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DECAMETHONIUM IN THE PERFUSED AND IMMERSED RAT DIAPHRAGM

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1 The water content and mannitol space of rat diaphragms which were perfused through the inferior vena cava was increased compared with immersed diaphragms. The potassium content of both preparations, when expressed in terms of dry weight, was maintained at similar values to that found *in vivo*.

2 Despite the application of a constant concentration of decamethonium, a steady level of neuromuscular block was not obtained in either the perfused or immersed rat diaphragm. The immersed preparation differed from the perfused preparation in that recovery from paralysis occurred despite the continued presence of the drug.

3 The rate of uptake of labelled decamethonium (100 μM) at the end-plate region was similar in the perfused and immersed diaphragm. The slopes of the regressions were 0.059 and 0.054 μl $mg^{-1} min^{-1}$ (based on dry weight) respectively which were not significantly different. This implies that the rate of uptake of the drug at the end-plate is slow and limited by the rate of entry into the fibre rather than by diffusion to the site of entry.

Introduction

Creese & Maclagan (1970) have obtained autoradiographic evidence that decamethonium enters muscle fibres in the rat. It has been suggested that the permeability of this organic ion at the end-plate is low and similar to that of sodium (Creese & England, 1970). If this is the case, then the uptake of decamethonium would be expected to be limited by entry at the cell membrane rather than by diffusion. If however, the permeability of decamethonium approached that of potassium then the uptake would be faster in a perfused muscle than in an immersed muscle because of delays due to diffusion in the latter (Klaus, Lullman & Muscholl, 1960; Keynes, 1954).

In the present study, rat diaphragms were perfused *in vitro* via the inferior vena cava with labelled drug to compare the rate of uptake with that in immersed muscles. In addition, the neuromuscular block produced by decamethonium was recorded in an attempt to confirm previous claims that a steady paralysis can be obtained with depolarizing drugs (Maclagan, 1962; Gibberd, 1966) when applied via the capillaries. The water, potassium and sodium content and also the mannitol space were determined in both preparations.

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Methods

The composition of the saline was (mM): K^+ 5.0, Na^+ 145.0, Ca^{2+} 1.3, Mg^{2+} 1.2, Cl^- 125.0, HCO_3^- 25.0, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2 (Creese & Northover, 1961). The glucose content was 200 mg/100 ml and the solution was gassed with 5% v/v carbon dioxide and 95% oxygen. The temperature was maintained at 38°C. Albino Wistar rats of 80-140 g were used. The animals were stunned and then decapitated.

For the recording of isometric muscle contractions, a strain guage (Statham micro-scale. model UL5) and a Statham transducing cell (model UC3) were connected to a pen recorder (Devices single-channel). The left phrenic nerve was stimulated with rectangular maximal pulses of 0.2 ms duration delivered at a frequency of 6/minute.

Immersed diaphragm

The left anterior portion of the diaphragm together with its rib was quickly removed, with or without the left phrenic nerve and transferred to a bath (100 ml) for recording of contractions, or to a tube (10 ml) for measurement of uptake of labelled decamethonium. Twitch tension was recorded by means of a strain gauge connected to the tendon by a thin platinum wire (0.4 g). The uptake of the labelled drug was measured in the manner described by Creese & England (1970). After immersion in labelled drug the muscle was

washed in saline for 10 min, attached to a metal strip and frozen on solid carbon dioxide.

Perfused diaphragm

The preparation is similar to that described by Gibberd (1966). The inferior vena cava was cannulated through the right auricle and the entire diaphragm with the left phrenic nerve was transferred to cold saline. The abdominal inferior vena cava was then tied below the diaphragm and the liver dissected to leave the tied stump of the vein. The diaphragm was then transferred to the perfusion chamber (200 ml) where the atmosphere was kept moist by a thin aerated layer of saline at the bottom. The diaphragm was perfused by means of a roller pump (Watson-Marlow flow inducer) at a rate between 3 and 4 ml/minute. The muscle could be perfused from one of two jacketed reservoirs. The change-over time (i.e. the time taken for the second perfusion of saline to reach the tip of the cannula) was 6 s at a flow rate of 3.2 ml/minute. Contractions of the left side of the diaphragm were recorded by connecting the tendon to a strain gauge by means of a platinum wire (0.4 g). For uptake studies, the entire diaphragm was perfused first with saline and then switched to the reservoir containing saline plus labelled decamethonium. After a known interval the muscle was again perfused for 10 min with saline and the perfusion chamber was filled so that the diaphragm was immersed. The immersion fluid was changed after 5 minutes. The left anterior strip was then quickly dissected and frozen.

