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SYNTHESIS, STORAGE AND RELEASE OF [^{14}C]ACETYLCHOLINE IN ISOLATED RAT DIAPHRAGM MUSCLES

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SUMMARY

1. Segments of rat diaphragms were kept in choline-free media for 4 hr and were then exposed to a physiological concentration of [^{14}C]-choline (30 μM) at 37° C. The synthesis, storage and subsequent release of [^{14}C]acetylcholine by the muscles was assessed by isotopic- and bio-assays after isolation of the transmitter by paper electrophoresis.

2. Replacement of endogenous acetylcholine (0·92 μ-mole/kg) with labelled acetylcholine proceeded slowly at rest, but rapidly during nerve stimulation. [^{14}C]Acetylcholine accumulated most rapidly when hydrolysis of the released transmitter, and thus the re-use of endogenous choline, was prevented by an esterase inhibitor. Fully replaced stores were maintained during nerve stimulation by synthesis rates sufficient to replenish at least 35 % of the store size in 5 min.

3 In the presence of hemicholinium-3, which inhibits choline uptake, acetylcholine stores declined rapidly during stimulation, and residual synthesis was slight, indicating little intraneural choline. Net choline uptake into nerve terminals was estimated from the highest observed synthesis rate and from previous measurements of the number and size of terminals, as 3–6 p-mole/cm^2 sec.

4. Transmitter synthesis was localized in the region of end-plates, and was reduced to a few per cent of normal 6 weeks after phrenic nerve section. Release experiments suggested that at least half of the acetylcholine in phrenic nerves is in their terminals; from this content and the morphology of the terminals, the average concentration of transmitter in the whole endings would appear to be about 50 m-mole/l. Homogenization of the muscles freed choline acetyltransferase into solution, but left some [^{14}C]acetylcholine associated with small particles, presumably synaptic vesicles.

5. Resting transmitter release was about 0·013 % of stores/sec. With

360 nerve impulses at 1–20/sec, release increased up to 0·43 % of stores/sec, and amounted to $3{\cdot}5–7 \times 10^{-18}$ moles per end-plate per impulse. The release rate was unaffected by the doubling of store size which occurred with eserine, but the extra transmitter did help to maintain releasable stores during prolonged stimulation. Experiments with fractional store labelling indicated that newly synthesized acetylcholine was preferentially released.

6. Preformed [^{3}H]acetylcholine was not taken up and retained by muscle or nerve cells in the absence of an esterase inhibitor. With eserine present, labelled acetylcholine was taken up uniformly by muscle segments; when eserine was then removed, radioactive acetylcholine remained only near neuromuscular junctions.

INTRODUCTION

The present experiments were begun in an attempt to obtain further information about the metabolism of acetylcholine (ACh) at neuromuscular junctions. The basic approach has been to follow the utilization of radioactive choline ([^{14}C]Ch) by phrenic nerves for ACh synthesis, and the subsequent turnover and release of the labelled transmitter. A similar approach to ganglia has recently been reported by Collier & MacIntosh (1969). The use of isotopes has permitted more reliable assays of ACh and choline stores, and release rates, than were possible by previous bio-assay procedures, and has made it possible to examine aspects of the turnover of ACh stores which would be difficult to study otherwise. The results obtained with diaphragms are very similar to those previously observed with ganglia (Birks & MacIntosh, 1961; Collier & MacIntosh, 1969). In addition, knowledge of the number, size and physiology of phrenic nerve terminals has made it possible to estimate the rate of net choline uptake by physiologically active nerve endings, and to correlate some results with the quantal storage and release of ACh.

Rat diaphragms were used because considerable information is available about the anatomy, physiology and basic transmitter biochemistry of these muscles. Hebb, Krnjević & Silver (1964) have provided clear evidence that ACh and the enzyme required for its synthesis, choline acetyltransferase, are present in phrenic nerve bundles and in parts of the muscles having nerve–muscle junctions. Several groups of workers have estimated the amount of transmitter release per impulse (Krnjević & Mitchell, 1961; Bowman & Hemsworth, 1965), and the end-plate potentials produced by low-frequency stimulation have been mimicked by the electrophoretic application of about ten times as much ACh at end-plates (Krnjević & Miledi, 1958). Hemicholinium-3 (HC-3) apparently interferes with the utilization of choline for transmitter synthesis in this tissue, as

elsewhere (cf. MacIntosh, 1963), since the size of miniature end-plate potentials gradually declines during nerve stimulation in the presence of this drug, unless excess choline is present (Elmqvist & Quastel, 1965).

Many of the techniques and approaches used in previous studies have been adapted for the present experiments, in particular those of Birks & MacIntosh (1961) with perfused superior cervical ganglia. They found that physiological amounts of choline were necessary to maintain moderate tissue levels and high release rates of the transmitter during prolonged nerve stimulation. HC-3 interfered with the utilization of choline for transmitter synthesis, leading to depletion of 85 % of the tissue content of ACh ('depot' ACh) during stimulation, and leaving a residual amount, apparently in axons rather than in nerve terminals, which was resistant to impulses. If transmitter stores were allowed to decline because of inadequate choline or blockade of choline uptake by HC-3, the release rate fell rapidly until a fifth of the total store was depleted, and then more slowly at a rate proportional to the amount of ACh remaining. It was concluded from these observations that part of the 'depot' store was more readily released by impulses than the rest. With eserine, 'surplus' ACh accumulated above the resting tissue level, with no change in release rates. Without eserine, ACh was hydrolysed as it was released, and less choline was collected instead (Perry, 1953), suggesting some re-use of choline in the ganglia. Experiments with several other tissues have contributed a clearer picture of the sites of ACh synthesis and storage. Choline is taken up into the cytoplasm of isolated cerebral boutons by a saturable 'carrier' mechanism, which is competitively inhibited by HC-3 (Marchbanks, 1968; Potter, 1968). Choline acetyltransferase behaves as a soluble cytoplasmic enzyme during subcellular fractionation of brain tissues, indicating that ACh synthesis occurs in the neuroplasm (Whittaker, 1965; Fonnum, 1968; Potter, Glover & Saelens, 1968). Acetylcholine storage has been shown to occur largely in synaptic vesicles in cerebral tissues (review: Whittaker, 1965) and in the electric organs of *Torpedo* (Israël, Gautron & Lesbats, 1968).

