40/sec, exogenous ACh, carbachol and KCl) on the patterns of release of both tracers were compared. It was found that the patterns of release were the same with either AChE inhibitor when electrical stimulation, ACh or carbachol were used to stimulate release (Fig. 13 and Fig. 14 panels A, B and C). For comparisons see also Fig. 16 row 3 and 4, panels A, B and C.

In these three pairs of panels, the increase in ^{3}H tracer was consistently greater than the increase in ^{14}C during the 30 min time course of stimulation (highly significant, comparison No. 1). The response curves were linear (comparison No. 2, also highly significant). The comparisons for curvature (No. 3) were non-significant, except in the eserine series when carbachol was used (Fig. 14 C). Here comparison No. 3 was found to be significant ($P < 0.05$). However, the more predominant pattern in this panel is a linear pattern (highly significant, for comparison No. 2). There was no slope difference in the response curves due to 40/sec and ACh stimulation (comparison No. 4: not significant). However, in the carbachol experiments the slopes of both response curves were different in both series. The ^{3}H slopes were consistently greater than the ^{14}C slopes.

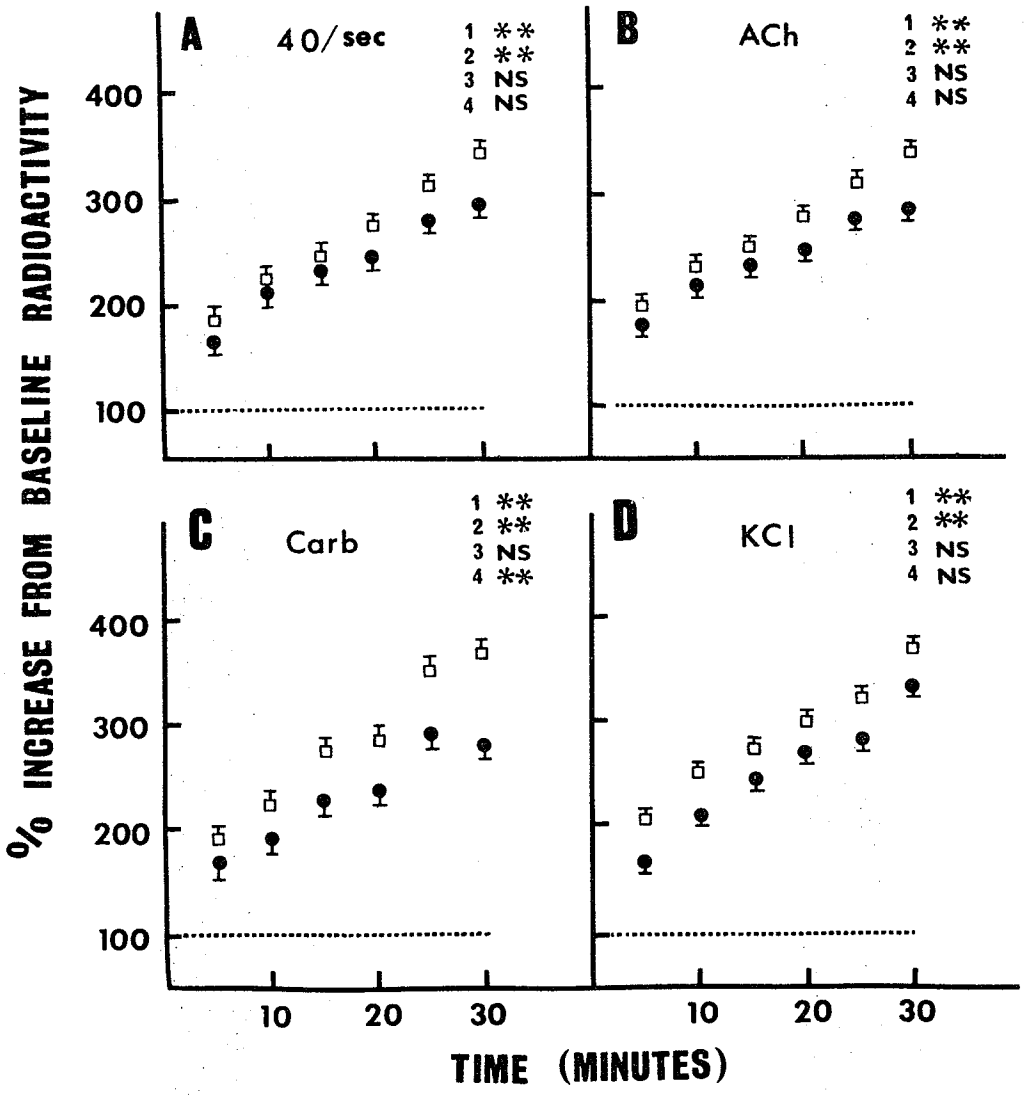
The results obtained with KCl stimulation (40 mM, 30 min)

Fig. 13. SURPLUS POOL EXPERIMENTS - Neostigmine as anti-cholinesterase agent (8 experiments).

The effects of nerve stimulation (30 min at 40/sec) and applications of ACh, (2×10^{-5} M); carbachol, (1×10^{-4} M); and KCl, (40 mM), on the release of ^{3}H and ^{14}C are shown in panels A to D respectively, corresponding to the sequence of the test periods in the experiments. The figures in the upper right hand corners of the panels refer to the comparison numbers in the analysis of variance table. The signs after the figures indicate the statistical significance of the comparisons employed.

