J. Physiol. (1970), **210**, *pp.* 363–386
With 7 plates and 9 text-figures
Printed in Great Britain

ENTRY OF DECAMETHONIUM IN RAT MUSCLE STUDIED BY AUTORADIOGRAPHY

BY R. CREESE AND JENNIFER MACLAGAN
From the Department of Physiology, St Mary's Hospital Medical School, London, W. 2

(*Received 7 May 1970*)

SUMMARY

1. When a steady plasma level of decamethonium was maintained by infusion in rats, the labelled compound became concentrated in the region of the end-plate of skeletal muscle, as shown by scintillation counting.

2. The distribution of tritium-labelled decamethonium in single muscle fibres was studied by autoradiography of frozen sections, with resolution less than 1 μ.

3. After intravenous injection of a dose of decamethonium which produced partial paralysis it was shown that the labelled compound had entered muscle fibres in the region of the end-plate, and for several hundred microns on either side of the end-plate.

4. Entry of decamethonium could be demonstrated as early as 30 sec after intra-arterial injection. There was no evidence of any redistribution of labelled drug for a period of 2 hr after the initial entry.

5. Previous administration of tubocurarine markedly reduced the entry of labelled decamethonium.

INTRODUCTION

The action of decamethonium has many similarities to that of the transmitter acetylcholine, and the depolarizing action of these compounds at the motor end-plate has been shown by iontophoretic application (Thesleff, 1955; del Castillo & Katz, 1957). The accumulation of labelled decamethonium in a region which corresponds to the band of end-plates has been demonstrated by contact autoradiography of dried diaphragm muscle (Waser, 1960). By measurement of the uptake *in vitro* it has been shown that the concentration of the compound in the tissue as a whole could markedly exceed that in the external solution (Taylor, Creese, Nedergaard & Case, 1965), and it does not seem likely that these results can be explicable in terms of surface adsorption of the drug.

In the present study the movements of tritiated decamethonium have

been followed by an autoradiographic method which allowed localization in single fibres. By using frozen sections of muscle it was possible to prevent the movements of the water-soluble decamethonium during processing, and to show that decamethonium enters the fibres of rat muscle at the end-plate region and at adjacent areas. Some of these findings have been briefly presented (Creese & Maclagan, 1967).

METHODS

Preparation of animals. Rats weighing 100 g were used in most experiments. Larger animals (250–500 g) were used in some cases for experiments which involved extensive recording. The animals were anaesthetized with pentobarbitone sodium (48 mg/kg) by intraperitoneal injection, or with a mixture of pentobarbitone sodium (12 mg/kg) and choloralose (80 mg/kg) which was injected into a tail vein. A tracheal cannula was inserted so that artificial respiration could be given, when necessary, from a small pump (Palmer).

The sciatic nerve was exposed in the popliteal space and shielded platinum electrodes were attached to the nerve, which was ligated central to the electrodes. The tendon of the tibialis anterior, peroneus longus or gastrocnemius muscle was attached by means of a rigid steel hook to a strain gauge (Statham 48 oz or 4 oz). The nerve was stimulated with maximal pulses of 0·1 msec duration, delivered at a frequency of 6/min, and isometric contractions were displayed on a Sanborn pen recorder. Blood pressure was recorded from the right carotid artery by a transducer (Statham). In some cases surface potentials were recorded from flexor hallucis longus by means of non-polarizable wick electrodes, the indifferent electrode being placed on the tendon.

Administration of drugs. Intravenous injections in anaesthetized animals were made into an exposed femoral vein by means of a glass tuberculin syringe of 0·25 ml. capacity. The volume injected was 0·1–0·2 ml. Continuous intravenous infusions were made into the right jugular vein through a fine polyethylene cannula by means of a roller pump (Watson–Marlow flow inducer).

Retrograde intra-arterial injections were made into the saphenous artery after ligating all the branches of the saphenous and femoral arteries except the popliteal artery. A rapid retrograde injection of decamethonium could then be made into the saphenous artery, while the femoral artery was occluded centrally during the brief period of injection. The solution was seen to pass rapidly into the popliteal artery, and caused paralysis of the hind limb muscles with a very rapid onset and short duration.

Drugs. The following were used: pentobarbitone sodium (Abbott Laboratories Ltd.); chloralose (British Drug Houses Ltd.); decamethonium dibromide (Syncurine, Burroughs Wellcome and Co.), mol. wt. 418; powdered tubocurarine dichloride pentahydrate (Burroughs Wellcome and Co.), mol. wt. 786; solution of tubocurarine chloride (Tubarine, Burroughs Wellcome and Co.), 10 mg/ml.

Radioactive compounds. [^{3}H-methyl]decamethonium dichloride (mol. wt. 329) was obtained from the Radiochemical Centre, Amersham, with a specific activity of 276–345 mc/m-mole. The radiochemical purity determined by paper chromatography with *n*-butanol:pyridine:water (1:1:1) was 98%. For intra-arterial injection the specific activity was reduced by addition of unlabelled decamethonium dibromide. Aqueous samples up to 0·1 ml. were added to 0·5 ml. N-KOH in methanol in polyethylene vials and counted by liquid scintillation (Creese & Taylor, 1967).

Sampling of tissues. Diaphragm muscles were removed from decapitated rats and frozen on a brass plate cooled on a block of solid CO_2. Condensation was prevented by means of a plastic transparent cover, and the end-plate region could then be identified as an irregular white line (Creese, England & Taylor, 1969; England, 1970). The muscle was sliced transversely at 1 mm intervals (Taylor *et al.* 1965). Each strip was weighed (1–5 mg) and transferred to a polyethylene vial containing 0·5 ml. N-KOH in methanol and then dissolved at 70° C and counted by liquid scintillation.

