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LABELLED DECAMETHONIUM IN CAT MUSCLE

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1 Tritium-labelled decamethonium was infused intravenously in 12 cats at final rates of 1.3–4.2 nmol kg^{-1} min^{-1} to produce a steady plasma concentration which ranged between 0.21–1.3 μmol/l in different experiments. Muscle contractions were elicited by nerve stimulation and the potential at the end-plate regions of superficial fibres was recorded by extracellular electrodes.

2 Under these conditions, it was not possible to obtain a steady degree of neuromuscular block. The initial decrease in muscle contractions was followed by recovery towards the original value although the concentration of decamethonium in the plasma remained constant, or in some cases rose. The initial depolarization of the end-plate region also waned during the constant infusion of the drug.

3 Once the twitch tension had returned to control values during infusion of the drug, prolongation of the infusion for a total of four hours did not produce a secondary neuromuscular block.

4 Scintillation counting showed that during infusion of labelled decamethonium the radioactivity of the muscles increased progressively with time. The uptake was less in the soleus muscle than in the fast-contracting flexor longus digitorum and extensor longus digitorum muscles. Muscles which had been denervated 12–13 days previously showed a greater uptake of labelled drug than control muscles from the contralateral limb.

5 The labelled drug was localized by autoradiography of frozen sections of leg muscles following intra-arterial injection of decamethonium. Grain counts in individual fibres showed that small amounts of decamethonium had entered the muscle fibres along their entire length, and there was increased uptake of the drug into the cell in the region of the end-plate.

6 The mechanisms underlying the waning of the pharmacological response during constant application of depolarizing drugs are discussed.

Introduction

It has been shown by contact autoradiography that tritiated decamethonium accumulates at the end-plate region of mouse (Waser, 1966) and rat muscle (Creese & Maclagan, 1967). In the latter case, by using the more precise localization available with frozen-section autoradiography, it was found that the drug had entered the cells at the end-plate region and in adjacent parts of the fibre (Creese & Maclagan, 1970). Entry of the drug appeared to be related to the increase in membrane permeability which the drug produced and did not appear to be due to the change in membrane potential, as entry could still be demonstrated in muscles which were completely depolarized in solutions containing a high concentration of potassium (Creese & England, 1970).

The depolarizing action of decamethonium is not identical in all species. For example, in the cat, decamethonium depolarizes the end-plate region by at least 55 mV and the effect is maintained throughout the period of application of the drug (Head, 1975). Thus the potential falls below the threshold for action potential generation (Boyd & Martin, 1956) and this is probably the cause of neuromuscular blockade (Burns & Paton, 1951). In the rat, however, when decamethonium is applied, the membrane potential falls only transiently (Thesleff, 1955); consequently, prolonged depolarization is unlikely to be the cause of neuromuscular blockade in the rat.

The reasons for these species differences in the depolarizing ability of decamethonium are unknown but may reflect differences in the underlying permeability changes caused by the drug. For this reason it was of interest to know whether decamethonium enters cat muscle fibres as it does in rat muscle. In the present experiments, the uptake of tritiated decamethonium into cat hind-limb muscle fibres was measured using scintillation counting and autoradiographic methods, and the pharmacological

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responses of the muscle were also recorded during intravenous infusion of the drug to give a constant plasma concentration which could be maintained for several hours.

At the same time these experiments provided an opportunity to determine whether the blocking action of the drug progressively waned during prolonged application *in vivo*. This effect is well-known in isolated muscles but has not been studied *in vivo* under conditions where the plasma concentration of the drug could be monitored to ensure a constant drug concentration.

Methods

Cats weighing between 2 and 4 kg were anaesthetized with an intravenous injection of chloralose (80 mg/kg) and pentobarbitone sodium (4 mg/kg). A tracheal cannula was introduced so that artificial respiration could be applied when necessary. The hind limb was immobilized with steel drills through the upper and lower ends of the tibia and fibula and the tendons of attachment of either the soleus, tibialis anterior, flexor longus digitorum or extensor longus digitorum were separated from the bones and securely tied to a strain gauge (Statham 80 oz). Shielded platinum electrodes were placed on the sciatic nerve in the popliteal space and the nerve was tied central to the electrodes. Muscle twitches were elicited with supramaximal square wave stimuli of 0.1 ms duration, delivered once every 5 s, and were registered on a Sanborn recorder.

