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THE EFFECT OF CURARE ON THE RELEASE OF ACETYLCHOLINE FROM MAMMALIAN MOTOR NERVE TERMINALS AND AN ESTIMATE OF QUANTUM CONTENT

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SUMMARY

1. Curarized and non-curarized rat hemidiaphragm muscles were indirectly stimulated *in vitro*.

2. The fluid bathing the active curarized muscles was eluted through a dextran gel (Sephadex G-10), effecting a complete separation of ACh from curare. The acetylcholine fraction was then assayed on an isometric leech muscle preparation.

3. Prostaglandin (PGE_1) in a concentration fifteen times that estimated to be released from the skeletal muscle preparation did not affect the response of leech muscle to ACh.

4. The amount of ACh released by curarized muscles ($4{\cdot}9 \times 10^{-18}$ mole/impulse.junction) was not significantly different from that released by non-curarized muscles ($4{\cdot}6 \times 10^{-18}$ mole/impulse.junction). These quantities are similar to those obtained by previous workers. It is concluded that curare in a paralytic dose does not affect the output of ACh from motor nerve terminals stimulated at low frequencies.

5. Spontaneous release of ACh from non-curarized muscles was estimated at 0·45–0·65 p-mole/min. hemidiaphragm. It is calculated that only 2% of this amount could give rise to post-synaptic electrical events, the remainder having a non-synaptic source.

6. The number of molecules of 'quantal' ACh released by stimulated muscle is calculated as $2{\cdot}5 \times 10^6$/impulse.junction, taking account of the non-synaptic release. The number of ACh molecules in one quantum was estimated to be 6250, an amount that could be easily accommodated in one synaptic vesicle.

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INTRODUCTION

Dale, Feldberg & Vogt (1936) found that the amount of ACh released into the fluid perfusing active muscles was not diminished when the muscles were completely paralysed by the addition of curare. They concluded that curare had principally a post-synaptic action, and this was soon supported by Cowan (1936) and Kuffler (1943). More recently, however, it has been proposed that curare may also exert its action pre-synaptically, interfering in some way with the release of transmitter (Lundberg & Quilisch, 1953; Lilleheil & Naess, 1961; Beani, Bianchi & Ledda, 1964; Standaert, 1964; Riker & Okamoto, 1969; Hubbard, Wilson & Miyamoto, 1969; Blaber, 1970; Galindo, 1971; Hubbard & Wilson, 1973).

Controversy has arisen over some of those results. For example, Chang, Cheng & Chen (1967) could not confirm the results of Beani *et al.* (1964) using the same type of preparation. Hubbard *et al.* (1969), monitoring end-plate potentials, claimed a reduction in transmitter release caused by tubocurarine in a modified rat hemidiaphragm preparation employing cut muscle fibres to abolish the twitch. This work was criticized by Auerbach & Betz (1971) on the grounds that the end-plate potential amplitude would be distorted due to alteration of the space constant in cut muscle fibres. Later, Hubbard & Wilson (1973) provided evidence that the alteration of space constant was not a critical factor in the assessment of end-plate potentials. Beranek & Vyskocil (1967), using rat hemidiaphragm, decided that curare had no pre-synaptic effect in doses that produced depression of end-plate potential amplitude. A similar conclusion was reached by Bowen & Merry (1969) and Bauer (1971).

In the present work the development of a gel filtration technique for the separation of ACh and curare has made it possible to assay ACh released from curarized skeletal muscle on the curare-sensitive leech preparation. Eserinized leech muscle is reputed to be one of the most sensitive and specific tests for ACh (MacIntosh & Perry, 1951) and the isometric preparation of this muscle offers a convenient and rapid method of assay (Forrester, 1966). The purification of ACh in the separation technique also provided the opportunity to re-assess the quantal content at low frequencies of stimulation since it is well known that the response of leech muscle and other bio-assay preparations may be altered by other substances released from tissues (Krnjević & Mitchell, 1961).