METHODS

Materials. [Methyl-^{14}C]Ch (40 c/mole) was obtained from the Radiochemical Centre and [acetate-^{3}H]ACh (400 c/mole) from the New England Nuclear Corp. Both were subjected to paper electrophoresis (Potter & Murphy, 1967) within a week of use. HC-3 was obtained from the Aldrich Chemical Co., eserine sulphate and neostigmine methylsulphate from British Drug Houses, and dipicrylamine (2,2′,4,4′, 6,6′-hexanitrodiphenylamine) from Eastman Co.

Animals. Albino rats (325–375 g) were used for all experiments. The left hemidiaphragms of a few smaller animals were denervated under ether anaesthesia by removal of a section of the left phrenic nerve through an incision in the anterior

chest wall. These rats were used 40–44 days post-operatively, when they had reached the above weight.

Muscle preparation. The rats were anaesthetized with ether at 8 a.m., the diaphragm was exposed from below, and the chest muscles overlying the left costal margin were removed. The animals were then exsanguinated for 30 sec, before the left phrenic nerve and hemidiaphragm were removed, which required an additional minute. Dissection was completed in an oxygenated medium at room temperature. The middle part of the left hemidiaphragm was selected for study because the neuromuscular junctions in this segment lie in a narrow band in the middle of the muscle fibres, mostly just lateral to the intramuscular branches of the phrenic nerve. This anatomy makes it possible to recover most of the neural elements in the tissue for

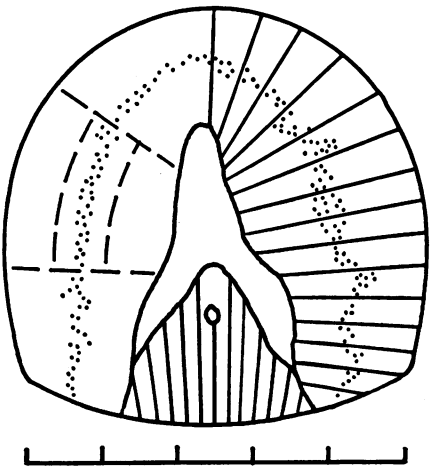

Fig. 1. A stretched whole diaphragm from a 350 g rat is shown diagrammatically against a scale in cm. Radial lines show the direction of muscle fibres, and dots indicate the positions of neuromuscular end-plates found by a histochemical stain for acetylcholinesterase. Dashed lines outline the part of the left hemidiaphragm used for all experiments, and its subsequent division into rib-end, end-plate region, and tendon-end parts for biochemical analyses.

biochemical assays, by dissecting out the middle third of the muscles (Fig. 1; cf. Hebb *et al.* 1964). Excess connective tissue and the intercostal muscles were removed from the ribs, leaving uncut muscle fibres running from two ribs to the central tendon. The phrenic nerve was trimmed to 3 cm and was tied at its free end. The ribs and tendon were tied to a triangular frame made of fine glass rod, so that each preparation could be held flat near the bottom of its bath.

Muscle baths. These were silicone-coated glass beakers held in holes in an aluminium block. Warm water was circulated through channels inside the block so that the temperature of the baths was maintained at $37 \pm 1°$ C. The volume of all baths was 5 ml.

The bathing medium for all experiments contained (mM): Na^+, 150; K^+, 5; Ca^{2+}, 2; Mg^{2+}, 1; Cl^-, 147; $H_2PO_4^-$, 1; HCO_3^-, 12; acetate$^-$, 1; and glucose, 11. It was equilibrated and vigorously bubbled with 5 % CO_2 in O_2 delivered from fine plastic tubing,

and its pH during use was 7·4. Bath changes were effected either by transferring the muscle preparations to fresh baths, or by tipping each dish and aspirating the fluid under gentle vacuum into test-tubes; fresh solutions were then added with a pipette. Drugs were added by changing the bath fluid to one containing the desired concentration of the drug. Unless otherwise noted, the concentration of all drugs was 30 μM.

Phrenic nerves were held just out of the baths by pairs of fine platinum electrodes, and were kept moist by the splattering of the fluid induced by the bubbler. Rectangular pulses of 20 μsec duration and 3–5 times the intensity initially required for vigorous muscle twitching were used for nerve stimulation. Electrodes were advanced along the nerves towards the muscles periodically during the experiments.

Assays of ACh and radioactive choline. The procedure for the radioactive substances was as follows. At the conclusion of an experiment each muscle was frozen instantly by immersing it in a beaker of ether surrounded by dry CO_2. The tissue was then freeze-dried overnight *in vacuo*. The ribs, central tendon, and extra-muscular parts of the phrenic nerve were removed, and the muscle was trisected into regions with or without end-plates (Fig. 1) yielding pieces weighing approximately 9 mg. These were powdered in small glass homogenizers and the powder was suspended in 50 μl. ice-cold ethanol containing 0·3 M acetic acid, 5 mM choline and 5 mM ACh. After 30 min the homogenizers were centrifuged, and 20 μl. of the clear extract were removed into disposable capillary micropipettes. The fluid was applied to and dried at the mid line of chromatography paper, and ACh was separated from choline by low-voltage paper electrophoresis (Potter & Murphy, 1967). The bands of separated substances were stained briefly in iodine vapour and their positions on the paper were marked in pencil before the iodine evaporated. Each band was then cut out, and the radioactivity on the paper was determined by liquid scintillation spectrometry in a toluene-base phosphor containing 10 % methanol. Counting efficiencies were determined with internal standards of [^{14}C]- or [^{3}H]toluene.

When endogenous ACh was to be assayed, ACh was omitted from the extraction fluid, and the position of the endogenous ACh on the electrophoresis paper was estimated from the position of the stained choline band. ACh was eluted from the paper into 1 ml. leech bath fluid, and was assayed biologically on eserine-treated strips of leech dorsal muscle (MacIntosh & Perry, 1950). Isometric contractions of the muscle were measured for 45 sec with the aid of a strain gauge, and test solutions were closely bracketed by ACh standards.

Results are expressed as μ-mole/kg wet tissue. Assay values were corrected as follows. Dry weight was converted to wet weight on the basis of the weight ratio between frozen and freeze-dried fresh muscles (100:24, Table 1). When only the end-plate region of a muscle was used for assays, the ACh content of this part was taken as 96 % of that of the whole muscle (Table 1). The data are corrected for average recoveries of 94 % during electrophoresis, for the overlap of radioactive choline into the ACh band (0·3 % of choline), and for isotopic counting efficiencies.