Blood samples were obtained either from a cannula in the carotid artery or from the tail vein. The tail was kept in a beaker of warm water (45° C) and samples of 0·2–0·3 ml. were obtained by cutting off the end. To prevent haemolysis the blood was collected in siliconized vessels and transferred to small siliconized centrifuge tubes by use of a fresh disposable syringe for each sample. The cells were rapidly spun down and 0·02 ml. plasma was removed with a Hamilton syringe and added to 0·5 ml. N-KOH in methanol in polyethylene vials and counted by liquid scintillation.

Freezing of tissue for autoradiograms. Muscles from the lower hind limb were rapidly dissected from the animal at known times after injection of labelled decamethonium. Small pieces of muscle, not more than 2–3 mm thick, were cut out and placed on cork disks or aluminium foil, with the fibres orientated in either the transverse or longitudinal direction. The muscles were then rapidly frozen in isopentane cooled in liquid nitrogen (temperature between -140 and -160° C). The frozen tissue was transferred to a cryostat (Bright and Co. Ltd.) in a dark-room.

Preparation of autoradiograms. The method was a modification of that described by Appleton (1964), and all processing of the muscles took place at low temperature to avoid movement of the water-soluble decamethonium. Sections 5 μ thick were cut in the cryostat at -20 to -30° C in full light. Test sections were picked up on glass slides at room temperature and stained for cholinesterase by the rapid azo-dye method (Barka & Anderson, 1963). When satisfactory sections through the end-plate region had been obtained, subsequent sections were cut under a red spot safelight (Kodak Wratten series I). The safelight was mounted 12 ft. from the cryostat knife and its position was adjusted so that its reflexion could be seen in the knife blade. The section then appeared as a dark shadow in the red glow on the knife. The section was picked up on a cold slide coated with a layer of emulsion which was 10 μ thick (Ilford G 5 or K 5). The temperature of the slides was controlled by storing them at -12 to -15° C in light-proof boxes in a separate refrigerator. When the slide is at this temperature, the section (whose temperature is between -20 and -30° C) will stick securely to the emulsion. No ice shadow was left on the knife if the section had been picked up without melting. The cold slide bearing the section was then rapidly transferred to a cooled light-proof box containing desiccant and stored in the cryostat at -30° C. In addition a control section, from a muscle treated with unlabelled decamethonium, was also picked up on the slide which contained the radioactive section.

After 2–5 days exposure the slides were warmed slowly to room temperature (2–3 hr) and histologically fixed, in the dark, in 5% neutralized formaldehyde solution (Pearse, 1953) for 5 min. After several washes in distilled water the emulsion was developed in Kodak D–19B at 16° C for 5 min and fixed in Johnson's Fixsol (diluted 1:6 with distilled water) for 30 min at 16° C.

The end-plate cholinesterase was then stained by the azo-dye method (Barka & Anderson, 1963). The sections were mounted in glycerine-gelatine, or they were dehydrated in ethanol and mounted either in Canada balsam or DePex (Gurr).

Photography. The completed autoradiograms were photographed by means of a microscope (Ortholux: E. Leitz and Co. Wetzler) fitted with an automatic camera. The slides were viewed with transmitted light, or with incident light and dark-field

illumination, which was provided by the Ultropak system (Leitz) at a magnification of 750 or 220. With dark-field illumination the silver grains appear as bright specks on a dark background, and there is a clear distinction between silver grains and stained material in the specimen (Rogers, 1967).

Estimation of grain density in autoradiograms. Visual grain counting was carried out at a magnification of 1000 with the use of an eyepiece graticule. The smallest grid was composed of squares $2{\cdot}2\ \mu \times 2{\cdot}2\ \mu$ at this magnification. The method was particularly suitable for measurements of resolution, for adjacent squares could be counted without moving the specimen on the microscope stage.

Photometric comparisons of grain density were made at a magnification of 1050 by a modification of the method described by Rogers (1967), in which the microscope was fitted with a dark-field illuminator (Pal-Opak, Leitz), photometric attachment (M.P.V., Leitz), and photomultiplier and photometer (Model 520 M, Photovolt Corpn., New York). By this method the area which was illuminated could be made to coincide with the area from which recordings were taken. Light was reflected from the grains into the photomultiplier tube and the current generated was recorded as a deflexion of a galvanometer. This procedure was particularly suitable for measuring the distribution of grain density along longitudinal sections of single fibres.

The nomenclature (autoradiography, autoradiogram) is that of Boyd (1955).