● □ Black dots represent the mean values for ^{14}C ('old' transmitter) and open squares for ^{3}H ('new' transmitter). Standard errors are derived from the error terms of the analysis of variance.

NEOSTIGMINE



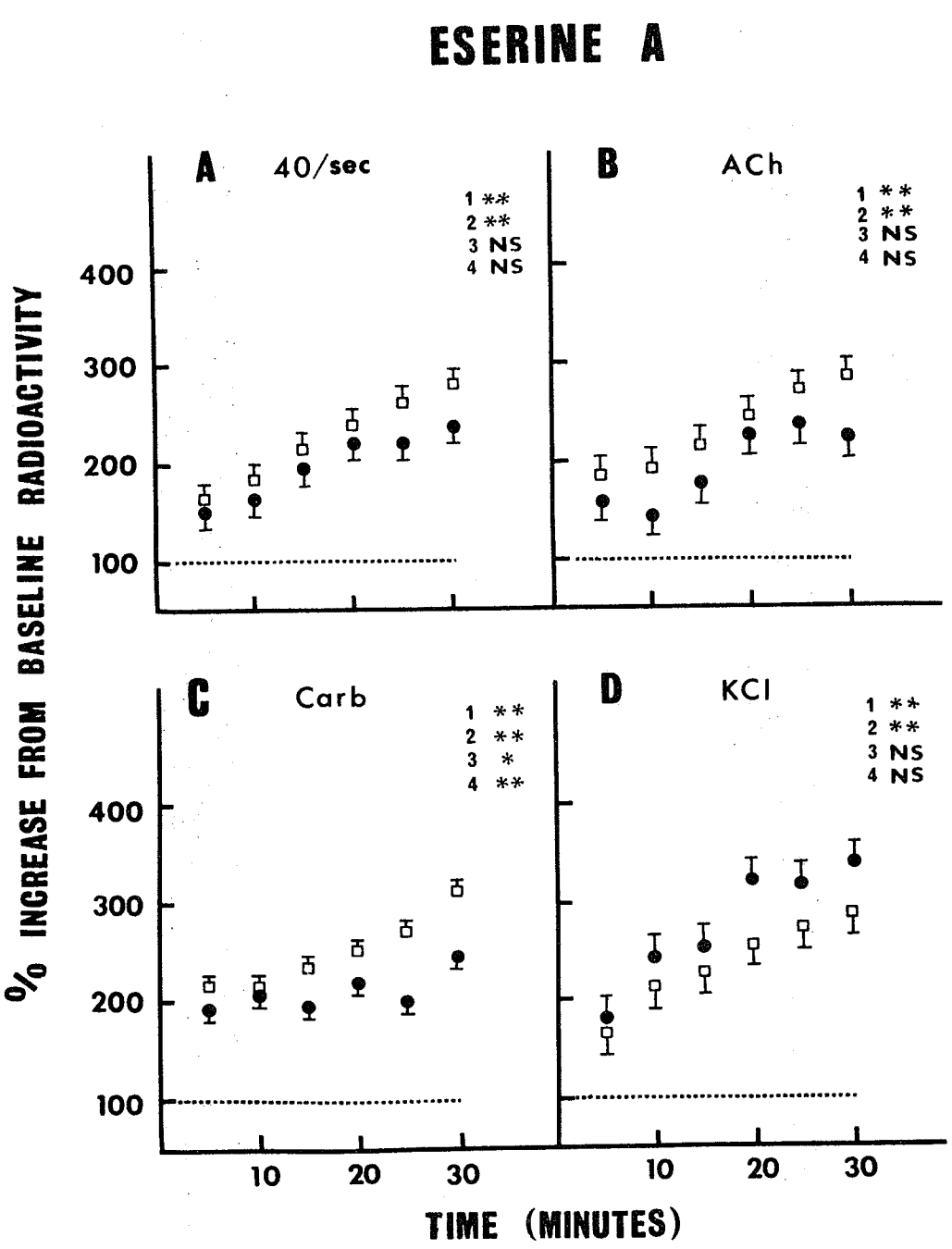


Fig. 14. SURPLUS POOL EXPERIMENTS - Eserine as anticholinesterase agent (6 experiments).

● ^{14}C tracer ('old'); □ ^{3}H tracer ('new')

The same legend given in Fig. 13 applies to this figure.

(Fig. 13D and Fig. 14D) show a greater amount of ^{3}H release in the neostigmine series, but the reverse is true for the eserine series. The four statistical comparisons employed on the data resulted in identical findings, i.e. a highly significant difference for the first two comparisons and non-significant for the third and fourth comparisons. Taken together, this indicates that both response curves are linear and parallel to each other.

In the third series of surplus pool experiments, the composition of the bathing fluid was changed to conform with the method described by Collier and Katz (1970). The first loading procedure was performed in the absence of an anticholinesterase in the bathing fluid. Eserine-Ringer solution was used only after the first half of the first washout period. The purpose of introducing eserine about 20 minutes before the second loading period was to assure that the tissue was well eserinized by the time the second tracer was loaded to label the surplus pool. After this loading under resting conditions, the bathing fluid used for the rest of the experiment contained unlabelled Ch (10^{-5} M). Similar test stimulations were used in this series of experiments. Results are shown in Fig. 15.

The release data for the two radioactive tracers shown in the four panels of Fig. 13, 14 and 15 were obtained during four consecutive test-periods. In contrast to the results shown in the first three panels from Fig. 13 and 14 (surplus pool experiments, Neostigmine and Eserine-A) no difference could be detected in this third series of surplus pool experiments (Eserine-B) between the amounts of tracers released by 40/sec electrical impulses, ACh or Carbachol (panels A, B and C of Fig. 15).