Freezing of diaphragms

The muscles were frozen on solid carbon dioxide in the manner described by England (1970) and sliced horizontally from tendon to rib in a direction parallel to the band of end-plates to produce a series of strips 1 mm wide. The frozen strips were rapidly weighted, transferred to polyethylene vials, dissolved and counted by scintillation methods (Creese & Taylor, 1967).

Analysis of muscles

Sodium, potassium and water content of muscles together with the mannitol space were measured by the method of Creese (1968).

Scintillation counting

Samples were counted in polyethylene vials at 6°C in a Packard 3000 Series Tri-carb Liquid Scintillation Spectrometer. The background was approximately 20 ct/min and the efficiency was 27.1% when estimated by a standard, tritiated *n*-hexadecane. The quenching produced by 6 mg of muscle tissue was less than 1%, and the counting rate was proportional to the radioactivity added to the vials.

Drugs used

Decamethonium dibromide (Syncurine, Burroughs Wellcome & Co., U.S.A.) mol.wt. 419; D(+)-mannitol (Hopkin & Williams Ltd., Chadwell Heath, Essex) mol.wt. 182; [^{3}H-methyl]-decamethonium dichloride (mol.wt. 329) with a specific activity of 1.1 Ci/mmol was obtained from the Radiochemical Centre, Amersham, Buckinghamshire. The compound was analysed at Amersham by thin-layer chromatography on alumina in chloroform-methanol (80:20 v/v) and the radiochemical purity was 98%.

Tritiated (+)-mannitol (mol.wt. 182) with a specific activity of 0.5 Ci/mmol was also obtained from the Radiochemical Centre where analysis by paper chromatography showed that the radiochemical purity was greater than 98%.

Results

Water and ion content

Table 1 shows an analysis of the water and ion content of rat diaphragms after immersion for 1 h and after perfusion for 1 h with saline. Although the whole diaphragm was perfused, only the left anterior portion was analysed.

As can be seen from Table 1 there is a small increase in the water content of the immersed muscle compared with the controls *in vivo*, similar to that obtained by Creese (1954) and Creese, El-Shafie & Vrbova (1968). There is a large increase in the water content of perfused muscles as compared with controls *in vivo*. The mannitol space is also markedly increased in the perfused muscle compared to the immersed muscle.

The potassium content (K^+/g wet tissue) is lower in the perfused preparation. This result is due to the high mannitol space, for when calculated on a dry weight basis the potassium content in immersed and perfused muscles shows little change when compared with controls *in vivo* (Table 1).

There is a large rise in the sodium content of the perfused diaphragm compared to the immersed preparation. From Table 1 it can be calculated that the increase in the mannitol space for the perfused diaphragm compared to the immersed

diaphragm is 214 ml/100 g dried muscle. The sodium associated with this increased mannitol space is 31.0 mEquiv/100 g dried muscle. The large increase in the sodium content of the perfused preparation could therefore be attributed to the increase in the extracellular fluid. The mannitol space and the sodium content were not estimated on the same piece of muscle and for this reason it is not possible to obtain a valid estimate of the fibre sodium whose estimation involves a small difference between two large values (see Creese, 1968).

Neuromuscular block

Both immersed and perfused preparations were allowed to contract for 30 min or more to ensure that the contraction height was steady before the application of drug. After perfusion with saline the solution was changed so that the muscle was perfused with saline containing a known concentration of decamethonium.