Recovery and assay of ACh and choline from solutions. ACh and choline were extracted from bath solutions and were concentrated for electrophoresis with the aid of dipicrylamine. This new method depends upon the ability of the anion dipicrylamate to form salts with quaternary amines (and potassium) which are insoluble in water but very soluble in ketones, and the fact that these salts are readily split in dilute acids. The acid dipicrylamine is a stable, detonatable, yellow compound, whose saturated aqueous solution at pH 2 is approximately 1 μM. (The concentration of solutions may be determined by their absorbance at 420 nm; the molar absorbance with a 1 cm light path is about 36,000.) A 0·1 M stock solution of the lithium salt was prepared by neutralizing an excess of dipicrylamine with 0·1 M-LiOH, yielding a brilliant crimson solution of pH 7–7·5, which was stored at 4° C.

Five-ml. samples of bath fluid containing labelled ACh and choline were collected in test-tubes containing 0·5 ml. 0·1 M lithium dipicrylamate. Carrier amounts of choline and often of ACh (about 0·1 μ-mole each) were added as required for electrophoresis, and the tubes were kept on ice until the dipicrylamates of the amines and potassium could be extracted. About 0·25 ml. 2-octanone was added to each tube and was vigorously mixed with the aqueous phase by swirling the fluids on a vortex mixer. After the phases separated, most of the red octanone was transferred to a smaller tube, and the remainder was collected by rinsing the surface of the aqueous phase with a further 0·25 ml. octanone. (The extraction is readily followed visually and is quantitative if the volume of octanone is more than a few per cent of that of the aqueous phase.) The octanone was vigorously mixed with 0·5 ml. of a solution containing 50 mM perchloric and 100 mM acetic acids. The red octanone layer was then aspirated and discarded, and the acid phase was freeze-dried. The residue of amine salts was dissolved in 50 μl. 90% acetone, leaving insoluble $KClO_4$, and 20 μl. was spotted for paper electrophoresis. Subsequent procedures and total recoveries were as for tissues.

Assays of choline acetyltransferase and endogenous choline. Muscles were homogenized in ten times their weight of ice-cold 20 mM-KCl, and after adjusting the suspensions to pH 8 with 1 N-NH_4OH they were centrifuged at 105,000 *g* for 30 min to remove insoluble material. The supernatant fluids were freeze-dried and redissolved in one-tenth their original volume. Twenty-μl. samples of the extracts were assayed for choline acetyltransferase activity by measuring the formation of [^{14}C]ACh from excess choline and [acetyl-^{14}C]CoA (Potter, 1969). The same assay system was used to measure choline in boiled extracts, but with choline as the limiting factor (Potter, 1969).

RESULTS

Freshly prepared muscles

These muscles were studied to provide pre-incubation control values. They were gently blotted before being frozen, and were dissected and weighed while frozen. Results are summarized in Table 1.

The segment of the left hemidiaphragm used represented approximately 36% of this hemidiaphragm, by weight, excluding crural fibres. The concentration of endogenous choline was similar in the three parts of a given muscle, but varied widely in different muscles; denervation had no observable effect on the choline distribution. The concentration of ACh in the muscle segments averaged 0·92 μ-mole/kg (an average amount of 98 p-mole). There was sufficient choline acetyltransferase in each preparation to synthesize 98 p-mole ACh in the test-tube, in 13 sec. Both the enzyme and the transmitter were localized in the region of neuromuscular junctions, and both were reduced to a few per cent of normal following denervation. These results confirm those of Hebb *et al.* (1964), who used whole hemidiaphragms from smaller rats; however, they found 2–3 times as much enzyme activity in normal muscles.

Esterase inhibition

It was found that 30 μM eserine or 30 μM neostigmine in the muscle baths reduced the hydrolysis of 10 μM [^{3}H]ACh by at least 97 %, when the transmitter was introduced 5–10 min after the esterase inhibitor. Tritiated acetate was extracted from the baths for these assays (Potter, 1969). It may be noted that eserine is a tertiary amine which presumably enters cells readily, whereas neostigmine, which is quaternary, probably remains extracellular.

TABLE 1. Incubated muscles were in choline-free media at 20° C for 4 hr and then in 30 μM [^{14}C]Ch at 37° C for 3 hr. For subsequent calculations, 'normal' ACh content will be considered 0·94 μ-mole/kg, the mean result of bio-assays and radio-assays; of this 96 % will be considered as in the end-plate region

			Distribution along muscle fibres (% of total)		
	n	Mean ± S.E. of mean	Rib end	End-plate region	Tendon end
		Fresh muscles			
Wet weight (mg)					
Normal	30	107 ± 13	35	29	36
Denervated	12	71 ± 9	37	28	35
Dry weight (mg)					
Normal	30	26 ± 3	35	29	36
Denervated	12	17 ± 3	35	30	35
Choline (μ-mole/kg)					
Normal	3	53 ± 31	35	31	34
Denervated	3	40 ± 34	35	29	36
ACh (μ-mole/kg)					
Normal	12	0·92 ± 0·13	*ca.* 2	95	*ca.* 3
Denervated	6	0·02 ± 0·01		(all)	
Choline acetyltransferase (μ-mole min^{-1} kg^{-1})					
Normal	6	4·32 ± 0·51	2	95	3
Denervated	6	0·11 ± 0·01	*ca.* 3	94	*ca.* 3
		Incubated muscles			
[^{14}C]Ch (μ-mole/kg)					
Normal	12	44 ± 23	33	33	34
[^{14}C]ACh (μ-mole/kg)					
Normal	12	0·96 ± 0·12	*ca.* 2	97	*ca.* 1
Denervated	6	0·03 ± 0·01		(all)	

Influx and efflux of [^{3}H]*ACh*

Experiments were first performed to see whether there is uptake and retention of ACh by cholinergic nerves in the absence of an esterase inhibitor. Although it is unlikely that extraneural acetate contributes

significantly to transmitter synthesis (Browning & Schulman, 1968), 1 mM unlabelled acetate was included in the baths to reduce any potential synthesis of intraneural transmitter from the [^{3}H]acetate produced by [^{3}H]ACh hydrolysis during the incubations. Twelve muscles were exposed to 10 μM radioactive ACh for 30 min, and were then transferred twice at 10 min intervals to ACh-free media. The nerves to nine muscles were stimulated at 5/sec during influx. No [^{3}H]ACh was found in any part of these muscles. It is unlikely, therefore, that phrenic nerve terminals normally take up and retain significant amounts of preformed ACh, in a fashion analogous to the recapture of noradrenaline by adrenergic nerves (cf. Iverson, 1967).