METHODS

Dissection of rat hemidiaphragm and setting up in bath

Wistar laboratory rats of either sex and in the weight range 170–200 g were used. The rat was killed by stunning and exsanguination. The thorax was opened and both phrenic nerves were dissected free, tied high in the cervical area and then severed centrally to the ligatures. The crura were separated from the diaphragm. Incisions were made in the abdominal wall and thorax such that the diaphragm could be removed intact, with remnants of the rib cage, and transferred to a dish of continuously oxygenated Locke solution. The diaphragm was then divided into its two halves and carefully dissected from the thoracic wall tissues, with the aid of a dissecting microscope. The preparations were then washed by being freely floated for 1 hr in at least 1 l. vigorously oxygenated Locke solution. Each hemidiaphragm was set up in a glass stimulation bath containing 4·0 ml. freshly oxygenated Locke solution. This was aerated with 95 % oxygen/carbon dioxide throughout each experiment. The muscle lay unstretched in the Locke solution. In the case of stimulation experiments, the phrenic nerve was placed loosely over a pair of silver wire electrodes which entered the bath through the stopper. The aerosol effect of the bubbling gas served to keep the nerve trunk adequately moist.

Assay with the leech dorsal muscle

The preparation was that of Forrester (1966). Dorsal muscle strips 2 × 40 mm were taken from the leech (*H. medicinalis*) and set up isometrically in a bath of 2·5 ml. capacity and periodically washed until a stable base line was attained. Tension was recorded via a tension transducer (Grass FTO 3C), and Everett-Edgecumbe pen-recorder.

Test solutions were placed in contact with the muscle for 45 sec. ACh-like activity was estimated quantitatively by a three-point assay. The response/$\log_{10}$ concentration curve was obtained and responses to test and standard solutions from the assay were superimposed on this. If the plots were parallel they were accepted. The quantity of ACh present was calculated.

Packing Sephadex columns

Sephadex G 10 columns were prepared using '*K*9/15' and '*K*9/30' plastic columns (Pharmacia Ltd). These were filled with gel slurry from a syringe, a quick method avoiding spillage or settling of the slurry in a beaker whilst pouring. The slurry was kept as a stock in distilled water at 5° C and equilibrated with leech saline before use. The respective void volumes were 7·5 and 3·0 ml. for columns of 26 and 9·5 cm in length, both 0·9 cm in diameter.

When a sample had entered the top of the gel bed, the collection of fractions was begun, with the initiation of flow from the eluant reservoir. This is defined in the text as the 'standard starting point'.

Gel filtration of ACh

The elution profile of very low concentrations of ACh through the gel filtration column was obtained in the following way. A volume 1·0 ml. AChCl was eluted through the column. The eluate fractions were then tested on the eserinized leech muscle strip. Fig. 1 shows the responses of a leech muscle strip to fractions collected from a Sephadex column (G 10) of 9·5 cm which had 1 ml. $5{\cdot}5 \times 10^{-7}$ M AChCl eluted