Recording of surface potential from tibialis anterior muscle

The method used was that described by Burns & Paton (1951). A non-polarizable wick electrode was placed at the end-plate region of a group of surface fibres, and the location was adjusted to the point of maximal depolarization produced by a test dose of suxamethonium (30 μg/kg i.v.). A second reference electrode was placed on the patella tendon or an exposed bone. The potential between these electrodes was recorded using a stable d.c. amplifier with an input resistance of 10 MΩ and was displayed on the pen recorder. The muscle was covered with liquid paraffin at 37°C contained in a pool formed from skin flaps.

Administration of drugs

Tritium-labelled decamethonium was diluted with 0.9% w/v NaCl solution (saline) and unlabelled decamethonium was added to bring the concentration to 30 μmol/l. The solution was maintained at 37°C and infused via a polythene cannula into a jugular vein by means of a Watson-Marlow peristaltic pump. The rates of infusion are given in the **Results**. On other occasions decamethonium was administered by rapid intra-arterial injection, in a small volume of saline (less than 0.2 ml) in a retrograde direction into the anterior tibial artery, while the femoral arterial flow was temporarily occluded in the thigh.

Blood samples

Blood samples were obtained from a cannula in the carotid artery and collected directly into small graduated centrifuge tubes containing heparin. The cells were rapidly spun down and two aliquots of 0.1 ml of plasma were removed from each sample, added to vials containing 1.0 ml hyamine hydroxide (1 mol/l in methanol) plus 0.5 ml KOH (1 mol/l in methanol) and counted by liquid scintillation.

Denervation

In five cats under pentobarbitone anaesthesia, the muscles of the left hind leg were denervated by removing 2 cm of the sciatic nerve in the popliteal space and reflecting the central stump to prevent regrowth of the nerve. The acute experiments were performed 12–14 days later.

Radioactive compounds and measurements

[Methyl-^{3}H]-decamethonium dichloride with specific activity of 1.1 Ci/mmol was obtained from the Radiochemical Centre (Amersham). The compound was analyzed at Amersham by thin-layer chromatography on aluminium in chloroform-methanol (80:20, v/v) and the radiochemical purity was greater than 98%.

Hind limb muscles were rapidly dissected free from connective tissue and frozen in isopentane cooled in liquid nitrogen. The muscles were weighed in crucibles, dried under vacuum with phosphorous pentoxide and reweighed. The dried muscle was powdered and samples of 4–8 mg were added to vials which contained 1 ml hyamine hydroxide (1 mol/l in methanol). The tissues were dissolved by heating for 30 min in a water bath at 70°C and after the vials had cooled 0.5 ml KOH (1 mol/l in methanol) and 14 ml of scintillator (Creese & Taylor, 1967) were added. Background counts were measured in vials containing 1 ml hyamine solution and 0.5 ml KOH solution plus scintillator.

Autoradiography

The methods used for freezing and sectioning muscles, for the preparation of the autoradiograms and for grain counting were identical to those described by Creese & Maclagan (1970).

Drugs

The following drugs and chemicals were used: pentobarbitone sodium (Abbott Laboratories Ltd.); chloralose (British Drug Houses Ltd.); decamethonium dibromide (Syncurine, Burroughs Wellcome and Co., mol. wt. 418); suxamethonium chloride injection (Anectine, Burroughs Wellcome and Co.); hyamine hydroxide (1 mol/l in methanol; Nuclear Enterprises, Ltd.); heparin injection 1000 iu/ml (Paines & Byrne Ltd.).

Results

Effect of decamethonium infusion on muscle contraction and end-plate depolarization

Decamethonium (30 μmol/l) was infused into anaesthetized cats at an initial rate of between 2.1–5.2 ml/min, a range found to produce a rapid onset of neuromuscular block. As soon as the contraction height began to diminish the infusion was reduced and a final rate of 0.17–0.43 ml/min was maintained (1.3–4.2 nmol kg^{-1} min^{-1}). This produced a transient period of steady block (approximately 90% in tibialis muscle) during which the first blood sample was taken. Figures 1, 2 and 3 show results from different experiments. The plasma concentration was subsequently steady (Figure 3), slowly decreased (Figure 1) or slowly increased (Figure 2). In all cases the muscle contractions recovered despite the continued presence of the drug.