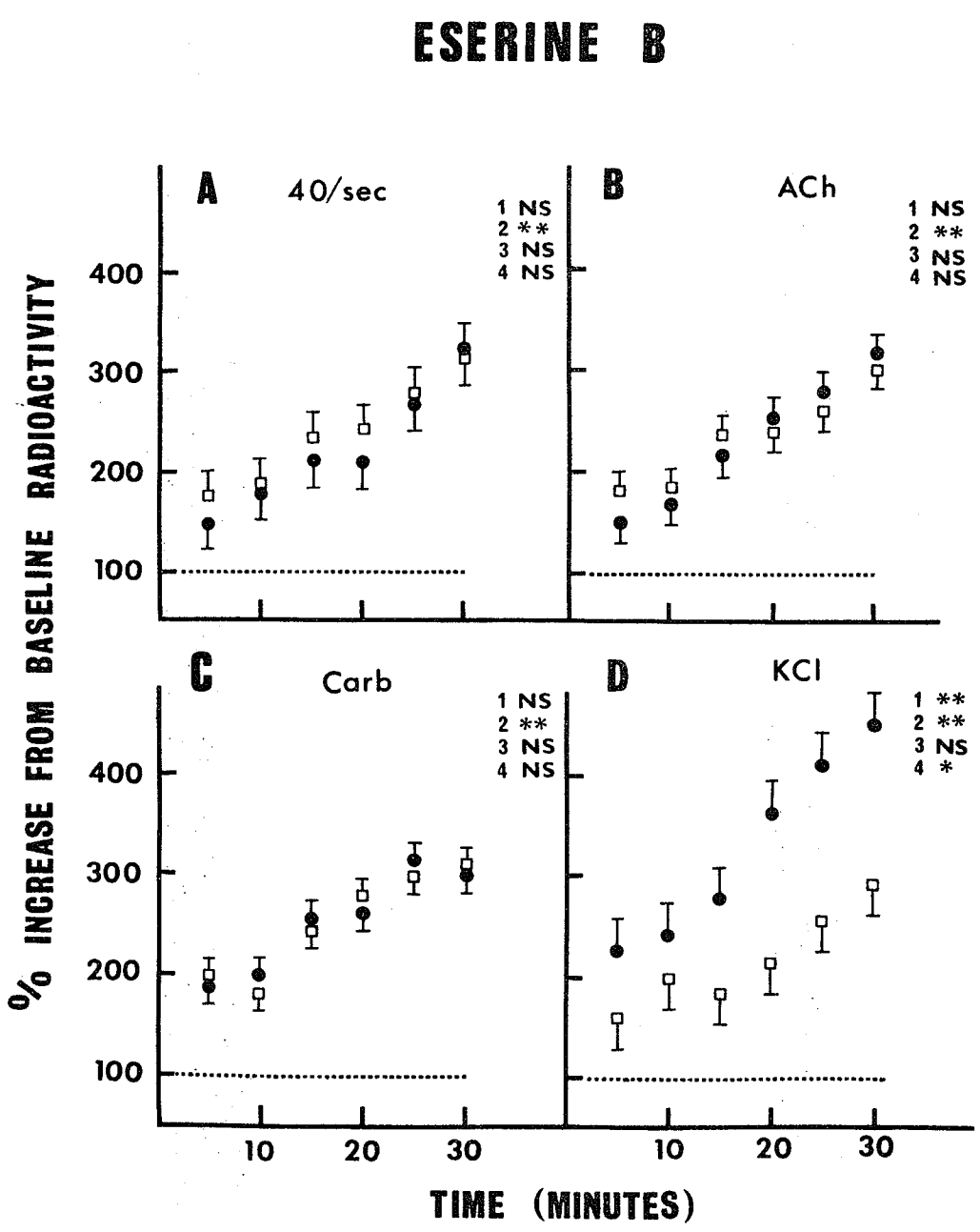


Fig. 15. SURPLUS POOL EXPERIMENTS - Eserine-B
(6 experiments)

• ^{14}C tracer ('old'); □ ^{3}H tracer ('new').

Same legend as in Fig. 13.

For comparisons see also Fig. 16, last three rows. The response curves (based on the findings of the orthogonal comparisons) are linear,(i.e. highly significant for linearity and not significant for curvature). This means that in this series of experiments (Eserine-B) the three stimulation procedures (40/sec, ACh, and Carbachol) resulted in linear response curves of the two tracers which are superimposed.

The effect of KCl on the release of the two tracers is shown in panels D of Fig. 16 for the three series of "surplus pool" experiments. In the experiments in which neostigmine was used as anticholinesterase, the response curves produced by KCl (40 mM) and electrical stimulation (40/sec) were similar. However, when eserine was used as anticholinesterase the pattern was reversed, ^{14}C rather than ^{3}H was released in preference. Increasing three times the concentration of KCl (120 mM), a divergence of the two response curves was observed in the Eserine-B series. The ^{14}C-slope became much steeper than the slope of the ^{3}H tracer (Fig. 15, D and Fig. 16, row V, D).

The loading conditions of the double tracer experiments and the release patterns of both tracers (^{14}C and ^{3}H) in response to stimulation are summarized in Table 3 and its accompanying Fig. 16.