In the presence of eserine, ACh was taken up and retained by the tissues. Muscles exposed to 10 μM [^{3}H]ACh required about 2 hr to equilibrate with it, whether or not their nerves were stimulated at 5/sec.Quantitatively, uptake in 30–120 min was about half that seen with choline under otherwise identical conditions (see below), in close agreement with a result of Saelens & Stoll (1965). Unlike the distribution of endogenous ACh, the amounts of tritiated ACh taken up per unit weight of tissue were virtually identical in the innervated and non-innervated parts of the muscles. Thus most of the uptake was due to non-neural tissue. The efflux of [^{3}H]ACh was studied in the manner used by Krnjević & Mitchell (1960), and the present results confirm their study with 500 μM ACh. Regardless of the period of influx, there was a very rapid washout phase followed by two exponential rates with average half-times (n = 5) of 2·4 min (efflux from the extracellular space) and 31 min (probably from muscle cells). On the basis of these experiments, a period of 5 min after nerve stimulation was allowed during collection experiments, to collect at least 80 % of the last-released transmitter.

With eight muscles, stimulated at 5/sec in eserine and 10 μM [^{3}H]ACh for 30 min, eserine as well as ACh was washed out for 30 min. In these tissues, labelled ACh was found only in the middle third of the muscles, apparently because ACh had entered phrenic nerves as well as other cells, and had been retained in normal ACh stores after the removal of eserine and the subsequent hydrolysis of ACh elsewhere. Since the amounts retained were only 4–9 % of the normal store size, this apparent method of replacing endogenous with labelled ACh was not pursued.

Influx and efflux of choline

Muscles exposed to 1–50 μM [^{14}C]Ch took up the amine evenly in all parts of the muscle. This pattern of distribution was not changed by nerve stimulation, denervation, or prolonged washout in choline-free solutions.

It was not possible, therefore, to study directly the kinetics of choline uptake by nerve terminals.

Since the plasma level of free choline in rats (Kahane & Levy, 1938), like other mammals (Bligh, 1952), is in the range 5–50 μM, all subsequent studies were performed with 30 μM choline. Muscles exposed to this concentration of [^{14}C]Ch equilibrated with it in 2–3 hr, when the tissue level was comparable to the normal endogenous content of choline (Table 1). Efflux studies then showed washout of at least two compartments of radioactive choline, with average half-times (n = 5) of 2·5 and 36 min.

In all of the following experiments, muscles were exposed to [^{14}C]Ch in order to replace stores of ACh with newly synthesized [^{14}C]ACh. To maintain a constant and high specific activity of [^{14}C]Ch in the baths it was necessary to minimize the amounts of endogenous choline leaving the tissues. This was achieved by keeping the muscles in a litre of choline-free bath solution at 20° C for 4 hr before use, so that the tissue content of choline fell to negligible levels. (The content of endogenous ACh did not change.) During experiments the level of endogenous choline was then so low that no change in the specific activity of added choline could be detected.

Replacement of endogenous ACh by [^{14}C]*ACh*

Resting diaphragms exposed to [^{14}C]Ch increased their content of radioactive ACh slowly; after 1 hr about a third of the normal store was labelled (Fig. 2). After 3 hr the average level reached 102 % of 'normal' (Table 1) and was equal to the amount of ACh found by bio-assays. No further increase in [^{14}C]Ch was found after longer incubations, or in the presence of 100 μM [^{14}C]Ch. Denervated muscles, incubated for 30 min in [^{14}C]Ch, also replaced their ACh stores, which were only a few per cent of normal. In both innervated and denervated muscles, [^{14}C]ACh was sharply localized in the middle third of the muscle fibres (Table 1).

Nerve stimulation greatly increased the rate of replacement of endogenous with radioactive ACh, even at impulse rates of 1–2/sec. Replacement was most rapid with intermittent stimulation at 20/sec in the presence of neostigmine, and appeared close to a limit after 30 min (Fig. 2). (Replacement in the absence of neostigmine is discussed in a later section.) However, the final content of [^{14}C]ACh under these conditions was 15–20 % less than the tissue level of ACh determined by bio-assay. When the bath fluid was changed to one without choline and with HC-3, intermittent nerve stimulation at 20/sec during 30 min was accompanied by nearly complete depletion of the labelled ACh (Fig. 2). Bio-assays then showed 23 $\pm$ 5 % of the normal store of ACh in the muscles. These results indicate that most of the normal store of ACh in diaphragms turns over

during nerve stimulation, while the remainder is only replaced during prolonged exposure to [^{14}C]Ch, and is not readily released during stimulation.

In the presence of eserine, resting muscles synthesized an amount of radioactive ACh equal to 17 % of the normal store in 5 min, and then continued to accumulate the transmitter at a progressively slower rate as the total store approximately doubled. After 1 hr, the amount of [^{14}C]-ACh was 128 ± 22 % of normal, although 68 ± 12 % of the endogenous

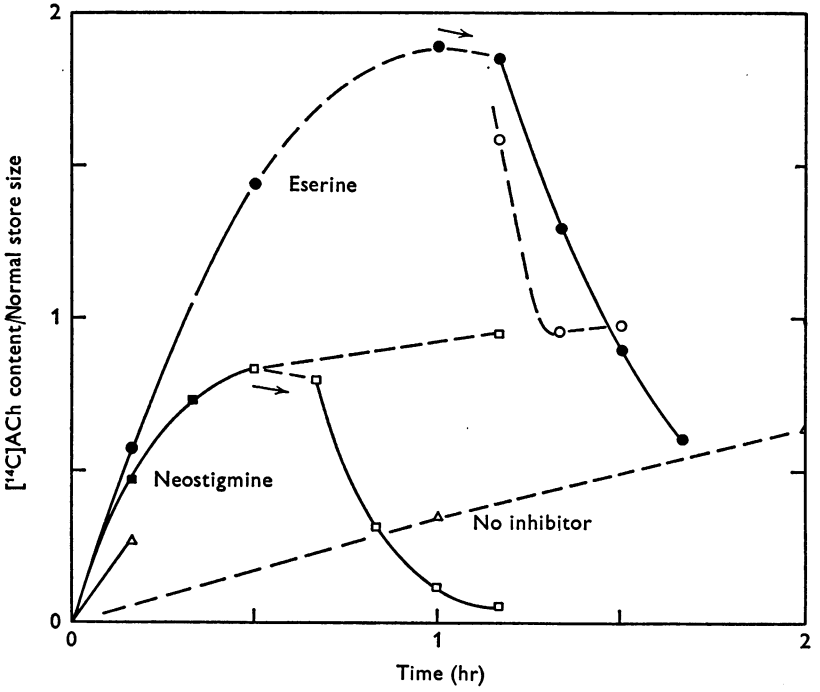

Fig. 2. Continuous lines represent nerve stimulation at 20/sec for the first 9 min of each 10 min period; dashed lines represent unstimulated muscles. Open symbols: esterase inhibitor absent or removed at preceding point. Arrows: introduction of HC-3 and removal of [^{14}C]Ch for points in the direction shown. Each point represents the mean result of assays of the end-plate regions of six or more muscle segments. Standard errors were less than $\pm$ 15 % of the mean values for muscles not in eserine, and less than $\pm$ 20 % for those in eserine. [^{14}C]Ch and the esterase inhibitors were introduced 5 min before zero time.