Figure 1 shows simultaneous records of contraction and surface recording of depolarization from the tibialis anterior muscle during a 90 min infusion of decamethonium. With this extracellular recording method, *changes* in potential can be recorded but absolute values of membrane potential cannot be obtained. In this experiment the maximum depolarization of the end-plate was reached 15 min after the start of the infusion, when the plasma concentration of the drug was 0.48 μmol/l. This was associated with 99% block of the tibialis twitches. As the infusion continued, the depolarization of the end-plate waned, until after 50 min infusion the surface recording returned to the value before the infusion commenced. The muscle contractions also gradually returned to normal but their rate of recovery lagged behind the return of the potential. This may be due to the fact that these two recordings were obtained from different groups of fibres—the electrical records being restricted to a few superficial end-plates. After 70 min infusion, both mechanical and electrical recordings had returned to control values despite continued infusion of the drug. During the following 30 min infusion, the muscle contraction became larger than the initial values. Such a potentiation of the twitch has been reported previously during the period immediately following a block caused by a single intravenous injection of decamethonium (Vrbová & Maclagan, 1966; Maclagan, 1976).

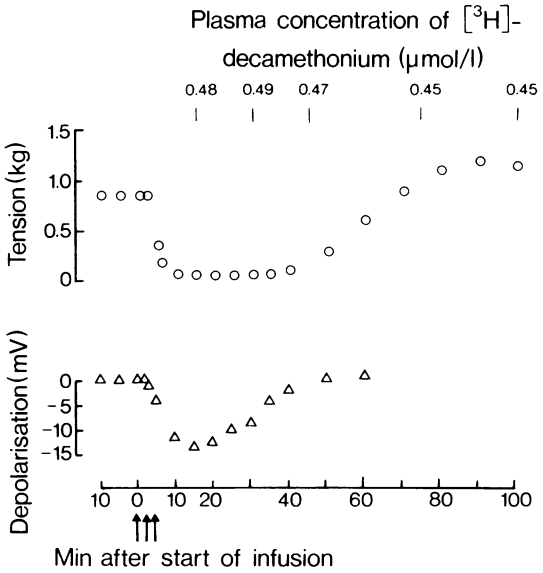

Figure 1 Infusion of [^{3}H]-decamethonium in a cat. The graphs show simultaneous measurements of contractions of the tibialis anterior muscle elicited by supra-maximal stimulation of the motor nerve (open circles) and potential at the end-plate region of surface fibres with reference to an indifferent electrode placed on the tendon (open triangles). At the first arrow infusion of [^{3}H]-decamethonium was started at a rate of 20.4 nmol kg^{-1} min^{-1}. The rate was adjusted at the second and third arrows and was then maintained at 4.2 nmol kg^{-1} min^{-1} from the third arrow until the end of the experiment. The values in the upper part of the figure indicate the plasma concentration of decamethonium in μmol/l. Time (min) is measured from the start of the infusion. Decamethonium caused initial depolarization and neuromuscular block followed by complete recovery to control values.

Similar results have also been obtained in the soleus muscle of the cat (Figure 2). This figure shows that a steady blockade was present 30 min after the start of the infusion. Two hours later the muscle twitch had returned to control values despite the continuous presence of the drug. In the case of the soleus muscle, surface recording was not possible as the scattered arrangement of the end-plates made it difficult to locate a discrete band of end-plates. The slowly contracting soleus muscle was always less sensitive to the drug than the fast contracting muscle (Jewell & Zaimis, 1951), but the time course of development of the blockade was similar in both types of muscle. This