TABLE 3

LOADING CONDITIONS OF THE DOUBLE-TRACER EXPERIMENTS

	EXPERIMENTS	N	LOADING 1	WASHOUT	LOADING 2	WASHOUT
I	PREFERENTIAL RELEASE	6	^{14}C-Ch, 60 min + E.S. 20/sec Neostigmine°	8 x 5 min	^{3}H-Ch, 60 min + E.S. 20/sec	8 x 5 min
II	+ HC-3 during second loading	4	" Neostigmine°	"	" +(HC-3, 10^{-2}M)	"
III	SURPLUS POOL -Neostigmine	8	+ E.S. Neostigmine°	"	Resting	"
IV	-Eserine A	6	+ E.S. Eserine°	"	Resting	"
V	-Eserine B	4	+ E.S. No anti-ChE	" + Eserine°	Resting	" + unlabelled Ch.°

E.S. electrical stimulation
N number of experiments
° indicates continuous presence for the rest of the experiment.

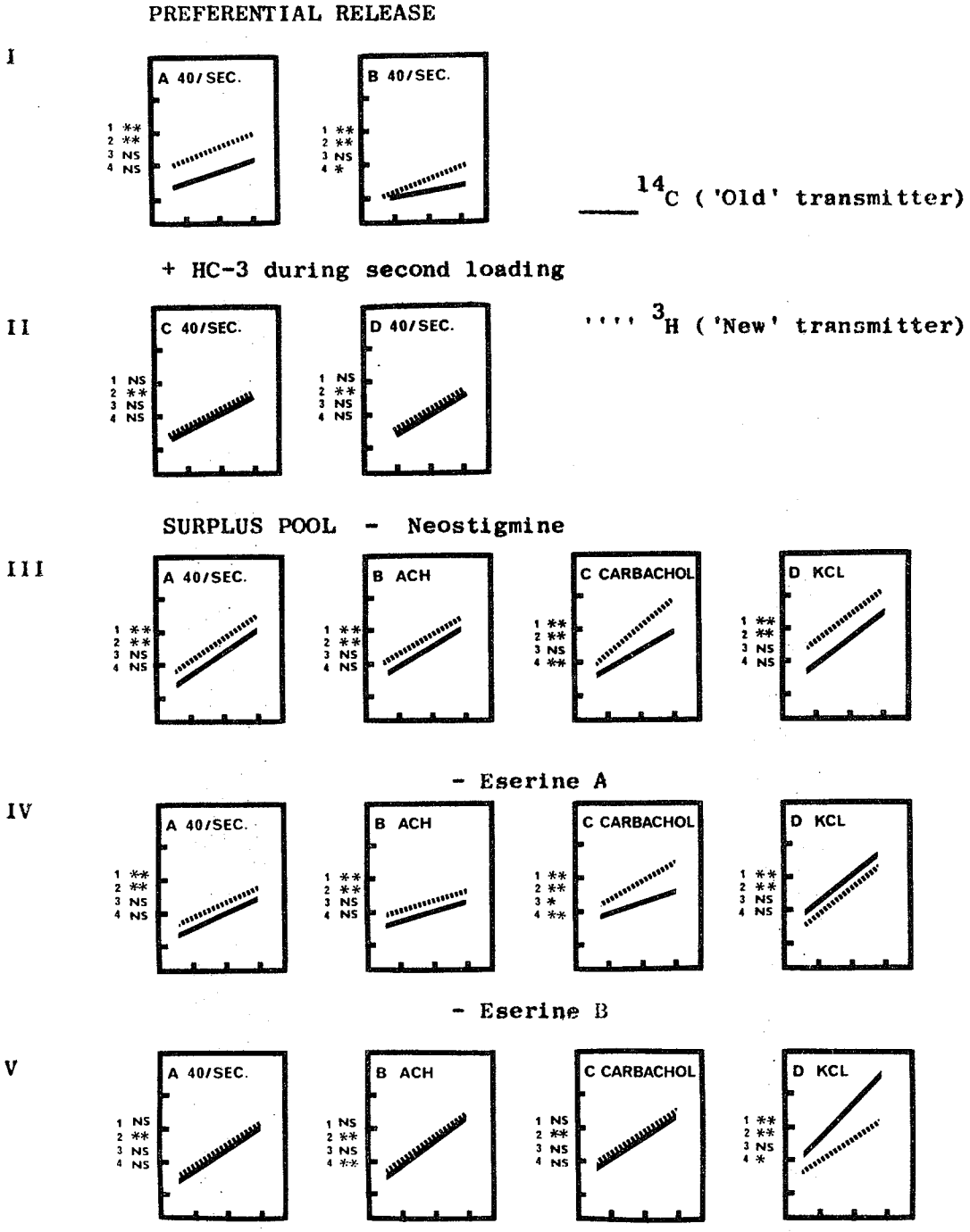


Fig. 16. Summarized release data of the double-tracer experiments.

DISCUSSION

Choline uptake in the motor nerve-skeletal muscle preparation reflects the total of the uptakes by the nervous- and muscular structures. Since the motor nerve terminals represent only a minute fractional volume of the muscle bulk, choline uptake by the muscles can be expected to obscure its uptake by nerves. It became necessary, therefore, to study choline uptake in the sciatic nerve-part of the nerve-muscle preparation.