ACh was still present. With intermittent nerve stimulation for 30 min at the beginning of the hour, the remaining endogenous ACh was also replaced, and nearly twice normal levels of [^{14}C]ACh were achieved (Fig. 2); further accumulation with longer periods of incubation with eserine was

negligible. Withdrawal of eserine at this point resulted in the disappearance of the supranormal or 'surplus' ACh, leaving a normal-sized store of radioactive ACh (Fig. 2). The apparent basis for these results is that phrenic nerves contain eserine-sensitive esterases (not necessarily acetylcholinesterase) which continuously hydrolyse excess cytoplasmic ACh.

TABLE 2. Results are given as % normal store size. All muscles were incubated as noted in the legend of Table 1. Some were then used after 10 min in 30 μM neostigmine alone, and others after 60 min in 30 μM eserine alone. A 30 min collection period was used to measure resting release. Tissue assays were of the end-plate region only, and the number of assays is given in parentheses

		Tissue [^{14}C]ACh		Released [^{14}C]ACh
		Initial	Final	
Muscles in neostigmine				
Control group	(12)	102 ± 13		
Resting release	(12)			0·76 ± 0·06/min
Nerve stimulation 5/sec, 5 min	(6)			21 ± 3
Nerve stimulation 20/sec, 5 min	(9)		105 ± 8	35 ± 5
Plus 30 μM choline	(7)			
Resting release				0·31 ± 0·04/min
5/sec, 5 min			71 ± 8	12 ± 2
Denervated muscles	(6)			
Resting release			2·5 ± 0·6	0·45 ± 0·09/min
Muscles in eserine				
Control group	(12)	189 ± 38		
Resting release	(6)		196 ± 46	1·21 ± 0·26/min
Nerve stimulation 5/sec, 5 min	(8)			22 ± 4
HC-3	(6)			
Resting release				0·56 ± 0·13/min

Effects of transmitter release and HC-3 on store size

In the experiments shown in Fig. 2, HC-3 was used to prevent ACh synthesis during prolonged nerve stimulation, and ACh stores consequently declined. The total amount of [^{14}C]ACh released into the baths from eighteen muscles in eserine and HC-3 during various periods of stimulation was only slightly greater than the calculated amount lost from the muscles, indicating little residual ACh synthesis (1·79 n-mole collected; 1·65 n-mole depleted).

Acetylcholine stores were well maintained during nerve stimulation when HC-3 was absent. The maximum observed rate of store replace-

ment during transmitter release occurred in a group of nine muscles which had been prepared so as to have normal stores of [^{14}C]ACh. These were stimulated at 20/sec for 5 min in neostigmine, and were then frozen immediately for assays (Table 2). Although the average amount of ACh collected during stimulation was 35 % of that normally stored, the amount of transmitter found in the tissues was normal. Thus ACh synthesis is as rapid as its release during periods of nerve stimulation of this duration. Fourfold higher release rates were attained in subsequent experiments during 18 sec bursts of impulses, but it was not practicable to determine whether transmitter stores were normal or somewhat reduced after stimulation because of the smaller total amounts of ACh involved. It may be noted that the rate of ACh synthesis in the above experiment was high even though the final bath concentration of [^{14}C]Ch was found to be only 0·8 μM.

Effect of 'surplus' ACh on transmitter release

The rate of release of [^{14}C]ACh from eserinized muscles containing twice normal ACh levels was compared to that from muscles in neostigmine, which contained normal ACh levels (Table 2). The resting rate of release appeared slightly higher in the presence of 'surplus' ACh. The increase in transmitter release which accompanied moderate and brief nerve stimulation was, however, unrelated to store size, i.e. 'surplus' transmitter did not cause an increase in the release rate. This result, which has also been observed with ganglia (Birks & MacIntosh, 1961), would occur if the rate of release were already at a maximum, or if 'surplus' ACh were not immediately available for release by impulses. Both of these possibilities are credible on the basis of the hypothesis that synaptic vesicles store and release the transmitter.

Two observations were made which appear to bear upon the question of how cytoplasmic ACh is released. First, HC-3 was found to reduce the relatively high spontaneous release rate of [^{14}C]ACh from muscles containing 'surplus' ACh (Table 2). Secondly, the rate of release of radioactive ACh was better maintained during prolonged nerve stimulation when 'surplus' ACh was present than when it was not; this was most apparent after the amount of transmitter released from each muscle exceeded the normal store size (e.g. the last filled circle in Fig. 2). This result implies that 'surplus' ACh can be released by contributing to the transmitter pool released by impulses.

Rate of release of ACh

The rate of release of [^{14}C]ACh from resting muscles in neostigmine was less than 1 % of their ACh content per minute (Table 2), and was com-

parable to that previously observed for whole hemidiaphragms from rats (Straughan, 1960; Mitchell & Silver, 1963; Krnjević & Straughan, 1964). The rate of release from denervated muscles was 60 % as great, again in confirmation of the above authors. The denervated muscles lost a much larger percentage of their ACh content/min, about 18 %, as expected from their small store size and the relatively rapid rate at which they replaced their endogenous transmitter stores at rest when incubated in [^{14}C]Ch.