Figure 1 shows a recording of such an experiment after perfusion with decamethonium, 150 μM. The block at this concentration commenced almost immediately and 100% block was achieved within 250-350 seconds. The paralysis could be quickly reversed by switching back to the saline reservoir; full recovery took about 250 seconds. Tachyphylaxis was not evident in the perfused rat diaphragm as can be seen from the figure. All the muscles used maintained full contractility during the experimental period which in some cases was 5 h or more.

The effect of smaller concentrations of decamethonium was also investigated. Concentrations of 45 μM and 10 μM decamethonium were infused from the second reservoir. The results obtained are illustrated graphically in Figure 2. With perfusions of 10 μM decamethonium there appears to be some potentiation of the twitch height which gradually wanes. There is later a small degree of block and it would seem possible that under the conditions of perfusion even small concentrations of decamethonium might eventually cause complete block. At no concentration was it possible to obtain a steady paralysis, and the block proceeded steadily to 100% with time. On washing out the drug, recovery took place quite rapidly in all cases (usually about 3-5 min), often with potentiation of the twitch height. In experiments with the immersed diaphragm it was found that partial recovery of the block occurred despite the presence of the drug, providing the dissection had been carried out sufficiently quickly. The recovery phenomenon is illustrated in Figure 3 with a concentration of 150 μM decamethonium. After washing out, full recovery occurred after 5 or more min with the immersed diaphragm, and as with the perfused diaphragm, there was some potentiation of the original twitch height. Again, tachyphylaxis was not evident.

Uptake of decamethonium at the end-plate

The histogram in Figure 4a represents the uptake of decamethonium in a rat diaphragm immersed in saline. The muscle was exposed for 20 min to the

Table 1 Water, sodium and potassium content and mannitol space of the immersed and the perfused rat diaphragm

Preparation	*Water content (mg/g)*	*Sodium content (m.equiv/ Kg wet wt)*	*Sodium content (m.equiv/ 100g dry wt)*	*Potassium content (m.equiv/ Kg wet wt)*	*Potassium content (m.equiv/ 100g dry wt)*	*Mannitol space (ml g^{-1})*
In vivo	769 (21) ± 14.3	33.9 (13) ± 5.8	14.4 (13) ± 2.3	95.2 (20) ± 5.9	41.2 (20) ± 3.4	–
Immersed (1 h)	789 (13) ± 14.7	44.6 (13) ± 9.9	21.2 (13) ± 4.6	84.0 (13) ± 8.1	39.8 (13) ± 2.5	0.298 (6) ± 0.063
Perfused (1 h)	837 (10) ± 11.1	70.6 (10) ± 5.8	43.5 (10) ± 3.8	67.9 (10) ± 4.3	41.8 (10) ± 1.8	0.578 (10) ± 0.141

Mean values are given ± s.d.
The number of estimates are shown in parentheses.
Each muscle was maintained in saline by perfusion or immersion for 1 hour.