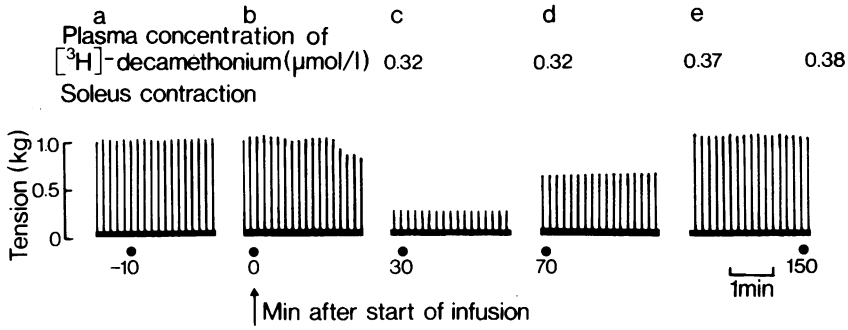

Figure 2 Indirectly elicited maximal contractions of the soleus muscle (a) before and (b) to (e) during infusion of [^{3}H]-decamethonium. The initial infusion rate (18.2 nmol kg^{-1} min^{-1}) was changed to 2.6 nmol kg^{-1} min^{-1} at 15 min and then maintained. The time in min after the start of the infusion is indicated below each section and the plasma concentration in μmol/l is given by the number above each section. Decamethonium produced a partial neuromuscular block. After 150 min infusion the block had completely recovered despite increasing plasma concentration of decamethonium.

is illustrated in Figure 3 which shows simultaneous recordings from soleus and flexor longus digitorum. In any one cat the onset of the recovery of the

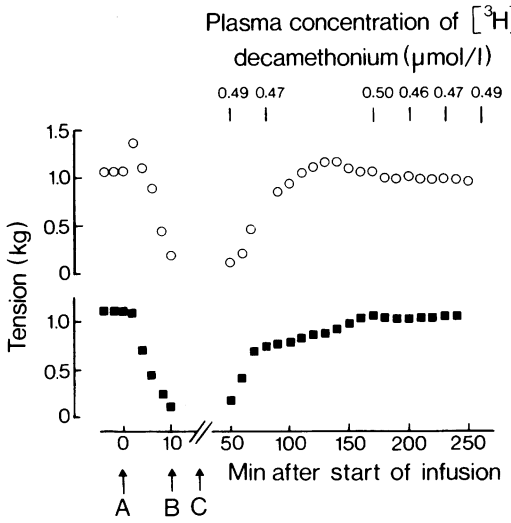

Figure 3 Simultaneous recording of contraction of soleus (○) and flexor longus digitorum (■) during prolonged infusion of labelled decamethonium. The infusion was started at a rate of 26.7 nmol kg^{-1} min^{-1} at A, adjusted at B and then kept constant at 2.7 nmol kg^{-1} min^{-1} from C until the end of the experiment. The time scale is different after the first 10 minutes. The plasma concentration showed only small variations and the values in μmol/l are given at the top of the figure. After an initial neuromuscular block the contractions of both muscles had returned to their control values by 150 minutes. The infusion was continued for a further 100 min but there was no sign of a secondary neuromuscular block.

contraction during the infusion was very similar in both fast and slow muscle, as shown in Figure 3.

In four of the cats, the infusion was continued after recovery of the twitch for a total period of 4 hours. There was no sign of development of a secondary blockade with continued application of the drug, and Figure 3 gives an example of one of these experiments.

Uptake of [^{3}H]-decamethonium by hind limb muscles

At various times during the infusion of decamethonium, individual muscles from the lower hind limb were rapidly dissected after ligation of the arterial branch supplying the muscle, and frozen in isopentane cooled in liquid nitrogen. The muscles were dried and powdered samples were weighed, dissolved and the radioactivity of each sample measured by liquid scintillation counting. Table 1

Table 1 Uptake of labelled decamethonium in fast-contracting hind limb muscles of cats infused intravenously at a constant rate

Time in min	*Uptake (ml/g wet muscle)*
7– 60	0.15 ± 0.021 (7)
140–160	0.28 ± 0.045 (7)
220–260	0.68 ± 0.079 (5)

The first column gives the duration of perfusion in minutes. The uptake in leg muscles (extensor digitorum longus and flexor digitorum longus) was measured by scintillation counting and is expressed as a clearance, in ml/g. This is the ratio of the (counts per g wet muscle) to (counts per ml plasma). The limits give the s.d., with the number of muscles in parentheses. There is a progressive rise in the radioactivity found in the muscle.