A. Uptake of Choline by the Sciatic Nerves

When single sciatic nerves of the frog were incubated in a medium containing choline, it penetrated into the nerves and the degree of this penetration was dependent on the concentration of choline in the medium (Fig. 7). With low concentration of choline (10^{-5} M), 60 min incubation at room temperature (20°C) resulted in an accumulation of choline in the tissue. The value obtained for per cent penetration into the intracellular water was 121 ± 14% (mean ± standard error for 5 experiments), i.e. the intracellular water accumulated choline on the average in concentrations 1.2 times higher than the choline concentration in the surrounding medium. This observation of uptake against a concentration gradient suggests an active transport system for choline. The accumulation of choline is probably an energy requiring process, as suggested by the finding that choline uptake is inhibited under anaerobic condition (Table 1). The values of per cent penetrations obtained, decrease progressively as the concentration of choline in the incubation medium increases (Fig. 7). In terms of the absolute amounts of choline crossing the cell membranes, however, the influx of choline increased progressively with increasing concentrations of choline in the external medium, as shown in Fig. 8.

Addition of hemicholinium-3 (5×10^{-4} M) to the incubation media resulted in strong inhibition of choline uptake especially apparent in the decrease of the per cent penetration when the concentration of choline present in the medium was low (Fig. 7 and Fig. 8). The decrease of the per cent penetration values of choline by 5×10^{-4} M HC-3 became progressively smaller with increasing concentrations of choline present in the medium, until finally at external choline concentration of 10^{-2} M and 10^{-1} M, no decrease in per cent penetration values could be detected (Fig. 7). It is shown in Fig. 8 that the influx of the different concentrations of choline was reduced by the presence of HC-3 (5×10^{-4} M) to values which increased linearly with increasing concentrations of choline in the medium. This linear component of choline entry indicates passive diffusion to be one pathway for choline uptake. The reduction of choline influx by HC-3 to values associated with the linear component probably represents the part of uptake mediated through a postulated 'carrier' mechanism. These findings are consistent with the existence of a two component uptake mechanism for choline, in the peripheral nerve, similar to that proposed by Hodgkin and Martin (1965) for the giant axons of loligo. Double reciprocal plots of the rate of uptake versus Ch concentration in the incubation medium in the absence and presence of HC-3 (Fig. 9) showed the same value of y-intercept. This is compatible with a competitive type of inhibitory action of HC-3 on the uptake of choline as postulated by Schuberth and Sundwall (1967). The apparent Km value for uptake obtained from the sciatic nerves was 6.2×10^{-4} M as compared to 1×10^{-4} M obtained for choline uptake by squid axons (Hodgkin and Martin, 1965). The negative value of Ki, however, needs some clarification.

This parameter was computed on the basis of data obtained from a very wide range of external choline concentrations (10^{-5} to 10^{-3} M). The two component mechanism of uptake for choline has been mentioned earlier to consist of 1. a carrier mediated transport and 2. a passive diffusion. As can be seen in Fig. 8, as one increases the concentration of choline in the medium, one reaches a point where carrier transport of choline becomes insignificant in comparison with the transport due to passive diffusion. Because of the higher passive diffusion at high concentrations of choline the total velocity of entry could then be expected to be in excess of the limiting velocity (Vmax) attainable by saturation of the carrier mediated transport. This would account for the negative Ki value obtained using derivations of the equations that apply to double reciprocal plots of competitive inhibition (Webb, 1963).

Repetitive electrical stimulation using different frequencies (1, 5, 20 and 40 pulses/sec during 60 min incubation) has no obvious effect on the uptake of choline, (Fig. 10). Although the analysis of variance on the differences in per cent penetration of choline (stimulated - unstimulated) does not reveal any significant difference from zero, the tendency under electrical stimulation using frequencies of 1, 5, and 20/sec is to decrease uptake, while 40/sec seems to increase it. Further work should be done to improve the incubation method under electrical stimulation so as to decrease the variability of the results, and to detect any differences in choline uptake due to stimulation. However, the present findings agree with the observations made in a non-cholinergic nerve, in the squid axon, by Hodgkin and Martin (1965). These authors also failed to show any effect of stimulation on the uptake of choline.

The presence of specific transport systems facilitating the entry of choline into the cells has been reported in various tissues studied, e.g. in frog skeletal muscle (Renkin, 1961), in ganglia (MacIntosh, 1963), in cortex slices of the kidney (Sung and Johnston, 1965), in squid axons (Hodgkin and Martin, 1965), in erythrocytes (Martin, 1967), in brain slices (Schuberth et al., 1966). This indicates that the specialized choline uptake mechanism is not a specific feature confined to the cholinergic nerves requiring exogenous choline for the optimal synthesis of acetylcholine.

B. Migration of Labelled Choline along the Axons of the Sciatic Nerves

Both a fast and a slow rate of axoplasmic transport system of materials have been found in cat motoneuron axons (Ochs and Johnson, 1968). A rate of approximately 930 mm/day was reported by these authors as the fast component for the transport of ^{3}H-leucine after uptake and incorporation into polypeptides, protein, and particulates.

After establishing the relative ease of choline penetration into the sciatic nerves, it was thought probable that by supplying the labelled precursor through the axons, axoplasmic transport of labelled choline in the direction of the axon terminals could provide a means to study transmitter turnover in the motor nerve endings. An advantage of this approach would be the selective labelling of the neural elements of the nerve-muscle preparation. However, exploratory studies in this direction did not provide encouraging results. Migration of labelled choline along the sciatic nerve axons does not seem to be fast enough to be important for the supply of choline in the axon terminals. A 24 hour incubation of the proximal segment of the sciatic under continuous elec-

trical stimulation did not cause a significant increase in radioactivity level of the distal segment some 3 to 4 cm away from the part which was incubated in labelled choline.