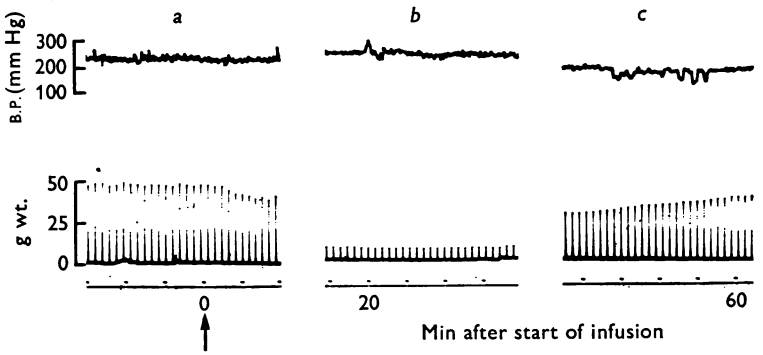
RESULTS

Pharmacological effects of single injections

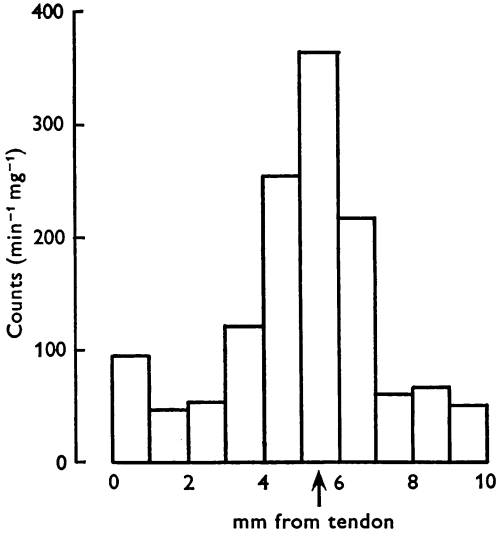
Decamethonium chloride (1·64 mg/kg) was injected into the femoral vein of anaesthetized rats. In animals of 100–150 g this produced a partial neuromuscular block (10–30 %) in the leg muscles, and in the majority of cases (thirty-one out of forty-one rats) there was no effect on breathing. A similar dose, given as the iodide, has been found to paralyse the righting reflexes in rats (Paton & Zaimis, 1949). In some animals a fall in blood pressure occurred 15–30 min after injection, at a time when the paralysis in the limb muscles was waning, and four animals died. In larger animals of 250–500 g this dose of decamethonium produced respiratory paralysis in all cases (ten animals) and artificial respiration was necessary. The limb muscles were also completely unresponsive to stimulation of the nerve trunk, and this paralysis was associated with depolarization of the end-plate region which was recorded by means of surface electrodes.

Continuous infusion of decamethonium

Text-fig. 1 shows a record of isometric contractions obtained during continuous intravenous infusion of labelled decamethonium (636 μM). The compound was run in at an initial rate of 0·46 ml./min (0·84 μ-mole min^{-1} kg^{-1}), and when neuromuscular block first appeared the rate was halved. Blood samples were taken at intervals, and the plasma level was constant from 15 to 60 min after infusion commenced. A neuromuscular block was produced which was steady initially, but later recovery took place (Text-fig. 1) in spite of the constant concentration of decamethonium in the



Text-fig. 1. Rat 350 g anaesthetized with chloralose and pentobarbitone. Records (upper trace) of B.P. (mm Hg), and isometric maximal contractions (g wt.) of the tibialis anterior muscle, elicited by stimulation of the sciatic nerve at 6/min (lower trace). *a*, I.V. infusion of tritiated decamethonium dichloride (0·84 μ-mole min^{-1} kg^{-1}) was started at arrow. Between *a* and *b* the infusion rate was halved and then kept constant. *b* 20 min and *c* 60 min after start of the infusion. Blood samples were taken at intervals and the radioactivity was similar in samples removed at 15 and 60 min. Partial block occurred at 20 min, with subsequent spontaneous recovery.



Text-fig. 2. Histogram which shows uptake in diaphragm muscle following infusion of tritiated decamethonium dichloride (636 μM) for 1 hr. The diaphragm was frozen and sliced and the arrow shows the position of the strip which contained the band of end-plates. The final concentration of labelled decamethonium in the plasma was 10·7 μM, with activity 195 counts min^{-1} $\mu l.^{-1}$, so the peak activity at the end-plate region was 1·85 ml. g^{-1}.

plasma. Muscles *in vitro* which are exposed to a steady concentration of the compound show neuromuscular block followed by recovery (Jenden, 1955). The concentration of decamethonium in the plasma which produced initial steady block varied from 11 to 43 μM in different animals.

Text-fig. 2 shows the uptake in the diaphragm muscle after infusion for 60 min. The muscle was removed, frozen, sliced with a razor blade at 1 mm intervals from the tendon to the rib, and counted by scintillation methods. The slice which contained the band of end-plates, as shown by the appearance of a white line (Creese *et al.* 1969) also showed a peak of radioactivity. In this case the peak uptake, expressed as (radioactivity per g muscle)/(radioactivity per ml. plasma) was 1·8 ml./g. In four muscles the peak value after 60 min varied from 1·4 to 1·8 ml./g (mean 1·6 ml./g), and this uptake could not be attributed to the extracellular space nor to the tubule system of the muscle. The value at the ends of the muscle (Text-fig. 2) was 0·28 ml./g and this radioactivity was partly attributable to the extracellular space. It was clear that the compound had become concentrated in the junctional region, but it was not possible to say whether the molecules were adsorbed on to the surface of the muscle or nerve or had entered the fibres.

In Text-fig. 2 it can be seen that there is also some increase in uptake in the slice of tissue nearest the tendon. This may be related to the increase in pharmacological sensitivity which has been found by iontophoretic application of depolarizing drugs at the muscle-tendon junction (Katz & Miledi, 1964).

Use of autoradiography

An autoradiographic method was needed which could be used to reveal the presence of drug molecules in muscle fibres. The decamethonium was labelled with tritium which produced β-particles of low energy, which is a requirement for good resolution. Frozen sections were prepared and placed in contact with an emulsion-coated slide. The results which can be obtained by this procedure are illustrated in Pl. 1, which shows a low-power view of an autoradiogram prepared from a diaphragm muscle which was removed 30 sec after injection of radioactive decamethonium. The photograph was taken with dark-field illumination and the outlines of muscle fibres can be seen cut transversely. In this method the light is reflected from the silver grains in the emulsion, and they appear as bright specks of light which indicate the position of the labelled drug. The muscle was removed shortly after injection had been made into the femoral vein, and the drug was largely confined to the main artery of the muscle. Silver grains are concentrated over the lumen of the artery; there is some activity also in the interspaces, but the drug does not appear to have reached the veins nor the muscle fibres.