ACh release in relation to the frequency of nerve stimulation was studied as follows. Muscles were incubated in [^{14}C]Ch for 3 hr to label their transmitter stores, and were then kept in neostigmine without choline during the experiments. After each period of nerve stimulation, 5 min were allowed for [^{14}C]ACh to diffuse from the muscles into the baths. The muscles were rested for 10 min or more between collection periods. Preliminary results showed that the lowest stimulation rate compatible with accurate assays was 1/sec, since at this frequency the amount of ACh collected was roughly twice that found at rest. The upper limit of frequency tested was 20/sec, since at higher rates the propagation of impulses to nerve terminals in the rat diaphragm is liable to particularly rapid failure (Krnjević & Miledi, 1959). Even at 20/sec the rate of release per impulse was comparable to that at lower frequencies for less than 30 sec (cf. Brooks & Thies, 1962, for comparable physiological data). Accordingly, the experiments were performed with a total of 360 impulses, and the stimulation period was varied from 18 sec (frequency = 20/sec) to 6 min (1/sec). Each of eight muscles was stimulated in random order at 1, 2, 5, 10 and 20/sec. The amount of [^{14}C]ACh collected during each period of stimulation, minus the amount expected on the basis of the resting release rate (Table 2), averaged $7{\cdot}8 \pm 2{\cdot}1$ p-mole, or 0·022 % store size/impulse. This rate is about half that observed by Krnjević & Mitchell (1961), who stimulated whole hemidiaphragms at 2–5/sec for 2–5 min, assuming that their tissues had the same total transmitter content as those from the rats of similar size studied by Hebb *et al.* (1964). The difference in results is partly due to the fact that they did not correct for the resting release of ACh, which was underestimated at the time. The present result compares more closely with the total release observed by Bowman & Hemsworth (1965) at 1/sec, which was 0·033 % of store size/impulse, again assuming the same tissue content found by Hebb *et al.* (1964).

Release of newly synthesized ACh

When muscles with only [^{14}C]ACh were placed in unlabelled choline, their content of labelled transmitter depended, as expected, on the rate of synthesis of non-radioactive ACh. But unexpectedly, when the store was nearly all [^{14}C]ACh the rate of release of the labelled amine at rest was less

than half that from muscles not exposed to unlabelled choline (Table 2). When the muscles were stimulated, the release rate was reduced to 57 % of that seen without unlabelled choline (Table 2). Thus, in proportion to the amounts present, these muscles apparently released newly synthesized non-radioactive ACh more than twice as rapidly as [^{14}C]ACh. Very similar results have recently been obtained by Collier & MacIntosh (1969) with superior cervical ganglia of cats. These data are compatible with the concept that a small part of the transmitter store in cholinergic nerve terminals turns over more rapidly than the rest (Birks & MacIntosh, 1961).

Re-use of choline from hydrolysed ACh

As previously noted, the rate of replacement of endogenous ACh in stimulated muscles exposed to [^{14}C]Ch was more rapid in the presence of neostigmine than without an esterase inhibitor (Fig. 2). With stimulation at 20/sec, 46 % of the normal store was replaced in 10 min with neostigmine, whereas without it the synthesis and retention of [^{14}C]ACh under the same other conditions was 27 % of store size. There was no indication in any of the present experiments that neostigmine caused an increase in ACh turnover, or a rapid increase in store size, either of which could explain the difference in these results. Therefore, the tentative conclusion is drawn that phrenic nerves can recapture and re-use nearly half of the choline (endogenous, in this case) produced by transmitter hydrolysis.

Subcellular fractionation studies

The intracellular localization of choline acetyltransferase was studied as follows. Each of three fresh muscles was finely minced with scissors and was homogenized in 10 ml. ice-cold 150 mM-KCl containing 1 mM potassium phosphate at pH 7. A motor-driven ground-glass pestle and a closely fitting ground-glass homogenizer were used. Homogenization was continued until a suspension free of visible particles was obtained; this procedure is considerably more traumatic than that required for brain tissues, or for the preparation of intact membranes with end-plates (Namba & Grob, 1968). The homogenates were centrifuged at 151,000 g_{av} for 30 min, and samples of the clear supernatant fluids and of the pellets were assayed for enzyme activity. Of the total activity in the three muscles, 86, 91 and 95 % were recovered in the supernatant fluids. These results are in keeping with the conclusion that brain choline acetyltransferase is a cytoplasmic enzyme (Whittaker, 1965; Fonnum, 1968; Potter *et al.* 1968).

Since little acetyltransferase sedimented under the conditions described above, it was concluded that few pieces of phrenic nerve terminals survived homogenization. Previous work with other tissues which require prolonged homogenization has shown that, although nerve terminals may be

damaged or destroyed, it is possible to isolate a considerable part of the neurotransmitter content of the intact nerves with homogeneous populations of small vesicles (noradrenaline from rat cardiac muscle: Potter, 1966; ACh from *Torpedo* electric organs: Israël *et al.* 1968). An attempt was made, therefore, to recover some [^{14}C]ACh, in association with small particles, from homogenates of diaphragms. Since recovery suffers with high purity, no attempt was made to separate small vesicles from other microsomes. Sixty per cent of the [^{14}C]ACh made by rat cortical tissue can be isolated with microsomes, and it is clear that ACh does not adsorb to them *in vitro* (Potter *et al.* 1968). Six muscles were studied as follows. They were prepared as for release experiments (Table 2; three in each esterase inhibitor), and each was then homogenized in 10 ml. 10 % (w/w) sucrose containing the same esterase inhibitor as in the baths. Particles were separated by centrifuging the whole homogenates into linear density gradients (50 ml. 20–50 % sucrose in Spinco SW 25·2 tubes) at 76,000 g_{av} for 30 min. Particle layers in the gradients appeared comparable to those previously observed with cardiac muscle (Potter, 1966). Each microsomal layer was removed with a pipette, diluted threefold with 150 mM-KCl, and was recentrifuged at 79,000 g_{av} for 30 min. The amounts of [^{14}C]ACh found in the pellets were 14, 16 and 22 p-mole from neostigmine-treated muscles, and 12, 17 and 30 p-mole from the eserine-treated tissues. The mean value of all six results represented 18 % of the normal store.

DISCUSSION

Choline uptake

Cholinergic nerves in the diaphragm, as in ganglia, appear dependent upon supplies of extraneural choline for ACh synthesis. In both tissues added choline is rapidly used for transmitter synthesis, and the utilization of either endogenous or added choline is inhibited by HC-3 (Birks & MacIntosh, 1961; Collier & MacIntosh, 1969). In ganglia, transmitter synthesis gradually declines during nerve stimulation unless choline is given, but the larger molar ratio of choline to ACh in diaphragms than in ganglia (about 58:1 versus 0·7:1, Friesen, Ling & Nagai, 1967; Collier & MacIntosh, 1969), and the apparently slower rate of chlorine efflux from muscles than from ganglia (Perry, 1953; Collier & MacIntosh, 1969), have the effect of providing enough choline to phrenic nerves *in vitro* to forestall a failure of ACh synthesis beyond the usual experiment. The free choline in muscle is largely lost in choline-free media, suggesting that the tissue does not synthesize it in significant amounts, and that the plasma ultimately provides what is required. Its concentration in the plasma of several mammals is 5–50 μM, and at least in man the level is held quite constant (Bligh, 1952). Because of this constancy, and because

neither decreased nor increased bath levels (1–100 μM) affected ACh stores or synthesis rates in muscles, it may be concluded that the availability of extraneural choline is normally fully adequate for ACh synthesis.