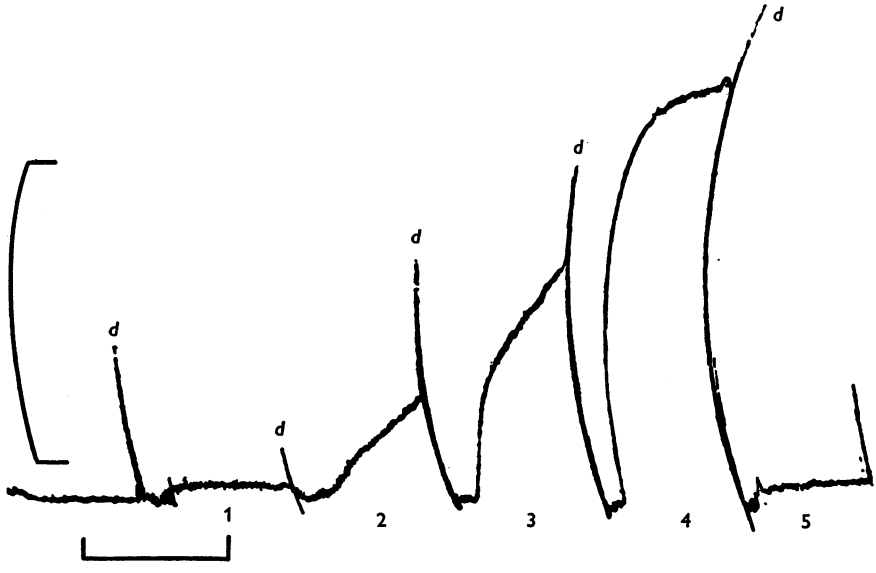

Fig. 1. Responses of a leech muscle strip to eluted ACh from the standard Sephadex column. *d*, pen recorder stopped, bath drained and refilled with test solution. (1) fraction 0–1·5 ml.; (2) ACh, $5{\cdot}5 \times 10^{-8}$ M; (3) fraction 1·5–4·5 ml.; (4) ACh, $2{\cdot}75 \times 10^{-7}$ M; (5) fraction 4·5–6·0 ml. Vertical scale, 1 g tension; horizontal scale, 60 sec. Virtually all of the ACh appears in fraction 1·5–4·5 ml. from standard starting point.

through it. It is evident that all of the ACh is detected in fraction (3) 1·5–4·5 ml. after the standard starting point. The mean percentage recovery by weight of ACh in eighteen experiments was 95·5 % (range 80–100 %).

Gel filtration of tubocurarine

When tubocurarine is applied to leech muscle, it produces a fall of tension, which is dose-dependent (Fig. 2). Also, the competitive blocking action reduces the response elicited from the leech muscle by ACh. This feature was used to detect the presence of curare in the fractions from the gel columns. Thus the elution profile of tubocurarine in a Sephadex column was investigated. After the response of a leech muscle strip to a standard concentration of ACh had been established, a column fraction was applied to the muscle strip for a period of 15 min. The standard concentration of ACh was then applied once more. If a significant reduction of tension in the response occurred, tubocurarine was concluded to be present in the fraction. The elution profile of tubocurarine in a Sephadex column (G 10), 26 × 0·9 cm, using this method, is given in Fig. 3. In each of six experiments the tubocurarine did not appear in any fraction earlier than 30 ml. after the standard starting point. The elution peak was 40–50 ml. In this column, ACh was eluted 6–10 ml. after the starting point. Thus in the relatively long column a very wide separation of ACh and tubocurarine could be achieved.

It was now necessary to find whether the same elution peaks were produced when a mixture of ACh and curare were put through the columns. Fig. 4 shows the

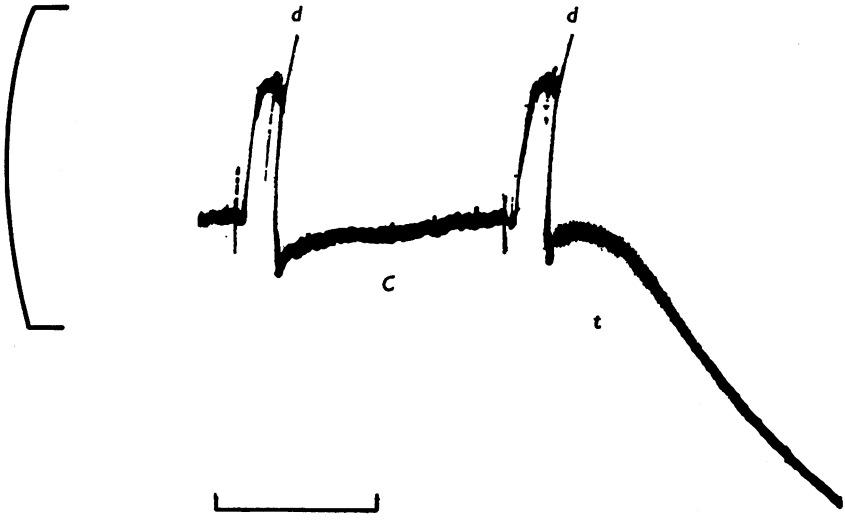

Fig. 2. Response of leech muscle strip to tubocurarine. *C*, Ringer control; *d*, as Fig. 1; *t*, effect of tubocurarine, $1{\cdot}3 \times 10^{-5}$ M.