Figure 4 Record of indirectly elicited twitches of the extensor longus digitorum muscles of the cat in response to an intra-arterial injection of [^{3}H]-decamethonium chloride (80 μg kg^{-1}, 0.6 mCi). Ninety minutes after the injection the muscle was rapidly frozen and prepared for autoradiography.

shows the uptake of [^{3}H]-decamethonium, expressed as a clearance in ml/g, in the fast-contracting muscles at various times after the start of the infusion. The uptake during the first hour is largely attributed to the extracellular space; thereafter, the uptake increased progressively with time to values which suggest that accumulation of the drug was occurring in the muscle fibres.

When the uptake in fast and slow muscles was compared it was found that at all stages of the infusion the uptake into the more sensitive fast-contracting muscles (extensor longus digitorum and flexor longus digitorum) was always greater than that in the slowly-contracting soleus muscle (Table 2).

Denervation of the muscles of one hind limb greatly increased the uptake compared to the unoperated muscles of the contralateral limb (see Table 3). After 140 min infusion, uptakes of over 1.0 ml/g were found in denervated muscles in some cases.

Autoradiography of frozen sections

The results obtained with scintillation counting of dried muscles was consistent with an uptake of [^{3}H]-decamethonium into a compartment larger than the extracellular space. However such experiments cannot give any further indication of the location of the drug within the muscle. For this reason, autoradiography of frozen sections of muscles loaded with [^{3}H]-decamethonium was undertaken. The method has been used successfully in rat muscle (Creese & Maclagan, 1970) but in this species much greater doses of decamethonium were needed to produce neuromuscular blockade than in the more sensitive cat muscle. This is shown by the plasma concentration in the rat which ranged from 11 to 43 μmol/l, whereas in the cat a comparable degree of neuromuscular blockade is produced with a plasma concentration below 1 μmol/l. Thus in the present experiments in the cat, much lower levels of radioactivity were expected than in the previous work on rats. For this reason [^{3}H]-decamethonium dichloride was injected intra-arterially in a dose (80 μg/kg) which caused complete blockade of extensor longus digitorum for 15 min (see Figure 4). After an interval of 90 min which allowed the extracellular concentration of the drug to diminish, the

Table 2 Uptake of labelled decamethonium in different leg muscles of cats after intravenous infusion at a constant rate.

	Uptake (ml/g wet muscle)		
Time in min	*Soleus*	*Extensor digitorum longus*	*Flexor digitorum longus*
135	0.20	–	0.26
135	0.28	–	0.53
144	0.11	0.21	–
144	0.11	0.20	–
164	0.19	0.31	–
224	0.30	0.34	–
250	0.28	0.64	0.68
260	0.33	–	0.80
260	0.30	0.73	–

The first column gives the duration of infusion. The expression ml/g is calculated as in Table 1. In each experiment the uptake into fast-contracting extensor longus digitorum and flexor longus digitorum muscles is greater than that in the soleus muscle.

Table 3 Effect of chronic denervation on uptake of labelled decamethonium in leg muscles of cats infused intravenously at a constant rate for 140 minutes

Uptake (ml/g wet muscle)		
Control	*Denervated*	*Muscle*
0.20	1.22	Soleus
0.11	0.26	Soleus
0.11	0.28	Soleus
0.26	1.30	Flexor longus digitorum
0.21	0.28	Flexor longus digitorum
0.19	0.49	Flexor longus digitorum
0.20	0.32	Extensor longus digitorum
0.20	0.38	Extensor longus digitorum

The muscles were denervated 12–13 days previously. The uptake is expressed as ml/g, as in Table 1. In each case the uptake in the denervated muscle is greater than that in the control muscle taken from the other leg.

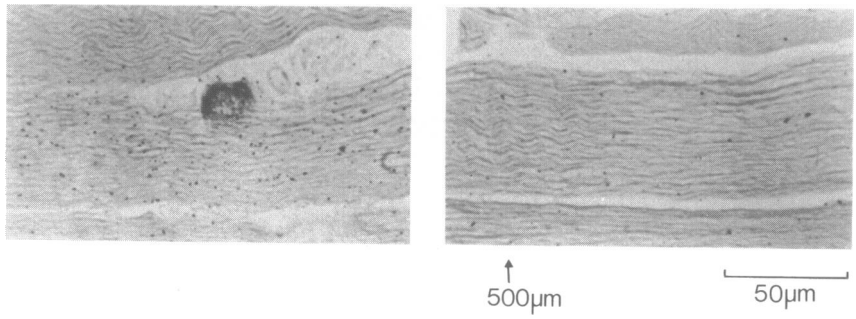

Figure 5 Autoradiogram prepared from muscle shown in Figure 4. The grain density over the fibres is high at the end-plate (left-hand section) but considerably lower at a distance of 500 μm from the end-plate region (right-hand section).