C. Double-Tracer Experiments

Ia. Preferential Release of Newly Synthesized Transmitter

Early experiments by Brown and Feldberg (1936), Kahlson and MacIntosh (1939) and Perry (1953) had shown that the cat's superior cervical ganglion, when stimulated continuously in the presence of an anticholinesterase, released ACh for long periods of time, but when no circulating Ch was present, re-synthesis of released ACh was slow and the ACh release declined to a low level. When exogenous Ch was supplied, the ACh output fell in the first few minutes to a steady level, but the release and ganglionic stores were maintained (Birks and MacIntosh, 1961). Based on these observations, the assumption was made that in the nerve endings of the sympathetic ganglia there is a functional separation of the transmitter stores. Birks and MacIntosh (1961) postulated that transmitter available for release can be divided into two components: 1. a small 'readily releasable' fraction and 2. another larger fraction which serves as a reservoir from which the readily releasable store can be replenished. These earlier studies on the perfused ganglion preparation which relied on bioassay procedures to assess transmitter turnover were supplemented with the use of radioactive choline to label the ACh stores. Collier and MacIntosh (1969) found that up to 85% of the ganglionic ACh could be labelled by perfusion with labelled Ch accompanied by continuous stimulation (20/sec) during 60 min. They used unlabelled Ch-Locke solution during the 20 min washout perfusion following the

loading procedure as well as during the rest of the experiment. After this washout perfusion, a 10-min period of preganglionic stimulation released, in the presence of eserine, labelled ACh into the effluent and also about twice as much unlabelled Ach. Only 36% of the ACh released by the 10 min period of stimulation was radioactive, even though 85% of ganglionic ACh was labelled at the end of the loading perfusion. Most of the unlabelled ACh must have been formed during the perfusion with unlabelled Ch-Locke solution after the loading procedure. When synthesis of ACh during this perfusion with unlabelled Ch was prevented by HC-3, almost all the released ACh was found to be labelled. This led Collier and MacIntosh (1969) to suggest that newly synthesized ACh is released before it equilibrates with preformed stores, as if the releasable transmitter were replaced partly by synthesis and partly by mobilization of the ACh store. As a corollary, during continuous activity, the ACh store does not turn over as a uniform pool but there is a preferential release of newly synthesized transmitter. In the experiments of Collier and MacIntosh (1969) eserine was added to the perfusion fluid containing unlabelled Ch at the start of the test period, after the end of the loading perfusion. Birks and MacIntosh (1961) had shown earlier that eserinization of a resting ganglion rapidly increases its total content of ACh if a supply of Ch is available. This excess ACh was called 'surplus' ACh (see INTRODUCTION). Thus the source of the unlabelled ACh in these experiments may have been either 'surplus' ACh formed before the test stimulation, or ACh synthesized during the test stimulation and released before mixing with the preformed store. The possibility exists that the labelled ACh loaded into the transmitter depot in the absence of eserine

may have subsequently become diluted by exchange with unlabelled surplus ACh formed in the presence of eserine during the period of rest preceding the test stimulation. If this exchange were rapid, it could also account for the release of unlabelled ACh in the experiments of Collier and MacIntosh. The concept of preferential release of newly synthesized transmitter was tested in the neuromuscular junction of the frog in the present experiments using a double tracer method. Two differently labelled precursors (^{14}C-Ch and ^{3}H-Ch) were sequentially used during two periods of loading(under continuous stimulation in neostigmine environment) to label the ACh stores of the motor nerve endings. Each loading period was followed by a washout procedure (see METHODS) to remove the loosely bound radioactive materials. It seems fair to assume that the remaining radioactivity in the tissues after the washout procedure consists predominantly of the radioactivity retained due to its incorporation into the cell. Due to the difference in time of loading for the two radioactive tracers, the transmitter formed from the first labelled precursor (^{14}C-Ch) can be considered as being the 'old' transmitter, while the transmitter formed during the second loading with ^{3}H-Ch represents the 'new' transmitter. It is realized that non-labelled transmitter, which cannot be detected by radiometric assay used in the present experiments, should also be present in the nerve-endings.

In interpreting the present release data it is useful to bear constantly in mind the concepts developed by Birks and MacIntosh for the ganglion (1961). A summary of the hypothetical model is presented in INTRODUCTION (Fig. 4 on page 32). Within this framework the present results of the preferential release experiments (Fig. 11, panel A and

Fig. 12, panel A) indicate the presence of two pools: 1. a small more readily releasable pool, and 2. a large less readily releasable pool, which can be visualized as being the storage pool serving as a reservoir for the more readily releasable pool. In this context, the output of transmitter during the first five minutes interval of the 30-min stimulation represents the virtual emptying of the readily releasable pool.

The present results of release during stimulation are expressed as % increase from baseline radioactivity for both the ^{14}C- and ^{3}H-tracers, which are taken to be equal to 100% at the start of the stimulation period. The jump in the radioactivity levels during the first five minutes of stimulation reflects the initial increase in the rate of transmitter release. The finding of a higher jump for tritium (^{3}H), which represents the new transmitter in comparison to ^{14}C (the old transmitter) (Fig. 11A and Fig. 12A) indicates a higher rate of release for tritiated transmitter. After the first 5-min period of release, the continuous stimulation releases both tracers at a constant rate for the remaining 25-min of stimulation as shown in Fig. 16, A. The two linear and parallel response curves in this panel indicate that there is a constant increment of radioactivity per 5-min interval for both tracers, and that they are released at the same rate. The rate determining step for the release during this last 25-minutes of continuous stimulation is most probably the rate at which transmitter from the storage pool is made available for release from the readily releasable pool. The latter process is further referred as 'mobilization' of the transmitter. The data discussed above supports the hypothesis of preferential release of newly synthesized transmitter. It is as if the tritiated transmitter, formed during the second loading

period from the supplied ^{3}H-Ch, replaced most of the ^{14}C-labelled transmitter present in the readily releasable pool. The observation that the two tracers have the same rate of release after the initial 5 min period of stimulation indicates that the mobilization is from the same storage pool. From the preferential release of newly synthesized transmitter it would follow that the readily releasable pool should be more closely related with the site of synthesis of the transmitter.