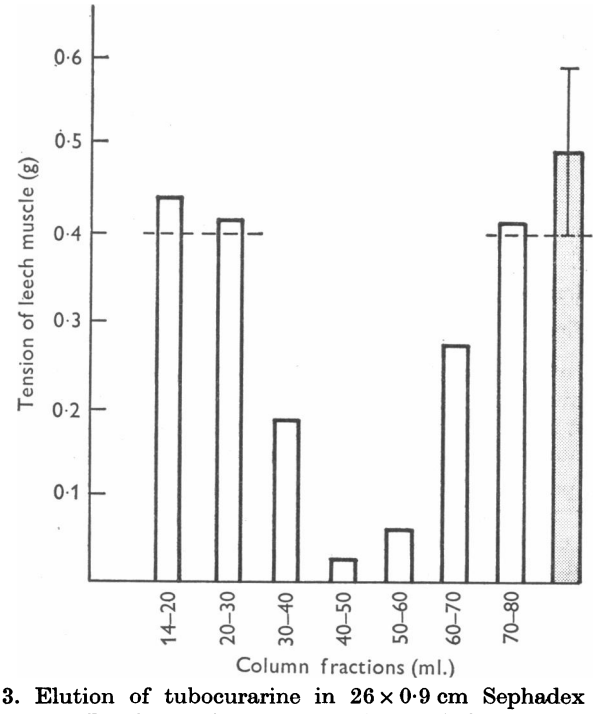

Fig. 3. Elution of tubocurarine in $26 \times 0{\cdot}9$ cm Sephadex column (one experiment). Leech muscle was exposed to each fraction for 15 min. Open columns, leech muscle responses to AChCl after each consecutive column fraction. Stippled column, mean response of $2{\cdot}75 \times 10^{-8}$ M AChCl prior to testing fractions (bar = ± S.D. of observation). The loss of tension indicates the position of tubocurarine in the elution profile.

emergence of ACh ($2{\cdot}74 \times 10^{-6}$ M) from a column $26 \times 0{\cdot}9$ cm, when applied with tubocurarine ($1{\cdot}3 \times 10^{-4}$ M) as a 1:1 volume mixture. In thirteen experiments the mean percentage recovery of ACh was 96·1% (range 85–100%). Peak recovery was in the fraction 6–10 ml. from the standard starting point. This was the same pattern as that given by ACh alone. Although the elution peaks for curare were not determined in all these experiments, nevertheless the results indicated that an effective separation of ACh and tubocurarine could be made.