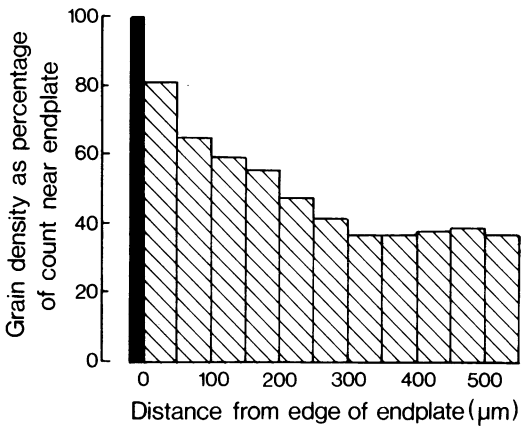

Figure 6 Distribution of grain-density along 8 fibres from the extensor longus digitorum muscle of Figures 4 and 5. The grain density at different points along the fibre has been expressed as a percentage of the value at the end-plate region. Each column represents the mean value of eight measurements after the background count had been subtracted (s.e. mean between 6.0 and 11.5).

muscle was removed from the cat and rapidly frozen. Autoradiograms were prepared from frozen sections which were cut longitudinally and stained with an azo-dye to show the position of the esterase at the endplate region. Figure 5 shows the autoradiogram prepared from the muscle referred to in Figure 4. There is a high grain density over the fibres at the endplate region (left-hand section), but the radioactivity is much reduced at a distance of 500 μm from the end-plate (right-hand section). The resolution of the method is of the order of 1 μm (Creese & Maclagan, 1970) so these results indicate that the drug is concentrated in the cells at the end-plate region and in the surrounding parts of the fibres. The grain density between the fibres was low, indicating that the extracellular concentration of the drug had indeed been reduced to low levels 90 min after the injection.

The longitudinal distribution of radioactivity was determined by measuring the grain density along the fibres in individual fibres obtained from several sections of the same muscle. Figure 6 gives the distribution in 8 fibres from the muscle shown in Figures 4 and 5. The grain counts show that a small amount of [^{3}H]-decamethonium had entered the muscle fibres along their entire length but there was an increased uptake of the drug into the cells in the region of the end-plate.

Discussion

In the present experiments it has been shown that prolonged infusion of [^{3}H]-decamethonium in the cat produces an initial paralysis of the indirectly elicited twitches of both fast and slow muscles, followed by recovery of the twitch response despite a plasma concentration of the drug which was maintained approximately constant. Extracellular recording of depolarization showed that this recovery of the twitch was accompanied by a waning of the end-plate depolarization produced by the drug. A similar effect has been reported in the cat tenuissimus muscle *in vivo* (Maclagan, 1962), but in the absence of measurements of plasma concentration of the drug it was attributed to a reduction in the infusion rate. These results in the cat are comparable to those obtained *in vivo* in the rat (Creese & Maclagan, 1970) and in man (Poulsen & Hougs, 1958). In all three species therefore application of a constant concentration of depolarizing drugs *in vivo* does not cause a maintained neuromuscular block.

The recovery from blockade is dose-dependent and can only be recorded within narrow dose limits. At the upper end of this restricted range it may be necessary to continue the infusion for more than one hour before the recovery occurs. If large enough doses are given, sufficient to cause complete blockade, recovery of the twitch may not occur.

Our results with prolonged application of decamethonium *in vivo* in the cat and in the rat (Creese & Maclagan, 1970) are comparable to those obtained by many authors in isolated muscle from the frog (Gissen & Nastuk, 1966), cat (Maclagan, 1962), rat (Thesleff, 1955; Gibberd, 1966; Harris & Leach, 1970), guinea-pig (Jenden, 1955), rabbit (Jenden, Kamijo & Taylor, 1954) and man (Creese, Dillon, Marshall, Sabawalla, Schneider, Taylor & Zinn, 1957). *In vitro,* as *in vivo,* the recovery of the twitch during prolonged application of the drug is critically dependent on the dose (Jenden *et al.,* 1954; Gissen & Nastuk, 1966).