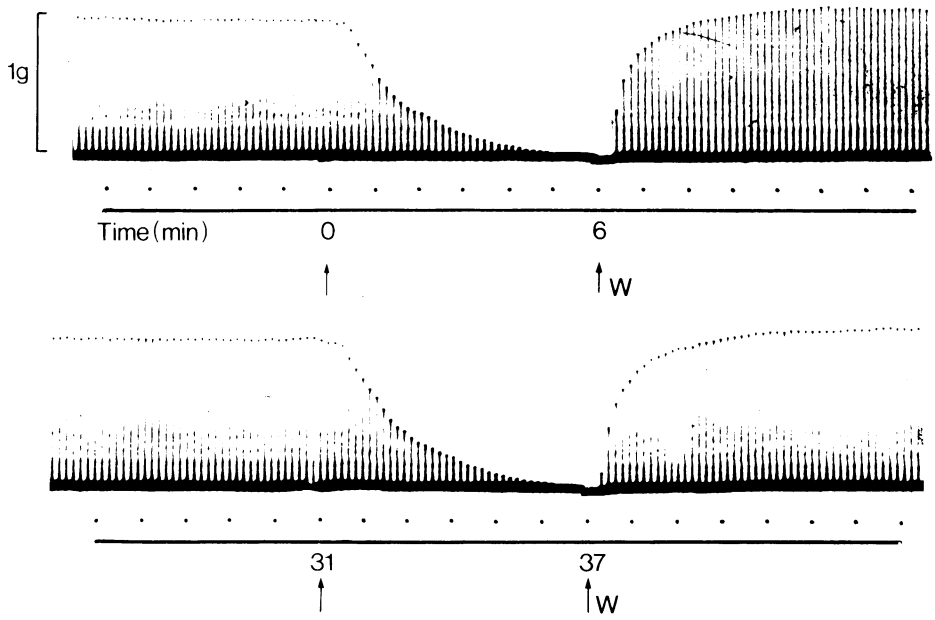

Figure 1 Rat 96 g. Record of isometric maximal contractions of the perfused rat diaphragm *in vitro* elicited by stimulation of the left phrenic nerve at 6/minute. At the arrow (time = 0) saline containing 150 μM decamethonium reached the tip of the perfusion cannula by perfusion from a second perfusion reservoir (see text). At W the muscle was again perfused with physiological saline. Thirty-one min after the first application the muscle was again perfused with 150 μM decamethonium (lower section). No tachyphylaxis was evident. A constant flow of 3.3 ml/min was used. Resting tension was equivalent to 5.3 g. The twitch tension produced is low since contraction of the left hemidiaphragm is recorded from the central tendon of the whole muscle.

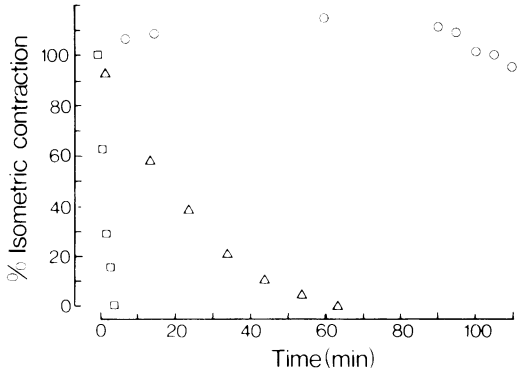

Figure 2 Graph to illustrate the effect of perfusion of the rat diaphragm with various concentrations of decamethonium. Ordinates represent the isometric maximal twitch tension expressed as a percentage of the control value. Abscissae represent the time of application of the drug. (□) 150 μM decamethonium, (△) 45 μM decamethonium, (○) 10 μM decamethonium. After changing back to perfusion with saline containing no decamethonium the muscle quickly recovered in all cases (see text).

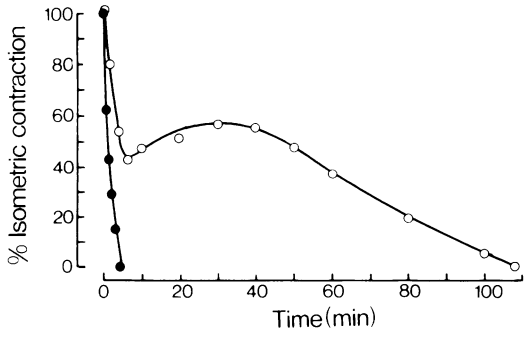

Figure 3 Comparison of effect of 150 μM decamethonium in the immersed and perfused rat diaphragm. Ordinates represent the percentage of the original maximal twitch. Abscissae give the time of application of the drug. (●) perfused diaphragm 150 μM decamethonium, (○) immersed diaphragm 150 μM decamethonium. Note the recovery phenomenon evident in the immersed muscle, but not in the perfused preparation.

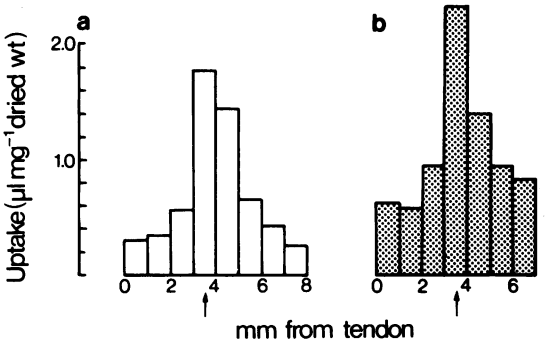

Figure 4 Uptake of labelled decamethonium in diaphragm of rat (20 min) in μl mg^{-1} dried weight. Concentration of 100 μM, with washout in inactive saline for 10 minutes. The arrows indicate the slice of muscle which contained the line of end-plates (see text). (a) Histogram with clear area shows uptake in an immersed diaphragm. (b) Histogram with stippled area shows uptake in a perfused diaphragm.