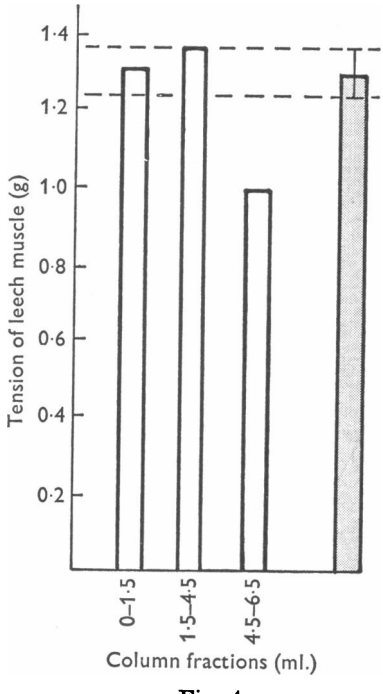

Fig. 4

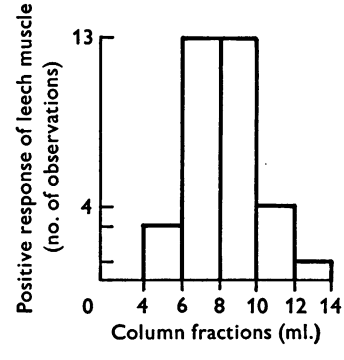

Fig. 5

Fig. 4. Elution of tubocurarine in the standard Sephadex column. Open columns, leech muscle responses to AChCl after each consecutive fraction; stippled column, mean response to AChCl, $2{\cdot}75 \times 10^{-8}$ M (bar = ± S.D. of observation). Curare first appears after the 1·5–4·5 ml. fraction.

Fig. 5. Elution pattern of ACh in G-10 Sephadex column of $26 \times 0{\cdot}9$ cm. ACh, $2{\cdot}75 \times 10^{-7}$ M, added along with tubocurarine, $6{\cdot}5 \times 10^{-5}$ M. Total ACh appears to eluate up to 14 ml. Appearance of tubocurarine starts at 30 ml. (Fig. 3) in this column.

Standard separation procedure

In general, the shorter a gel column, the less dilution there is of substances under elution. Since the amounts of ACh in the muscle bathing solutions were anticipated to be very low, as short a column as possible was the ultimate aim. It was found by trial and error that a column of dimensions $9{\cdot}5 \times 0{\cdot}9$ cm could just effect a separation of ACh from tubocurarine. Fig. 4 shows the elution pattern of curare from this shorter

column. No test for the disappearance of curare in later fractions was made as it was necessary to define only the leading boundary containing the curare. It appears first in the fraction 4·5–6·5 ml. after the standard starting point. In this column ACh is eluted just ahead of curare in the fraction 1·5–4·5 ml. after the starting point (Fig. 1). It was concluded that ACh could be completely separated from tubocurarine in this column with the minimum of dilution (× 3) taking place.

Solutions used

Phrenic nerve–hemidiaphragm preparations were dissected and stimulated in Locke solution of the following composition; (mM) NaCl, 154; $NaHCO_3$, 6; KCl, 5·6; $CaCl_2$, 2·2 and Glucose, $5{\cdot}5 \times 10^{-3}$ M. Eserine (physostigmine sulphate: Macfarlane Robson, Edinburgh) $1{\cdot}5 \times 10^{-5}$ M, pH 7·0, adjusted and maintained by O_2/CO_2, 95/5 %, room temperature (20–22° C).

Leech dorsal muscle strips were maintained in 'Leech saline', a 1:1·4 dilution of the eserinized Locke solution; composition (mM): NaCl, 110; $NaHCO_3$, 4·3; KCl, 4; $CaCl_2$, 1·6; and glucose, $3{\cdot}9 \times 10^{-3}$ M. Eserine, $1{\cdot}07 \times 10^{-5}$ M.

Drugs

Actylcholine was AChCl (Roche) and values in the test are as chloride. Tubocurarine was the chloride (Burroughs-Wellcome); text values as such. Prostaglandin E_1 was kindly supplied by Professor D. A. van Dorp, Unilever, Vlaardingen, and Dr H. C. McKirdy, Department of Physiology, University of Glasgow. The PGE_1 was stored as an ethanolic solution ($2{\cdot}5 \times 10^{-3}$ M) at 5° C. In this work, its activity was qualitatively checked by a rat fundus strip preparation (Vane, 1957) before use in the leech experiments.