Autoradiography of muscle fibres

Pl. 2*a* shows an autoradiogram of a transverse section of diaphragm muscle removed 10 min after intravenous injection of tritium-labelled decamethonium (1·64 mg/kg). The section was made in the region which contained the end-plates, and some of the fibres show the dark stain of the azo-dye which was used to demonstrate the presence of the esterase. This section was photographed by transmitted light, and the grains are seen as small black dots. There is a high grain density over all the fibres in the

TABLE 1. Grain counts over end-plate region of single fibres of peroneus longus after injection of tritiated decamethonium (1·64 mg/kg)

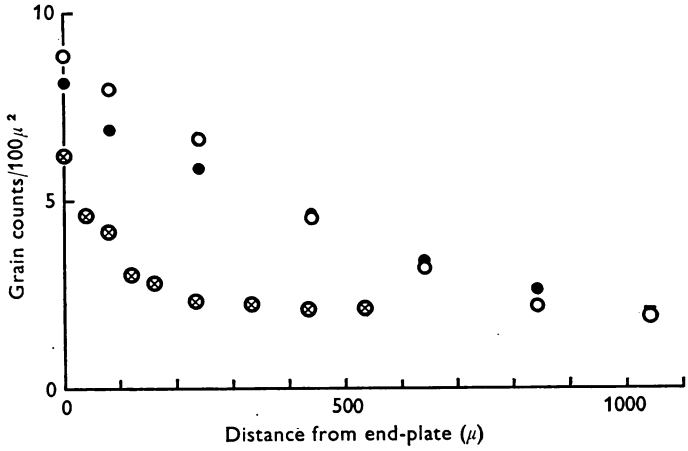
Time after injection	Counts/100 μ^2	Background/100 μ^2
3 min	11·90 ± 2·29 (50)	0·56 ± 0·50 (108)
2 hr	11·26 ± 2·13 (50)	0·56 ± 0·50 (108)

Rat 117 g. The left muscle was removed 3 min after injection and the right muscle was removed after 2 hr. Mean grain counts are shown, and the limits give the S.D., with no. of fibres in parentheses. The background count has not been subtracted.

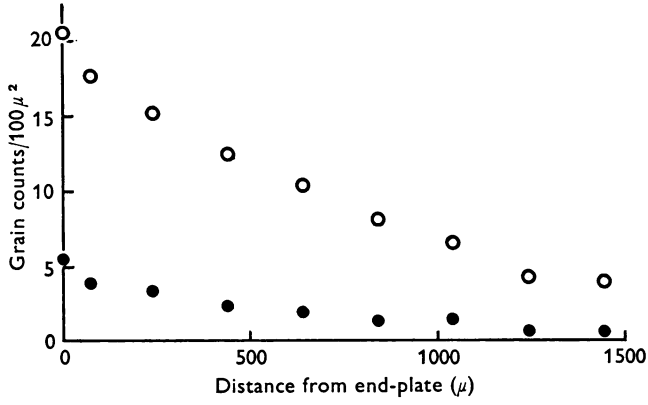
end-plate region, and this is contrasted with a control section from a muscle which received no radioactivity (Pl. 2*b*). There was no obvious indication of surface adsorption. The fibres were approximately 20–30 μ in diameter, and it is unlikely that radioactive compound found over the centre of the fibres could have reached this position by translocation during processing. This question is considered below in the section on the measurement of resolution.

The distribution of the drug within the fibres can be studied more easily in longitudinal sections, as shown in Pl. 3 which was prepared from a peroneus muscle removed 2 hr after injection. The end-plate region of the fibre contains a high grain density, and the radioactivity is much reduced at a distance of 600 μ from the edge of the end-plate.

Single intravenous injections of labelled decamethonium produced a transient peak of radioactivity in the arterial plasma which fell to half in 5–10 min. The drug entered the fibres at an early stage, and although the plasma level fell subsequently to low values the radioactive compound remained in the fibres. Table 1 shows a comparison of results obtained in peroneus muscles removed 3 min after injection (left side) and 2 hr after injection (right side). Transverse sections were prepared and the slides were processed together. The grains were counted in fibres which showed esterase (see Pl. 2), so that the radioactivity in the fibre in the vicinity of the end-plate was estimated. No significant difference was found between



Text-fig. 3. Distribution of grain-density in four fibres of peroneus longus removed 3 min (○) and in six fibres removed 2 hr (●) after injection of tritiated decamethonium dichloride (1·64 mg/kg). The lower curve (⊗) shows the distribution in a fibre from an animal which had received half this dose of decamethonium. Ordinate: grain counts/100 μ^2 minus background. Abscissa: distance from centre of end-plate in μ.



Text-fig. 4. Effect of tubocurarine on the uptake of tritiated decamethonium. The open circles show the distribution of radioactivity in four fibres of peroneus longus from a rat which had received an I.V. injection of decamethonium dichloride (1·64 mg/kg). The closed circles show six fibres from the peroneus muscle of another rat which had received tubocurarine dichloride (0·8 mg/kg) prior to injection of labelled decamethonium. Both muscles were removed 90 min after injection of decamethonium. These results were obtained from the last pair of rats listed in Table 2. Ordinate: grain count/100 μ^2, minus background. Abscissa: distance from the centre of the end-plate in μ.

muscles removed after 2 hr and those removed 3 min after injection (Table 1).

The background (Table 1) was estimated by counting over sections prepared from muscles which had received no radioactive compound. The control and radioactive sections were processed together. The counting area was 400 μ^2 and the count varied from 0 to 10 grains, with a mean value of 2·3 in 108 areas. The S.D. was similar to the mean, as expected of a Poisson distribution. A similar relation is seen in the background counts in Table 2. In Tables 1 and 2 the values are expressed as grains/100 μ^2.