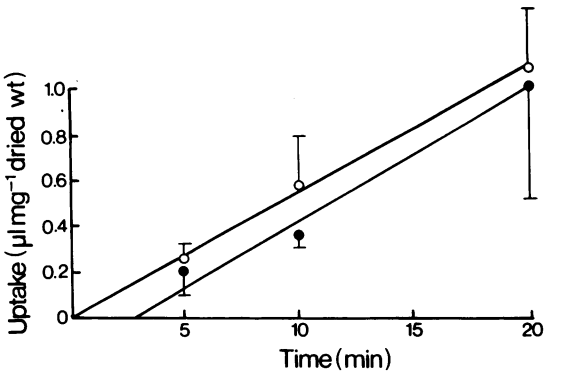

Figure 5 Specific uptake of labelled decamethonium (μl mg^{-1} dried wt.) at the end-plate region in rat diaphragms after immersion with 100 μM decamethonium (○) and after perfusion with 100 μM decamethonium (●) at various times. Each point represents the mean of at least four muscles and the limits give ± s.d. The rate of uptake is not significantly different in the two preparations (see text).

presence of labelled decamethonium (100 μM) and then washed with saline for 10 minutes. The uptake is expressed as clearance in μl mg^{-1} or (radioactivity per mg dried muscle)/(radioactivity per μl saline). Figure 4 shows the distribution of radioactivity along the muscle from tendon to rib, and the arrow indicates the slice which contained the line of end-plates, as shown by the white line which is seen when the muscle was frozen before being sectioned (England, 1970). The maximum radioactivity was at the end-plate, with a smaller uptake at the end of the fibre. This effect is similar to that described by Taylor, Creese, Nedergaard & Case (1965) and Creese & England (1970).

Table 2 Uptake of ^{3}H-decamethonium in the perfused and immersed rat diaphragm at various times

Preparation	*No.*	*Time (min)*	*Asymptotic uptake (μl mg^{-1} dried wt)*	*Specific uptake at end-plate (μl mg^{-1} dried wt)*
	4	5	0.10 ± 0.01	0.26 ± 0.03
Immersed	4	10	0.22 ± 0.01	0.57 ± 0.11
	4	20	0.32 ± 0.01	1.08 ± 0.13
	5	5	0.20 ± 0.01†	0.20 ± 0.05
Perfused	5	10	0.32 ± 0.03*	0.36 ± 0.03
	5	20	0.71 ± 0.04†	1.01 ± 0.22

Mean values are given ± s.e.
* Significantly different from immersed diaphragm $P < 0.05$
† Significantly different from immersed diaphragm $P < 0.001$
The specific uptake on the end-plate region was obtained by subtracting the non-specific asymptotic uptake at the ends of the fibres from the total peak uptake at the end-plate region. See text.

Figure 4b represents the uptake of decamethonium in a diaphragm perfused for 20 min with saline containing labelled decamethonium (100 μM) and then washed for 10 min by perfusion with saline. The distribution of radioactivity along the muscle is qualitatively similar to that in the immersed muscle except that the uptake is somewhat larger. The non-junctional uptake is significantly larger in the perfused muscle at all times (Table 2). The uptake at the ends of the fibres (when expressed as a clearance) is independent of concentration and is probably attributable to diffusion (Creese & England, 1970). The significance of this increase in the non-specific uptake in the non-junctional region for the perfused diaphragm remains to be determined. In contrast, the peak uptake at the end-plate region (when expressed as a clearance) is concentration-sensitive and saturable (Creese & England, 1970; Humphrey, 1970) and is not significantly different in either the perfused or immersed diaphragm (Table 2).