TABLE 1. Output of acetylcholine from indirectly stimulated rat hemidiaphragm

		Stimulation		
		Frequency	Duration	ACh output (mole × 10^{-18}/impulse. junction (± S.D.))
	n	(Hz)	(min)	
Non-curarized	16	1	60	4·6 (1·09)
Curarized	18	1	60	4·9 (1·03)
Non-curarized	13	10	20	1·2 (0·60)
Non-curarized	7	1	30	5·8 (1·90)

RESULTS

Release of ACh from non-curarized, indirectly stimulated muscle

Rat hemidiaphragm muscles were stimulated indirectly in a 4·0 ml. volume at 1 Hz for 60 min. The bathing fluid was then immediatley assayed for ACh on leech muscle. Table 1 compiles the results of sixteen experiments. There was a mean output of $4{\cdot}6 \times 10^{-18}$ mole/impulse. junction. It has been assumed that the number of end-plates per hemidiaphragm is 10^4, the number found by Krnjević & Mitchell (1961) in rats of similar weight to those used in this work.

Experiments of 30 min produced lower *total* outputs of ACh, but the

assay of this quantity of transmitter fell on that portion of the dose–response curve where discrimination is less reliable. Consequently this shorter experiment was carried out only when extra sensitive leeches were available. Table 1 gives the results of seven experiments of this duration. A mean value of $5{\cdot}8 \times 10^{-18}$ mole/impulse.junction was obtained. This value is significantly different from that using stimulation at 1 Hz for 60 min ($0{\cdot}01 > P > 0{\cdot}001$). When the muscles were stimulated at 10 Hz for 20 min, the ACh output dropped to a value of $1{\cdot}2 \times 10^{-18}$ mole/impulse. junction. This is significantly different from the release where stimulation took place at 1 Hz for 60 min ($P < 0{\cdot}001$).

Release of ACh from curarized indirectly stimulated muscle

Muscles were stimulated at 1 Hz for 60 min in 4 ml bathing fluid which was then eluted through the standard Sephadex column (Methods). The fraction containing all the curare-free ACh was assayed on leech muscle. The results are shown in Table 1. In sixteen experiments a parallel control was run, using the contralateral hemidiaphragm in bathing solution without curare (results included above). The mean output was $4{\cdot}9 \times 10^{-18}$ mole/impulse.junction. This is not significantly different from the output from the non-curarized muscles (Student's t-test for difference: $P < 0{\cdot}05$).

Spontaneous release of ACh from non-curarized muscle

Muscles were soaked in 4·0 ml. solutions for 30 or 60 min duration. Fewer results were obtained from the 30 min periods, because the smaller quantities of ACh so obtained could not be reliably assayed unless extra sensitive leeches were available. For the same reason it was not possible to investigate the spontaneous release of ACh from a curarized muscle, since the requirement to separate the curare in the gel column leads to dilution beyond the threshold of assay. The rate of release of ACh, was smaller in the 60 min experiment than in the 30 min but did not differ significantly; $P > 0{\cdot}05$ (values of $0{\cdot}45 \pm 0{\cdot}03$ and $0{\cdot}65 \pm 0{\cdot}04$ p.mole/min respectively).

Study of the effect of prostaglandin E_1 on the leech muscle

In view of two reports describing the release of prostaglandin E_1 from mammalian skeletal muscle (Ramwell, Shaw & Kucharski, 1965; Laity, 1969), it was necessary to check whether the response of the leech muscle to ACh was altered in any way by the presence of prostaglandin. Most prostaglandins are capable of producing effects on smooth muscle (Horton, 1971; Bergström, Carlson & Weeks, 1968; Pickles, 1969), but any effects on leech muscle have apparently not been investigated.