Longitudinal sections of peroneus muscle were also prepared from the same muscles as those used for Table 1, and in some cases the sections extended for 1 mm or more from the end-plate. Text-fig. 3 shows the distribution of radioactivity in four fibres removed after 3 min and in six fibres removed 2 hr after injection of labelled decamethonium dichloride (1·64 mg/kg). The distribution is similar and in each of these small series the counts fell to a low level 1000 μ from the end-plate. There was no evidence for any redistribution of labelled drug along the fibres after the initial entry for a period of at least 2 hr.

Muscles removed 3 min or more after intravenous injection showed a grain-density in the non-junctional region of the fibre which was appreciably above background. In the results shown in Text-fig. 4 the grain-density at the region of the end-plate was 5–6 times as high as the value obtained 1·3–1·5 mm from the end-plates. The count was still declining at this distance, and the true ratio of peak to asymptote is probably greater than that indicated in Text-fig. 4. In some sections obtained from muscle 2 hr or more after injection there was also an appreciably grain density in the region of the nerve trunk, which indicated an uptake of decamethonium either by nervous tissue or by the sheath.

Text-fig. 3 also contains a plot from a rat which received 0·82 mg/kg of decamethonium, which was half the usual dose. The peak value at the end-plate falls steeply and the asymptote is reached approximately 400 μ from the centre of the end-plate. In eight fibres from three rats (107–150 g) which received a low dose of decamethonium (0·164–0·82 mg/kg) the distance required for the count of the end-plate region to fall half way to the asymptote varied from 40 to 90 μ (mean 70 μ), while for twenty-four fibres from eight rats (105–140 g) which received a large dose of decamethonium (1·64 mg/kg) the half-way point was 155–520 μ from the centre of the end-plate (mean 275 μ). The peak activity which is found at the end-plate region extends along the fibres for a variable distance, depending on the dose.

Attempts were made to determine the shape of the distribution curve from single fibres, as shown in Text-figs. 3 and 4. The distribution did not in general fit Gauss curves or simple exponential curves, and it was found that the sum of two exponential terms could be used to fit the results. The half-distance may have been underestimated in fibres in which the counts were still declining at the end of the section.

The density of grains immediately over the stained region of the end-plate was also studied. The azo-dye tended to obscure the grains situated beneath the end-plate in autoradiograms such as those shown in Pl. 2. In some experiments the sections

were picked up on emulsion-coated coverslips and later inverted on to slides so that the silver grains appeared above the tissue instead of below it. In such inverted autoradiograms no difference in grain density was detectable between areas immediately over the stained esterase and adjacent parts of the fibre. There was no evidence of a concentration of grains over the area which corresponded to the synaptic folds.

TABLE 2. Effect of tubocurarine on uptake of labelled decamethonium in the end-plate region

Weight (g)	Tubocurarine (mg/kg)	Decamethonium (mg/kg)	Time after injection (min)	Grains/ 100 μ^2	Background grains/ 100 μ^2
152	—	0·164	30	8·65 ± 1·74 (35)	0·38 ± 0·38 (112)
153	0·8	0·164	30	3·63 ± 1·33 (35)	0·38 ± 0·38 (112)
105	—	1·64	60	13·1 ± 2·93 (82)	0·37 ± 0·39 (112)
125	0·8	1·64	60	5·9 ± 2·32 (82)	0·37 ± 0·39 (112)
140	—	1·64	60	14·8 ± 3·30 (70)	0·37 ± 0·39 (112)
120	0·8	1·64	60	7·9 ± 2·45 (76)	0·37 ± 0·39 (112)
210	—	1·64	90	23·9 ± 4·81 (34)	0·88 ± 0·81 (128)
200	0·8	1·64	90	6·8 ± 1·57 (34)	0·88 ± 0·81 (128)

In the first pairs of animals the diaphragm muscle was removed: in other pairs peroneus muscles were counted. When tubocurarine was used it was injected I.V. 10 min before the injection of labelled decamethonium. Grain counts were made over muscle fibres in transverse section which showed end-plates. Mean grain counts are shown, and the limits give the S.D., with no. of fibres in parentheses. Tubocurarine reduced the grain density ($P < 0·01$ for each pair of animals). The background count has not been subtracted.