It is reasonable to suppose that nerve terminals can also make use of some of the choline which becomes available at synapses when released ACh is hydrolysed. Perry (1953) and Collier & MacIntosh (1969) found that the sum of choline and ACh release from stimulated ganglia was roughly halved by the presence of eserine, indicating some retention of choline in the tissue, although not necessarily in nerves. The present results show that endogenous ACh in phrenic nerves is more rapidly replaced by [^{14}C]ACh when hydrolysis of the endogenous ACh, and thus the re-use of endogenous choline, are prevented by neostigmine. This result may mean that nearly half of the choline provided by transmitter hydrolysis is re-used for ACh synthesis.

It is known that HC-3 competitively inhibits choline uptake into isolated cellular elements, including nerves, and does not significantly affect ACh synthesis otherwise (Hodgkin & Martin, 1965; Martin, 1968; Potter, 1968; Gardiner, 1961; MacIntosh, 1963). Results obtained with HC-3 indicate that choline stores in nerves are small. During prolonged nerve stimulation, muscles in eserine and HC-3 released only a little more ACh than was depleted from the tissues, an amount equivalent to about 8% of the normal store. Similar experiments with ganglia indicated a residual synthesis of 9% (Birks & MacIntosh, 1961: table II, F, G; see also Collier & MacIntosh, 1969). It appears from these results that the amount of choline in nerves which is available for ACh resynthesis is less than 10% of the ACh content, and is probably no more than a few per cent, since choline uptake is not fully arrested by HC-3.

If the foregoing conclusion is correct, it follows that the rate of net transmitter synthesis during the turnover of a large fraction of ACh stores is an approximate measure of the rate of net choline uptake by the nerves. During nerve stimulation at 20/sec for 5 min, 35% of the [^{14}C]ACh in muscles was released and resynthesized. Choline was therefore provided for transmitter synthesis at the rate of 0·12 p-mole/sec. The number of end-plates in the muscles studied was about 3000, since 36% of the left hemidiaphragm was used (by weight, the middle muscle fibres being the longest), and this whole muscle has about 10,000 end-plates (Krnjević & Mitchell, 1961). R. Miledi (unpublished observations) has estimated the cross-sectional area of nerve terminals in osmium-fixed rat diaphragms as 2·2 μm^2, and their length as about 150 μm. Considering the terminals as cylinders, and assuming that they are responsible for 50–100% of the ACh release and resynthesis observed during 5 min of nerve stimulation, net choline uptake by the terminals may be estimated as 3–6 p-mole/cm^2.sec. In 30 μM choline the uptake rates into squid axons (Hodgkin & Martin, 1965) and human red blood cells (re-calculated from Martin, 1968, using Ponder's values, 1934, for cell dimensions) are only 0·03 and 0·0005 p-mole/cm^2.sec, respectively. In relative terms, therefore, phrenic nerve terminals have a very well developed mechanism for choline uptake.

ACh synthesis

Transmitter synthesis in innervated diaphragms appears limited to phrenic nerves. The present results confirm and extend those of Hebb *et al.* (1964) to the effect that both choline acetyltransferase and ACh are localized in the region of neuromuscular junctions. Following section of the nerves, the small amounts of enzyme and transmitter which lie outside the main junctional region disappear to the same degree as the larger amounts in the usual region; there is therefore no reason to suspect synthesis outside of nerves in normal muscles. After denervation, however, the residual enzyme and ACh may be present in Schwann cells (cf. Miledi & Slater, 1968).

ACh synthesis was found to keep pace with transmitter release at a release rate one-quarter that of the maximum observed rate (compare synthesis = release of 35 % of store size in 5 min with a release of 7·8 % in 18 sec). The sustained rate of replenishment of ACh stores during nerve stimulation at 20/sec, 7 %/min, may be compared to a rate of 10 %/min with comparable stimulation of plasma-perfused ganglia (Birks & MacIntosh, 1961: table II, H). Unfortunately it is not possible to say whether the higher release rates observed with both tissues are accompanied by correspondingly high rates of transmitter synthesis. Present data are also not sufficient to determine what fraction of the choline acetyltransferase of diaphragms is in nerve terminals. Nor is it likely, because the terminals are readily disrupted, that a direct estimate can be made, comparable to observations that 50–80 % of the enzyme in brain tissues can be isolated in identifiable nerve terminals (Whittaker, 1965).

A good case can be made that the rate of ACh synthesis is not constant in phrenic nerve terminals, but is at least partially regulated in response to transmitter release. The slowest rate of synthesis observed was in resting muscles which were maintaining nearly twice normal ACh stores in the presence of eserine. In these muscles the synthesis rate was presumably equal to the spontaneous release rate of 1·2 % of normal stores per minute. A higher rate of synthesis occurred in muscles in neostigmine during stimulation at 20/sec, when stores remained constant during release of 7 % of the store size per minute. These results indicate that synthesis can be related to release. But this simple picture is complicated by the observation that resting diaphragms placed in eserine gained considerable 'surplus' ACh in 5 min, in addition to maintaining spontaneous release; they accumulated an amount equal to 17 % of the normal store when only 2–3 % of the store turned over without eserine. The implication of this result is that transmitter synthesis in resting muscles is more rapid than required to maintain stores and to balance spontaneous release, and

that the extra amount synthesized is wasted by hydrolysis intraneuronally. The ACh which would become 'surplus' at rest may contribute, however, to releasable stores in stimulated muscles, since during nerve stimulation only a little more [^{14}C]ACh accumulated in 10 min in eserine than in neostigmine (Fig. 2). In any case, synthesis was at least twice as rapid in stimulated muscles as at any time in muscles at rest in eserine.