Ramwell *et al.* obtained a total recovery of PGE_1 of 125 ng (± 33) per g

wet wt. of rat hemidiaphragm. An average weight for hemidiaphragm in the present work was 0·35 g; this would give a total PGE_1 content of 44 ng per hemidiaphragm. On the unlikely assumption that all of this would diffuse out during stimulation, a bathing solution of 4·0 ml. would contain 11 ng/ml. ($3{\cdot}1 \times 10^{-8}$ M) PGE_1. Laity (1969) obtained from indirectly stimulated rat hemidiaphragms 0·25 ng/ml. ($7{\cdot}0 \times 10^{-10}$ M) PGE_1 in the bathing solution. The present experiments showed no immediate response by leech muscle strips to PGE_1 in leech saline in concentrations up to $2{\cdot}8 \times 10^{-5}$ M PGE_1. The strip was then soaked for 15 min periods in PGE_1 $2{\cdot}8 \times 10^{-5}$ M. No unusual changes of tension took place during these periods, or in the intervening periods when leech saline replaced the drug solutions. In five experiments, dose–response curves of leech muscle to ACh were obtained and repeated using the same concentrations of ACh in the presence of PGE_1, $1{\cdot}4 \times 10^{-6}$ M. No significant difference was found. It is concluded that PGE_1, in concentrations fifteen times higher than those amounts released from active muscle, neither affected the leech muscle directly nor altered the sensitivity of the leech muscle to ACh.

DISCUSSION

The principal finding in this work is that curare, in a paralytic dose, does not affect the release of ACh from stimulated motor nerve terminals at low rates of stimulation. The amounts of ACh released are in accord with those estimated by other workers.

It has been assumed in calculating the output that every impulse delivered to the nerve bundle reached every nerve terminal but it is probable that this ideal situation did not pertain. As shown by Creese (1954), hypoxia readily occurs in the rat diaphragm preparation and only the most vigorous oxygenation can prevent it. Impairment of conduction, especially at points of axon bifurcation, has been found by Krnjević & Miledi (1959). Paul (1961) later showed this to be due to hypoxia in the hemidiaphragm preparation. The decline in the ACh output with increased frequency of stimulation (Table 1) would be explained by the increased demand for oxygen, precipitating an unknown degree of hypoxia and nerve conduction block. In this context it is useful to compare our results with those of Beani *et al.* (1964). If it is assumed that the guinea-pig diaphragm has a similar end-plate population to the rat, they found that at rates of 100/min, 5, 20 and 50/sec, the ACh output was 4·0, 2·0, 1·0 and $0{\cdot}45 \times 10^{-18}$ mole/impulse.junction respectively. Only at the lowest frequency of stimulation does their output approach the $4{\cdot}6 \times 10^{-18}$ mole/impulse. junction obtained in this work, when the frequency was 1 Hz. This suggests that the number of impulses reaching the nerve terminal is highly

dependent on conditions of oxygenation. It is interesting to note that some workers who have concluded that curare has no presynaptic action, used either amphibian preparations with low stimulation frequencies (Beranek & Vyskocil, 1967), or mammalian preparations under optimal conditions of oxygenation (Hutter, 1952). It would seem difficult to assess any effect of curare together with hypoxia on ACh release in preparations where the stimulation frequency is high. Another well-documented factor contributing to the decline in output with increasing frequency would be the 'depression' of the release mechanism seen after a single stimulus (Takeuchi, 1958; Thies, 1965).

The frequency 1 Hz used in this work was the minimum required to produce sufficient ACh in the bathing solution for satisfactory assay after gel filtration. Whatever criticisms may be made of the general experimental conditions, however, the present experiment was a direct comparison of the two preparations differing in one parameter only, the curarization. It could be argued that the abolition of the twitch by curare diminishes the oxygen demand. The improved conditions might then enhance the output of ACh slightly, offsetting any small decrease in ACh release that curare may produce. This seems unlikely, however, since curare does not have any effect on the spontaneous release of ACh (Beani *et al.* 1964).

TABLE 2. Spontaneous release of ACh from isolated hemidiaphragm of rat

	Temperature (° C)	ACh (p-mole/min. hemidiaphragm)
Krnjevic & Mitchell (1961)	'Room'	1
Hayes & Riker (1963)	37	2
Mitchell & Silver (1963)	37	2–3
Krnjevic & Straughan (1964)	37	2·3
Forrester (1967)	'Room'	1–3
This work	18–22	0·45–0·65

Spontaneous release of ACh

The results for spontaneous release of ACh are somewhat lower than those found by other workers using bio-assay methods (Table 2). This perhaps indicates that our preparations were in at least as good a condition as those of previous investigators, since it is well known that deteriorating preparations give rise to an increased frequency of m.e.p.p.s. It is evident, however, from the calculations below that the amounts of ACh giving rise to m.e.p.p.s is apparantly only a small fraction of the total spontaneous release.