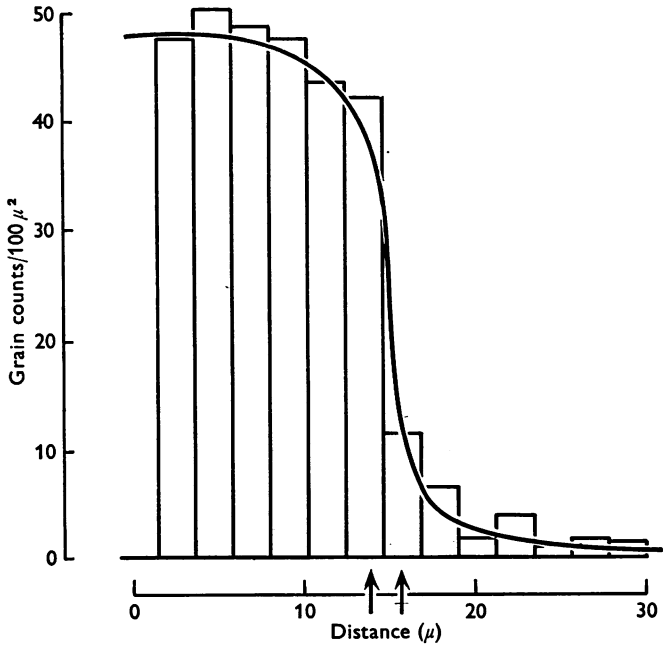
The effect of tubocurarine

Tubocurarine is known to prevent the action of decamethonium, and it also inhibits the uptake of labelled decamethonium and similar compounds in muscles *in vitro* (Creese, Taylor & Tilton, 1963; Taylor *et al.* 1965). Table 2 shows the effect of tubocurarine on the uptake of decamethonium in four pairs of rats. Both animals in each pair received labelled decamethonium, and one of the pair had previously been injected with tubocurarine. Artificial respiration was needed for all the animals which received tubocurarine, and also for some of the controls. Transverse sections were prepared from that part of the peroneus muscle which contained the band of end-plates, and the slides from each pair were processed together. The presence of the antagonist diminished the entry of decamethonium in each case, as shown by grain counts at the end-plate region of single fibres. In some cases longitudinal sections were obtained, and Text-fig. 4 shows the distribution of grains in fibres from the last pair of rats listed in Table 2. The grain-density in the controls was high at the end-plate and diminished over a distance of 1 mm or more from the peak, whereas fibres from

animals which were pretreated with tubocurarine showed much smaller peaks.

Autoradiography following arterial injection

In order to follow the movements of labelled decamethonium at a comparatively early stage it was necessary to use the method of close arterial injection. The drug was introduced in a rectrograde manner into the saphenous artery while the femoral artery was temporarily occluded.



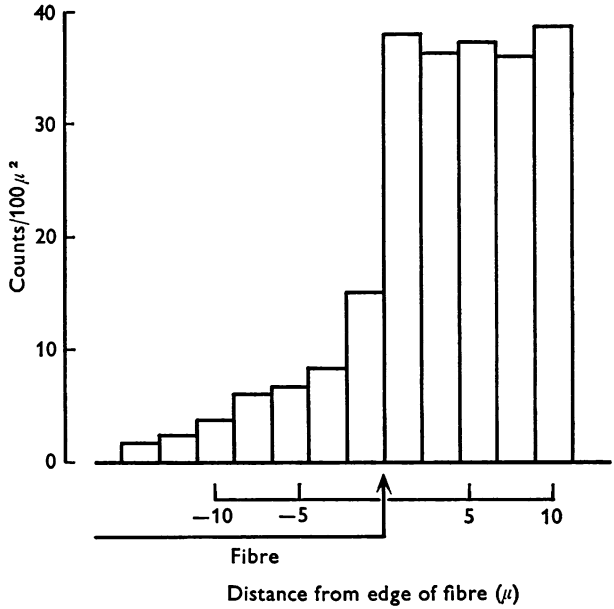
Text-fig. 5. Distribution of grains over layer of radioactive ice adjacent to diaphragm muscle (see text). The method of counting is illustrated in Pl. 6. Ordinate gives mean grains/100 μ^2 (mean of ten series). Abscissa gives the distance in μ from an arbitrary origin. The curve has been fitted by means of eqn. (6) (see Appendix). The arrows mark the points at which the grain density has fallen to 75 and 25 % of the original value.

Injection of decamethonium (0·63–0·49 mg/kg) produced a transient partial paralysis to nerve stimulation. The muscles were removed 10 sec after the end of injection and they were frozen within 30–45 sec after injection.

Pl. 4 shows a longitudinal section from the end-plate region. The position of the grains indicates that at this time the drug was in the interspaces and had also entered the muscle fibre. Pl. 5 was taken from a non-junctional region and there is a high grain density over the interspaces but little over the fibres, indicating that no entry had yet occurred in the region of the fibre which was distant from the end-plate.

The resolution of the autoradiograms

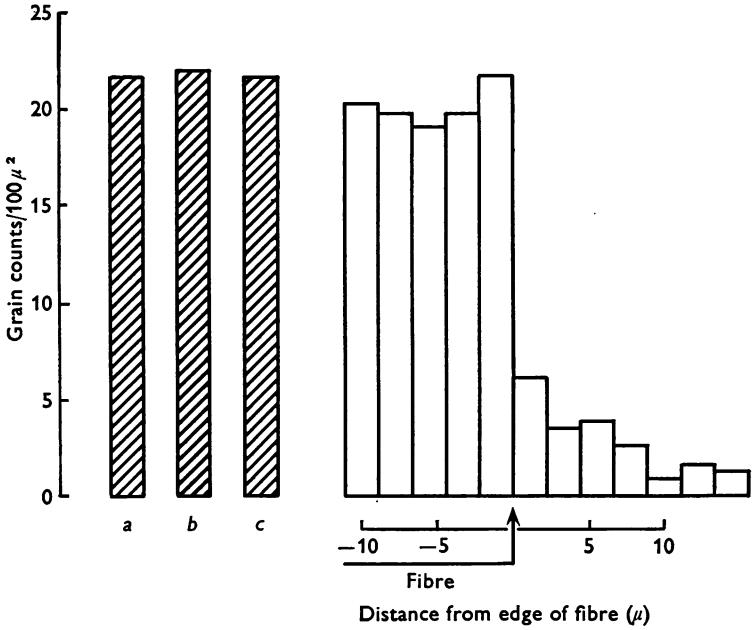
From the autoradiograms it was concluded that the drug rapidly entered the fibres at the end-plate and adjacent regions and thereafter remained without redistribution for several hours. It is necessary to show that the drug is not transported during handling and processing of the tissue, and for this purpose the resolution of the autoradiographic process was studied in several different circumstances.



Text-fig. 6. Distribution of grains over the interspaces and the adjacent muscle fibres from experiment shown in Pl. 5. The mean grain density/ 100 μ^2 from thirty-five fibres is plotted against the distance in μ from the edge of the fibre (shown by arrow). The grain density falls to half within 1 μ of the edge of the cell.