Potential measurements are not available for most experiments on isolated muscles but it is known that in frog (Gissen & Nastuk, 1966) and in rat muscle (Thesleff, 1955) the membrane potential returned towards control levels while the drug was still in contact with the muscle. It appears, therefore, that recovery of both depolarization and muscle contractions during prolonged application of low doses of depolarizing drugs is a widespread phenomenon both *in vivo* and in isolated muscle preparations. There was, however, no sign of the secondary blockade which has been found *in vitro* and termed Phase II block by Taylor and his colleagues (Jenden *et al.,* 1954; Jenden, 1955).

When the radioactivity of the cat muscles was measured by scintillation methods at various times during the infusion, the results showed (1) that the drug accumulated in the muscle, (2) that the uptake increased progressively with time, (3) that muscles which were less sensitive to the action of decamethonium (soleus muscle) showed a smaller uptake of the drug than the more sensitive fast-contracting muscles, and (4) as might be expected, a procedure such as denervation of the muscle, which increases its sensitivity to all depolarizing drugs, greatly increased the uptake of the drug into the muscle. Similar results in rat and guinea-pig muscles have been obtained by Creese, Taylor & Case (1971).

The results of the experiments with labelled decamethonium also showed that the drug accumulated in the muscle to a greater extent than could be accounted for by its presence in the extracellular space. With this method of expressing uptake as a clearance in ml/g, the uptake into the extracellular space would be of the order of 0.1–0.2 ml/g, but in our experiments uptakes much greater than this were found in normal muscle and after denervation the values increased further. By use of frozen section autoradiography, which prevents movement of the water soluble drug and which permits more precise localization, the drug was found within the fibres. The resolution of the method was 1 μm (Creese & Maclagan, 1970) and there is the possibility that some of the drug is attached to the tubule system within the fibre. Longitudinal sections showed grains all along the fibres, with a concentration in the region of the end-plate and for several hundred microns on either side. There is some indication that decamethonium is entering the cells via the receptor-activated sodium channels; Creese & England (1970) and Humphrey (1975) both found that the permeability of rat muscle to decamethonium was similar to that of sodium and much lower than that of the potassium ion. In addition, tubocurarine prevents both the entry of the drug into the cells and the pharmacological action on the receptors.

Our finding that the uptake of decamethonium into the muscle cells increases progressively with time was of particular interest as the pharmacological effects of the drug on contraction and on the membrane potential were only transient. It has been suggested by others that the transient blockade and potential changes in isolated muscle preparations are due to the development of receptor desensitization (Thesleff, 1955; Gissen & Nastuk, 1966). However, this suggestion is difficult to reconcile with the finding that the drug continues to enter the cells, presumably via the sodium channels, even when the blockade has disappeared.

There are several possible ways to explain the waning pharmacological effect without postulating receptor desensitization. Stimulation of outward sodium pumping from the cell could explain the repolarization of the membrane while the sodium channels remain open. The stimulus to the sodium pump might be either an increase in the intracellular concentration of sodium, caused by decamethonium, or the resulting increase in potassium ion concentration in unstirred layers just outside the cell. Both of these mechanisms are known to be effective activators of the sodium pump in other tissues (Glynn, 1957, 1962; Glynn & Karlish, 1975).

Some evidence to support the presence of sodium pumping during the recovery phase comes from the work of Head (1975). He showed that in isolated cat muscles, ouabain, applied during the recovery phase after decamethonium-induced depolarization, slowed or prevented the normal recovery of the membrane potential. This effect may be species dependent as, in the frog under similar conditions, strophanthidin was ineffective (Terrar, 1974).

This problem cannot be resolved with the available evidence but the results obtained in the present experiments showing progressive uptake of decamethonium into cat muscle fibres do not appear to support the theory that receptor desensitization occurs during prolonged application of blocking doses of depolarizing drugs. At much higher concentrations, however, such as those produced during iontophoretic application, receptor desensitization may be an important factor (Thesleff, 1955; Gissen & Nastuk, 1966).

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