A theoretical figure for spontaneous release can be calculated from m.e.p.p. frequency, number of end-plates and the number of ACh molecules computed to be contained in one synaptic vesicle. Liley (1956) estimated

m.e.p.p. frequency in rat hemidiaphragm as 3/sec at 37° C. The average end-plate population of a hemidiaphragm is 10^4 (Krnjević & Mitchell, 1961). An estimate of 4000 molecules per vesicle has been made (Hubbard, 1973). Thus the theoretical amount of ACh spontaneously released is:

$$\frac{\text{molecules}}{\text{m.e.p.p.s}} \times \frac{\text{m.e.p.p.s}}{\text{min}} \times \frac{\text{end-plates}}{\text{hemidiaphragm}} = \frac{4 \times 10^3 \times 180 \times 10^4}{N}$$

moles/min.hemidiaphragm (N = Avagadro's no.)

= 0·01 p-mole/min.hemidiaphragm.

The actual amount measured for spontaneous release over a 60 min period was 0·45 p-mole/min.hemidiaphragm. If all of this were to come from vesicles, then each vesicle would need to contain 150,000 molecules. This is clearly untenable, since even if the vesicle contained a saturated solution of ACh, the number of molecules would amount to only 45,000 (Allen, 1962; Canepa, 1964). If the figure of 4000 molecules/vesicle is accepted, then it would appear that of the 0·45 p-mole/min.hemidiaphragm detected, only 0·01 p-mole/min have a 'quantal' origin. The source of the remaining 0·44 p/mole is presumably from intramuscular nerves (Brooks, 1954; Mitchell & Silver, 1963) and can be regarded as 'non-synaptic' that is, not giving rise to post-synaptic electrical events. As will be seen below, when this non-synaptic portion is taken into consideration for the stimulated output of ACh, no great discrepancy of quantal content arises.

Stimulated release of ACh

The total output of ACh from a hemidiaphragm stimulated at 1 Hz for 60 min was assessed at 170 p-mole. Assuming that the non-synaptic (spontaneous) output of ACh is unaffected by indirect stimulation, the total synaptic output becomes $170 - (0{\cdot}44 \times 60) = 144$ p-mole. For a total of 3600 impulses delivered, with 10^4 end-plates, it is calculated that there are $2{\cdot}5 \times 10^6$ molecules ACh released per junction per impulse. This compares favourably with previous results obtained from assay experiments (Straughan, 1960; Krnjević & Mitchell, 1961; Forrester, 1967).

Quantum estimation

Hubbard & Wilson (1973), using end-plate potential recordings estimated the mean quantal content in the rat hemidiaphragm at 200 per impulse when stimulation was at 5 Hz. The quantal content rose to 334 when the frequency was lowered to 2 Hz. Blaber (1970), using cat tenuissimus muscle, calculated the quantal content of the first few end-plate potentials to be 400, at stimulation frequencies ranging between 25 and 100 Hz. It therefore seems reasonable to assume that the quantal content

at 1 Hz would be about 400. From the figure $2{\cdot}5 \times 10^{-6}$ molecules per impulse.junction previously calculated, the number of molecules per quantum is 6250, an amount that can easily be accommodated in one vesicle. The excess amount calculated per vesicle over the isosmotic amount of 4000 molecules per vesicle (Hubbard, 1973) could be explained through intramuscular nerves releasing more non-synaptic ACh in the active state. Thus, having accounted for non-synaptic release, the amounts of ACh assayed from the stimulated muscle would strongly support the hypothesis that the contents of one vesicle provide enough ACh for one quantum. Added strength to the vesicle hypothesis has been proved recently by Heuser (1974) who has correlated the presence of 'giant' vesicles with the recording of large m.e.p.p.s.

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