Pl. 6 shows an autoradiogram from a diaphragm which was mounted on a holder and dipped momentarily in solution which contained labelled decamethonium and then rapidly frozen. The tissue was later transferred to the cryostat for sectioning. When the diaphragm was removed it carried some of the solution which formed a thin layer of radioactive ice, and Pl. 6 shows the grains over the layer of frozen ice which had adhered to the surface of the muscle. Counts were made across the edge of the band of grains, as indicated on Pl. 6, and the results of ten different series are shown in Text-fig. 5. The density of the grains does not fall abruptly to the background value but declines over a distance of several microns. A radio-

active source with a sharp edge would produce a grain density in the emulsion with a curve similar to that fitted in Text-fig. 5, with a blurring of the edge as seen in the autoradiogram. Similar curves have been found experimentally by Hill (1962) and derived theoretically by Salpeter, Bachmann & Salpeter (1969, Fig. 12). The two arrows mark the points at which the grain density has fallen to 75 % and to 25 %. The resolution may



Text-fig. 7. Open rectangles show the distribution of grain-density across the outside fibres of diaphragm muscle in experiment illustrated in Pl. 7. The arrow marks the edge of the fibre and the histogram gives mean grains/ 100 μ^2 from thirty cells. Shaded histogram shows counts from forty fibres in the deeper layers of the diaphragm. *a* and *c* counts over outer area of fibre. *b* counts over centre of fibre. There is no increase in counts at the edge of fibres in the deeper layers.

be expressed operationally as the distance from the edge of a source at which the grain density falls to half (Nadler, 1951; Appleton, 1966). In Text-fig. 5 this is half the distance between the arrows (see Appendix), and the resolution expressed in this way is 0·9 μ.

Text-fig. 6 is a histogram which shows counts made on thirty-five cells similar to those of Pl. 5, in which the muscle was removed shortly after arterial injection and the labelled drug was in the interspaces and apparently not in those parts of the fibre which were several mm from the end-plate region. The arrow marks the edge of the fibres. In this histogram the

grain count is similar in all areas of the interspaces, and it falls to half in less than 1 μ.

Pl. 7 was taken from a diaphragm muscle removed 90 min after intravenous injection of tritium-labelled drug. By this time the decamethonium in the plasma has fallen to low values while the muscle fibres retain their radioactivity. In Pl. 7 the grains are viewed by incident light in a dark field, and cell detail is not displayed. Counts were made across thirty surface fibres at the outer surface of the muscle and the results are shown in the histogram of Text-fig. 7 (right side). The half-distance, measured from the edge of the fibre, is less than 1 μ.

In Text-fig. 7 the count at the edge was greater than that over the rest of the fibre. This was found in twenty of the thirty fibres which were counted, and the differences are unlikely to be fortuitous ($P < 0{\cdot}01$ by one-tail t-test). It is well known that increased grains are liable to occur at the edge of tissues (Rogers, 1967), and this is attributed to difference of pressure on the emulsion during the preparation of the autoradiogram. The effect fortunately does not occur at the edges of cells in the depth of the muscle. The shaded histogram in Text-fig. 7 shows counts made on forty fibres in the deeper layers of the diaphragm, from autoradiograms similar to that shown in Pl. 2. Counts were made over the outer segments and also over the centre of each fibre. The result is shown in Text-fig. 7 and there is no significant difference between counts at the edge and at the centre of the cells in the depths of the tissues.

In some cases the autoradiograms were calibrated by measurement of the autoradiographic efficiency, which may be expressed as the ratio of grains to the disintegrations produced in the section (Oja, Oja & Hasan, 1966). The results in Text-fig. 7 were obtained from a diaphragm which was removed 2·5 hr after intravenous injection of labelled decamethonium. The lateral strip was frozen in isopentane so that transverse sections could be prepared in the cryostat. The sections were taken from the region which contained the band of end-plates and some of the fibres showed stained esterase. In Text-fig. 7 the grain density of the fibres in the centre of the diaphragm may be taken as 22 grains/100 μ^2, and if the background is subtracted this is 20 grains/100 μ^2. The medial strip of the diaphragm was frozen with solid carbon dioxide and strips of muscle 1 mm wide were cut and counted by scintillation methods. A distribution was obtained similar to that shown in Text-fig. 2 and the peak count in the strip which contained the end-plate was 520 counts min^{-1} mg^{-1}.

The activity in the end-plate region was 520 counts min^{-1} mg^{-1} and since the counting efficiency was 10 % the activity was $5{\cdot}2 \times 10^6$ disintegrations min^{-1} g^{-1} or 5·56 disintegrations min^{-1} $ml.^{-1}$ if the specific gravity was 1·07. The extracellular space was 0·3 ml. $ml.^{-1}$ and showed only negligible grains in the autoradiograms, so

the radioactivity was contained in 0·7 ml. The activity in the fibres was therefore $8{\cdot}0 \times 10^6$ disintegrations min^{-1} $ml.^{-1}$. The exposure was 7 days or 10^4 min, so the activity in the fibres was $8{\cdot}0 \times 10^{10}$ disintegrations in 1 ml. or 10^{12} μ^3. Hence the activity was 8·0 disintegrations in each 100 μ^3 fibre during the exposure.

If the sections were 5 μ thick a surface area of 100 μ^2 would be associated with 500 μ^3 fibre or 40 disintegrations. The grain count was 20 grains/100 μ^2 so the autoradiographic efficiency was 50%. A frozen section originally 5 μ thick would become rapidly dehydrated during exposure and would be expected to shrink to 1 μ or less, which is within the range of the β-particles. A thick emulsion was used which would produce a high chance of capture of β-particles, and in most cases the chance of a β-particle producing a silver grain on entering the emulsion was close to 100%.