Transmitter storage

The ACh content of diaphragms was quite stable, neither increasing nor decreasing with rest, nerve stimulation, long incubations, or major changes in the bath choline concentrations. A possible explanation for this is that most of the ACh in phrenic nerves is stored in vesicles, which on the average are kept at a constant content. Alternatively it is possible that considerable amounts are in the neuroplasm (especially in pre-terminal axons), and that the concentration there is held quite constant by the combined effects of those factors which regulate synthesis, and intraneural esterases. As yet no method has been devised to establish how much of the total store of any transmitter is in vesicles, although direct measurements show that at least half of the amount in some tissues is so held (ACh: Israël *et al.* 1968; noradrenaline: cf. Iversen, 1967). The general trend of such measurements has been towards higher recoveries in the vesicles, as improved media and procedures for homogenization and centrifugation are used. It is to be expected, therefore, that more ACh will be recovered with phrenic nerve vesicles in the future, than the relatively low average of 18 % of the total, in microsomes, achieved in the present study.

The relative amounts of ACh in phrenic nerve axons and their terminals are unknown (cf. Hebb *et al.* 1964). Unfortunately it cannot be assumed that all of the ACh which is released during nerve stimulation comes from nerve terminals, since the total ACh content of the nerves, including the portion not in terminals, does turn over during prolonged incubations with or without stimulation, and because isolated cholinergic axons are known to release and resynthesize large amounts of ACh (Saunders, 1965). In the 30 min period of intermittent stimulation used for replacement studies (Fig. 2), 83 % of the endogenous store was replaced, when without stimulation 17 % was; with HC-3, total normal stores were reduced by 77 % with prolonged stimulation. At best these results permit only a guess that at least half of the ACh in the nerves studied was in their terminals.

Cole (1957) found that the width of end-plates in rat diaphragms varied between 19 and 28 μm. If the average thickness of the terminals is 1·67 μm (R. Miledi, unpublished), the total volume of the end-plate disk varies between 600 and 1000 μm^3. The appearance of stained end-plates suggests that the terminals occupy less than half of this; taking 150 μm as their average length gives a volume of 330 μm^3. From this and the estimate that 50 p-mole ACh are present in 3000 terminals, the con-

centration of ACh in the whole terminals (expressed as if in uniform solution) would appear to be about 50 mM. If the vesicles occupy 5–10 % of the terminal volume and contain 50–100 % of the ACh in terminals, then the tonicity of ACh in each vesicle should be several times the tonicity of rat plasma. A vesicle core 30 nm in diameter containing 0·5–1·0 M ACh would have 4000–9000 molecules. This amount is less than that required to mimic quanta by micro-injection (about 10^5 molecules: Krnjević & Miledi, 1958), but is greater than the amount found in isolated vesicles from brains (roughly 1500 after corrections: Whittaker, 1965. A two to threefold higher concentration was found in the larger vesicles of electric organs by Sheridan, Whittaker & Israël, 1966).

Transmitter release

The amount of [^{14}C]ACh released during 360 nerve impulses was independent of the frequency of stimulation between 1 and 20/sec. Per impulse the amount was 0·022 % of store size, so if half of the tissue ACh is in nerve terminals, the store there is theoretically adequate for 2300 impulses. The maximum observed release rate was 0·43 % of total stores/sec. These figures may be compared with a constant volley output of 0·013 % of store size from ganglia stimulated at 1–64/sec for 500 impulses, and a maximum release rate of 0·87 %/sec (Birks & MacIntosh, 1961). Thus although the ACh content of ganglia, 1460 p-mole, is about 15 times that of the muscle segments studied, the ratio between store size and initial release rate is similar in the two tissues.

As in previous investigations (Straughan, 1960; Krnjević & Mitchell, 1961; Birks & MacIntosh, 1961; Bowman & Hemsworth, 1965), less ACh was collected during prolonged nerve stimulation at high than low rates, per impulse. This result is in accord with neurophysiological evidence that release declines progressively during stimulation at rates above 1–5/sec, and much more rapidly at high than low frequencies (Brooks & Thies, 1962; Thies, 1965).

Assuming that 50–100 % of the ACh collected came from nerve terminals, the amount was 3·5–7 times 10^{-18} mole per end-plate per impulse. It is tempting to divide this amount by published figures for the number of quanta released per impulse in order to estimate the number of molecules of ACh per quantum. Such estimates (e.g. 100 quanta/impulse, Liley, 1956, gives 20,000–40,000 molecules per quantum) are higher than anticipated, a discrepancy which may be the result of (*a*) repetitive firing of nerve impulses in the presence of neostigmine, (*b*) non-quantal release of ACh during nerve stimulation, and/or (*c*) the possibility that present techniques underestimate the number of quanta released at a distance from the post-synaptic membrane.

The fact that HC-3 reduced the spontaneous release of ACh from eserinized muscles suggests that the transmitter released at rest may come primarily from the cytoplasm. (A related result is that choline efflux from red blood cells is reduced by HC-3 (Martin, 1968).) Spontaneous quantal release does not appear to contribute much to resting release, since even if

quanta represented 40,000 molecules of ACh each, 60/sec would be required to account for the observed release. The results do show that 'surplus' ACh helped to sustain a high rate of release during prolonged stimulation. Either the 'surplus' contributes to refilling quanta, or it contributes to non-quantal release during stimulation (in excess of resting release) at a rate which is not quantitatively different from non-quantal release in the presence of neostigmine.

Neurophysiological evidence clearly indicates that there can be depression of the number of quanta released at neuromuscular junctions even after a single impulse which releases less than 1% of the total available quanta (cf. Martin, 1966). In terms of the vesicle hypothesis, this can be visualized as due to the depletion of those vesicles nearest the nerve terminal membrane; if anything, the release mechanism at this time is still facilitated by movements of calcium (Katz & Miledi, 1968). Maintenance of transmitter output is then dependent upon the rate of replacement of quanta which, because of the low Q_{10} of the recovery process from a single impulse (Takeuchi, 1958), appears at first less dependent upon new synthesis than upon other factors. New synthesis soon takes a major part in maintaining release, however, since newly synthesized ACh is released in relatively large quantities well before over-all turnover. Birks & MacIntosh (1961) presented excellent quantitative data concerning the effects of diminishing store size on release rates, which showed that release was faster at first than after some store depletion, when release became proportional to content. The fact that quantal size decreases with store size indicates that there is diffusion of intra- and extravesicular ACh into those vesicles nearest the membrane, and not simply a mobilization process for full vesicles (Elmqvist & Quastel, 1965). The present experiments, and those of Collier & MacIntosh (1969), show in addition, from measurements of replacement of endogenous with [^{14}C]ACh, that most of the transmitter store does slowly contribute to release even when the stores are kept quite fully stocked.

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