In these experiments neostigmine was used as anticholinesterase throughout the whole time-span of the experiment, starting from the first loading period till the end of the experiment. No unlabelled-Ch was added to the incubation fluid. From the data in Fig. 11, A or Fig. 12, A, it can be concluded that there is a preferential release of newly synthesized transmitter. Panels B of the same Figures show the response curves during the second stimulation period. Comparing the results in the two panels (A and B), it appears that the release of ^{3}H continued at the same rate, while the ^{14}C response curve in the second panel (B) is almost flat. Most probably this is due to depletion of ^{14}C-labelled transmitter.

Ib. Effect of HC-3 addition during the Second Loading Period

If during the second loading period (in the presence of about 10^{-5} M ^{3}H-Ch) 10^{-2} M of HC-3 were added to the incubation medium, the two test-periods did not display a preferential release of ^{3}H-tracer (Fig. 12, C and D or Fig. 16, second row, C and D). Both response curves in these panels are linear and superimposed. There is no difference in rate during either the initial 5 min of stimulation or during the remaining 25 min of this test-stimulation. It appears that under HC-3 no significant incorporation of ^{3}H-tracer occurred, and that the ^{3}H-tracer is

randomly distributed in the stores. This would explain why ^{3}H and ^{14}C have the same release patterns.

II. Surplus Pool Experiments

The results of the so-called surplus pool experiments using neostigmine or eserine as anticholinesterase are essentially identical in both series of experiments when continuous electrical impulses (40/sec) or ACh (2×10^{-5} M) were used to stimulate release (Fig. 13, A and B, Fig. 14, A and B, Fig. 16, third and fourth row, A and B). In these surplus pool experiments the second loading period (using ^{3}H-Ch as precursor) was conducted under resting condition to provide optimal conditions for the formation of excess ACh, or the surplus pool. No stimulation was delivered during the second loading period, so that the old (^{14}C) transmitter should be conserved. However, the pattern of release indicating preferential release of ^{3}H labelled transmitter means that during the second loading period (under resting condition using ^{3}H-Ch), the newly synthesized transmitter had a significant turnover with the readily releasable pool and the storage pool. A rapid exchange between these pools, even under resting conditions, explains this pattern of release similar to the series of experiments discussed earlier (preferential release experiments). When ACh (2×10^{-5} M) was used as stimulant during the same length of time (30 min), the release curves for both tracers are identical for the Neostigmine and Eserine-A series (Fig. 16, third and fourth row, panels B). These response curves are similar to the response curves due to electrical stimulation (40/sec). Thus, ACh in the concentration used seems to release both labelled transmitters as effectively as electrical stimulation. If such is the case, this would support the

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hypothesis suggested by Koelle (1961) for the ganglion that the presynaptic terminals are also sensitive to ACh, and that, during normal transmission, the ACh initially released by the preganglionic nerve impulse might depolarize the terminals and further release ACh, so amplifying the transmitter release process. The findings in panels B of the third and fourth rows in Figure 16 (for the Neostigmine and Eserine-A series) showing the same rate of release for both tracers after the initial 5 mn of stimulation, like in the preferential release experiments, suggest that the two radioactive tracers moved out from one same pool, in this case most probably from the storage pool. If a surplus pool was present, which in the ganglion was found to be released by perfusion with ACh or injection of carbachol, there should have been a greater release of ^{3}H than of ^{14}C, and a consequent divergence of the two response curves. This divergence is, however, absent in both series of experiments (using neostigmine or eserine as anticholinesterase).

Using carbachol (a stable analogue of ACh) at a concentration of 1×10^{-4} M, as the releasing agent (panels C, row 3 and 4 of Fig. 16), divergent response curves were obtained in both cases (Neostigmine and Eserine-A series). The discrepancy between the patterns of release in response to ACh and to carbachol might be due to a greater releasing ability of carbachol from the "surplus pool". This additional release from the surplus pool can explain the increase in the rate of release for the ^{3}H. In order to form a more definite opinion about these observations a dose response curve should be established using a wide range of concentrations of ACh and carbachol, especially since in the present experiments the concentrations of carbachol used were 5 times greater than the con-

centration of ACh (10^{-4} M as compared to 2 x 10^{-5} M of ACh).

There are various possible mechanisms by which carbachol might release ACh from the nerve terminals, e.g. by activation of presynaptic ACh receptors that have been proposed (in the ganglion) to participate in ganglionic transmission (Koelle, 1961, 1962). The principal evidence originally advanced for the presence of specific, presynaptic cholinoceptive sites was that the effect of carbachol on ACh release was markedly reduced by chronic preganglionic denervation (Volle and Koelle, 1961). From this it was concluded that the stimulant action of carbachol on normally innervated ganglia results principally from the liberation of ACh from the preganglionic fibres. An alternative process proposed (McInstry and Koelle, 1967) is that carbachol can enter the axonal terminals, either by passive diffusion across the high concentration gradient between the extracellular and intracellular fluid, or by a specialized transport system (in the red blood cells it has been shown to be different from the choline transport system, Martin, 1969) and then to displace ACh from binding sites.