Figure 5 illustrates this specific component of decamethonium uptake at the end-plate, which is obtained by subtracting the non-junctional value from the peak. The results from 15 perfused and 12 immersed muscles were expressed as μl mg^{-1} (dry weight) and plotted against time. The slopes of the simple regressions (Figure 5) are 0.0541 and 0.0586 μl mg^{-1} (dry weight) for the immersed and perfused muscles respectively. The difference

between the slopes is small and it was concluded that the deviation from parallelism were not significant ($0.6 > P > 0.5$). The combined slope is 0.0566 (95% confidence limits 0.0429 and 0.0703). In Figure 5 the variance increases with time and hence weighted regressions were also calculated with a weighting factor equal to $^1/Y^2$ where Y is the expected value. The slopes of these regressions after three iterations were 0.057 and 0.048 μl mg^{-1} (dry weight) for the immersed and perfused muscle respectively, and again, the values of the slopes were not significantly different ($0.3 > P > 0.2$).

The simple regression for the immersed muscle has a small positive origin at 0.004 ml min^{-1}, while the simple regression for the perfused muscle has a negative origin at 0.16 ml min^{-1}. This indicates an apparent lag in the peak uptake of the perfused diaphragm which could be, in part, attributable to the increase in the extracellular space. However, the origins of the two regressions are not significantly different at the 5% probability level. In control experiments the perfusate was collected over 15 s intervals for 10 min during perfusion. It was found that over 90% equilibration occurred within 2 min with a half time of 32 s and no lag phase was evident.

Discussion

The rat diaphragm can be maintained by immersion so that the loss of intracellular potassium is small with only a moderate rise in the sodium content (Creese & Northover, 1961). In this study, the potassium content of the perfused muscle (K^+/g wet tissue) is low, but this is to be expected considering the high water content of the tissue. Calculated on a dry weight basis, the potassium contents of the perfused and immersed muscles do not differ markedly from their contents *in vivo*. There is, however, a very definite increase in the sodium content of the perfused muscle, but this is no more than expected as a result of the increased mannitol space.

Maclagan (1962) found that with cat tenuissimus muscle *in vivo*, infusion of depolarizing drugs produced a steady level of neuromuscular block, but no measurements of drug plasma levels were taken. In contrast, the same muscle *in vitro* exhibited a biphasic neuromuscular block similar to that described by Jenden (1955), but at no time was the level of block steady despite the application of a constant concentration of drug. Maclagan (1962) suggested that the difference between responses *in vivo* and *in vitro* might be due to deterioration. Later Gibberd (1966) suggested that a steady level of block with decamethonium was characteristic of administration via the capillaries both *in vivo* and *in vitro*. However, in this study it was not found possible to produce a steady block in the perfused diaphragm *in vitro*. This is in agreement with the finding of Creese & Maclagan (1970) who did not find a steady level of block in rat muscle *in vivo* despite constant plasma levels of decamethonium. It would therefore appear that a true steady level of block cannot be achieved in the rat by a constant concentration of decamethonium in contrast to the neuromuscular block produced by competitive blocking drugs such as tubocurarine (Holmes, Jenden & Taylor, 1951).

A neuromuscular block in which the muscle wholly or partially recovers despite the continued application of the drug has been demonstrated both in rat muscle *in vivo* (Creese & Maclagan, 1970) and in the immersed muscle *in vitro* (Gibberd, 1966). It is therefore surprising that in this study and previously (Gibberd, 1966), recovery from the block has not been demonstrated in the perfused rat diaphragm *in vitro*. The difference is probably not due to deterioration since the potassium content of the perfused and immersed preparations do not differ significantly. It has been suggested that the recovery phenomenon is due to repolarization of the muscle membrane (Gissen & Nastuk, 1966). Repolarization of the end-plate region in rat diaphragm despite a constant concentration of decamethonium has been shown by Thesleff (1955). It is of interest to speculate that repolarization is dependent upon the active transport of sodium which is stimulated by high concentrations of potassium (Horowicz & Gerber, 1965; Garrahan & Glynn, 1967). Following the depolarizing action of decamethonium the efflux of potassium is increased and the external potassium concentration is raised (Paton, 1956). It may be that, due to the kinetics of potassium washout (see below), an abnormally high external potassium concentration exists for some time in the immediate vicinity of the sodium 'pump' in the immersed preparation but not in the perfused. Hence local variations in external potassium concentrations might explain the difference in the type of responses to decamethonium. However, there is some doubt as to whether decamethonium produces a block by prolonged post synaptic depolarization in the rat (Derkx, Bonta & Lagendijk, 1971; Humphrey, 1973). It may be that in the rat depolarizing agents produce neuromuscular block by a pre-synaptic action on motor nerve terminals (Galindo, 1971).

It is well known that isolated mammalian muscle does not under normal conditions, give a

twitch response to acetylcholine when added to the fluid bathing the external surface of the muscle. However, retrograde injection of acetylcholine in the isolated, normally innervated, rat diaphragm will provide a twitch (Paterson, 1965) similar to that produced *in vivo* by "close-arterial" injection (Brown, Dale & Feldberg, 1936; Brown, 1937). The difference in response to acetylcholine when applied via the capillaries may be associated with the rate of change of concentration at the motor end-plate (Paterson, 1965). This implies that the concentration rises more rapidly in the first few seconds with the perfused preparation where the diffusion path is shorter. However, the present study has been concerned with the entry of drug at the end-plate region and for this process the time uptake curves for both preparations are similar and the rates of uptake do not differ significantly.

This is in accord with the conclusion that the permeability of the end-plate region to decamethonium is similar to that of sodium (Creese & England, 1970). It is known that muscle cells are considerably more permeable to potassium than sodium and in rat muscle the estimated sodium/potasium permeability ratio is 0.006 (Creese, El-Shafie & Vrbová, 1968). The high permeability to potassium ions results in a marked discrepancy between the overall rate of exchange of ^{42}K in a whole muscle and the rate at which the isotope would exchange if all the fibres had free access to the medium (Keynes, 1954; Creese, Hashish & Scholes, 1958). The fact that exchange rates for potassium ions may be diminished by intracellular diffusion was demonstrated by Creese (1960). In rat diaphragms, Creese (1960) was able to show marked differences in the ^{42}K content of the superficial and deep layers after loading the muscle for various times with ^{42}K. Similarly, Klaus, Lullman & Muscholl (1960) demonstrated that the rate constant of the ^{42}K loss was increased up to 4-fold by retrograde perfusion of the rat diaphragm. Hence, the finding that the rate of uptake of decamethonium is *not* increased in the perfused preparation indicates that measurements made on immersed muscles do not need corrections for lags produced by intracellular diffusion. The present results therefore suggest that decamethonium behaves not like potassium, which has a high permeability, but like sodium in which the permeability is low and extracellular diffusion relatively fast, so that the uptake is usually not affected by diffusion lags. This is further supported by the autoradiographic finding that decamethonium uptake is uniform throughout the whole muscle after immersion in labelled decamethonium for 60 minutes (Humphrey, 1972).

The significance of the entry of decamethonium which has been shown *in vivo* (Creese & Maclagen, 1970) and *in vitro* (Humphrey, 1972) is still not clear. Cookson & Paton (1968) have suggested that entry occurs as a result of a non-specific change in permeability at the end-plate region caused by the agonist. Against this concept is the work carried out by Mackay & Taylor (1970) with labelled C_2 and C_6 (homologues of decamethonium with two and six methylene groups respectively). Both these compounds have been shown to have no significant uptake in mouse muscle even in the presence of pharmacological concentrations of decamethonium. It would therefore appear that decamethonium does not increase the permeability of the end-plate region to all ions. A second fact which suggests that entry is not the result of a non-specific permeability change is the finding that the ethyl analogue of decamethonium, decaethonium, is taken up almost as rapidly as decamethonium and yet has almost no effect on membrane potential (MacKay & Taylor, 1970). The kinetics of decamethonium entry show carrier-like properties (Creese & England, 1970), but the relationship between entry and pharmacological response remains to be determined.

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