The observed increase in rate of release of ^{3}H in response to carbachol in two series of the experiments presented, using either neostigmine or eserine, indicates an extra source of ^{3}H being liberated; probably this extra source is the surplus ACh formed during the second loading period while in resting condition (under the influence of the anticholinesterase). From these results it is apparent that the surplus pool cannot be labelled selectively. This seems to be due to the existing rapid turnover, or exchange, between the newly synthesized transmitter and the existing transmitter pools.

Collier and Katz (1970) have attempted to label selectively the transmitter pool or the surplus pool in the cat's superior cervical ganglion. In their procedure no anticholinesterase was used when the ganglion's transmitter store was to be labelled, while when the surplus store was to be labelled eserine was used. Unlabelled Ch containing eserine was used during the rest of the experiment. One series of the present studies were performed adapted to the procedure described by Collier and Katz (1970) using unlabelled choline in the bathing fluid after finishing the second loading procedure. The release patterns for both labels in response to electrical stimulation, ACh and carbachol in this series of experiments are identical (Fig. 15, A, B, and C and Fig. 16, row Eserine-B, panels A, B and C). Both response curves in these three panels overlap each other, ^{3}H choline release follows exactly the same release pattern as ^{14}C choline. Most probably this is due to a rapid turnover of the labelled transmitters, and dilution of the stores by uptake of the non-labelled choline used in the bathing fluid after the loading procedure.

Stimulation using potassium ions gave the results depicted in Fig. 16, third, fourth and fifth row, panels D, for the three series of surplus pool experiments. Using neostigmine as anticholinesterase, the pattern of response curves for both tracers is similar to that obtained with electrical stimulation (40/sec); ^{3}H-labelled choline was released preferentially. Using eserine as anticholinesterase, however, (panel D of Eserine-A row in the same Figure), ^{14}C-labelled transmitter was released preferentially. The linearity of the responses and the rates of release were similar in both series. In the last series of experiments

(Eserine-B, panel D of the same Figure), using unlabelled Ch after the loading period, ^{14}C-labelled transmitter is also being preferentially released (like in the Eserine-A series). The only difference observed is that in the Eserine-B series, the rate of release of ^{14}C is higher. It is to be expected that the release pattern of both tracers follows the same pattern with potassium stimulation (40 mM) as with electrical stimulation (panels A and D of the Neostigmine series), since it is known that in both cases the nerve terminals are depolarized. Increasing the external potassium ion concentration, the frequency of the miniature end-plate potentials (m.e.p.p.s.) at the neuromuscular junction is greatly increased (del Castillo and Katz, 1954c; Liley, 1956c). The finding that the release pattern, using eserine as anticholinesterase, is the reverse to the one obtained with neostigmine (i.e. the old transmitter is now being released in preference to the new transmitter, as shown in panels D of last two rows of Fig. 16) indicates that under eserine, mobilization of the old transmitter is preferred. Using a more intense potassium stimulation (120 mM), the rate of release of the old transmitter is also increased, as indicated by a steeper slope of the ^{14}C response curve (shown in panel D, Eserine-B series of Fig. 16). It is as if another pool of old transmitter outside the storage pool is utilized to account for the divergence of both curves. With the use of this non-specific stimulant in high concentration (120 mM), it might well be that even the old transmitter in the "Stationary" pool was released (see model of Birks and MacIntosh, Fig. 4). Another possibility is that potassium ions in that high concentration play an important role in a cationic exchange mechanism. In short, no satisfactory explanation can be offered

without further work for full interpretation of these results.

In most of the literature there is an implicit acceptance of the ganglion model for the dynamics of transmitter release at the neuromuscular junction (Eccles, 1964; Desmedt, 1966). The present work provides some experimental evidence indicating that the superior cervical ganglion (Birks and MacIntosh, 1961) is a suitable model for the transmitter dynamics at the neuromuscular junction.

The experimental results of the present studies in the neuromuscular junction of the frogs discussed above are compatible with the overall model known for the ganglion (Birks and MacIntosh, 1961). This conclusion was arrived at on the basis of the release data presented using the double-tracer technique, a method which has not been used previously for the study of transmitter dynamics. The existence of a readily releasable pool and a storage pool are apparent from the release data obtained. A surplus pool seems also to be formed under the influence of either neostigmine or eserine. The release of this pool seems to be more effective with carbachol than with ACh. Selective labelling of the surplus pool with a second tracer does not seem to be successful. There seems to be a rapid turnover (exchange) between the newly synthesized transmitter and the old transmitter, even under resting conditions. The linear patterns of all these release data are also consistent with the quantal nature of transmitter release, i.e. release in packages of uniform size.

Full interpretation of the results discussed depends to some extent on the knowledge of the ACh content in the tissue, as well as in the medium, at the different stages of the experiments. ACh identification procedures should be used for further studies at the neuromuscular junction

to quantitate the actual amount of ACh present in the tissue, or released in the medium, and also to obtain information as to what part of the released radioactivity is due to labelled ACh. In view of the fact that the amount of ACh coming from the nerve terminals of the nerve-muscle preparation would be small, a very sensitive assay of ACh would be necessary.

SECTION V

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