DISCUSSION

The pharmacological effects of decamethonium and other depolarizing agents on skeletal muscle are extremely complex, whether measured as depolarization or as neuromuscular block. It has not proved possible to obtain a pharmacological effect which is steady for long periods (e.g. Jenden, 1955), and tachyphylaxis, dual block and desensitization are some of the terms which have been used to describe this action. Behaviour of this kind led to the suggestion that these compounds were entering muscle cells (Jenden, Kamijo & Taylor, 1951, 1954), and this was supported by measurements of the uptake of labelled decamethonium-like compounds *in vitro* (see Taylor & Nedergaard, 1965). A similar concept was originally advanced by Burns & Paton (1951) but later repudiated (Paton, 1962).

Substantial amounts of labelled decamethonium are taken up *in vivo*, and Lüthi & Waser (1965) have extracted the compound from cat muscle without evidence of break-down products in this tissue. Waser (1966) found by contact autoradiography that decamethonium at the region of the end-plate became concentrated to an extent at least 100 times greater than that of labelled curarine alkaloids. The method did not permit further localization and the effect was attributed to fixation on to the muscle membrane.

The present results have indicated that labelled decamethonium rapidly enters muscle fibres in the region of the end-plate. The findings are not attributable to movement of the labelled drug during processing, for entry is found at the end-plate region in conditions in which no entry can be detected elsewhere (Pls. 4 and 5), and in other circumstances the grain-density decreases in single fibres as the distance from the end-plate is increased (Pl. 3, Text-fig. 3). The resolution, as measured by the distance for the grain-density to fall to half, was less than 1 μ. The spread of grain-density at the edge of a source can be seen in Text-fig. 5, and this degree of overlap could not account for the activity found over the centre of cells of

thickness 30 μ or more as seen in Pl. 2 and Text-fig. 7. In the case of water-soluble compounds it is desirable to avoid fixatives and embedding media, and this may be achieved if frozen tissues are used throughout for sectioning and exposure. Sections of thickness 5 μ were used in the present study, and consistent results were obtained providing the sections were not allowed to thaw when they were picked up on the emulsion-coated slide. In other cases when fixation and embedding is possible a resolution of 0·21–0·3 μ has been obtained by Hill (1962, 1964) who used sections of frog muscle of thickness 0·45–0·60 μ.

The distribution which was found inside muscle fibres with a peak at the end-plate region has some resemblance to the results obtained when the sensitivity of the fibre to depolarizing drugs was measured (Miledi, 1962). The pharmacological effects were found to be maximal at the end-plate and extended for 0·5 mm or more along the fibre. Labelled decamethonium inside the cell probably becomes rapidly attached to some intracellular constituent, for the radioactive compound remained in the tissues when the external concentration had fallen to low values and no change in distribution could be detected over the next 2 hr. The compound can be found days or weeks after injection and there is a very low internal diffusion coefficient (Taylor, Dixon, Creese & Case, 1967). The characteristic distribution is achieved at an early stage, and the decamethonium appears to enter both at the end-plate and also at a large area in the vicinity of the junctional region. There was no evidence to suggest that the characteristic distribution seen soon after injection was produced by entry at the end-plate and subsequent diffusion laterally along the fibre. The compound appears to travel readily in a radial direction on entry, and movement along the transverse tubule system of the fibre (Peachey, 1965; Schiaffino & Margreth, 1969) is an obvious possibility. The results do not exclude some degree of surface adsorption, but it would appear that the bulk of the labelled compound which is found in the junctional region of the fibre is within the muscle cell.

It is characteristic of the behaviour of stimulant drugs that the response is dependent on concentration, and the dose-response curve shows saturation and increases with concentration to a maximum value. Waser (1966) found no evidence of saturation in experiments in mice, and this has led to doubts regarding the interpretation of studies with labelled depolarizing drugs (e.g. Waud, 1968). However, Creese & England (1970) have measured uptake *in vitro* as a clearance and it was possible to show saturation at low concentrations of decamethonium. In the present experiments the major portion of the uptake could be prevented by the antagonist tubocurarine, and this criterion may be used as a safeguard to distinguish non-specific uptake (Creese *et al.* 1963).

The significance of the entry of stimulant drugs is not at present clear. Rapid entry of labelled nicotine has been found in the superior cervical ganglion of the cat (Brown, Hoffmann & Roth, 1969), and it is possible that entry occurs in other excitable tissues and with other drugs. Some authors (Paton & Rang, 1965; Brown *et al.* 1969) have attributed the entry of labelled agonists to depolarization. However, depolarization when produced by potassium was found to slow the uptake of decamethonium, and the characteristic distribution with a peak at the end-plate region was obtained in muscles in which further voltage changes were prevented by immersion in a solution composed largely of potassium methyl sulphate (Creese & England, 1970). Cookson & Paton (1969) have suggested that the entry of labelled stimulant drugs is a consequence not of depolarization but of the non-specific change in permeability which accompanies pharmacological activity. If this is the case it should be possible to determine whether the saturable uptake of decamethonium runs parallel to that of inorganic ions such as that of potassium. It is characteristic of the movements of labelled organic ions that the kinetics show carrier-like properties (Creese & England, 1970; Creese & Taylor, 1967). There is also convincing evidence that the receptors for stimulant drugs are on the outside of the tissues (del Castillo & Katz, 1955), and it may be that the receptors are also the carrier molecules which are activated by a rise in the concentration of the stimulant drug.

The authors are grateful to the Medical Research Council for their generous support; to Professor F. Goldby and the Staff of the Anatomy Department, St Mary's Hospital Medical School for much help in the early stages; and to Miss Anne Boddy for technical assistance.

APPENDIX

Distribution of grains produced by standard sources

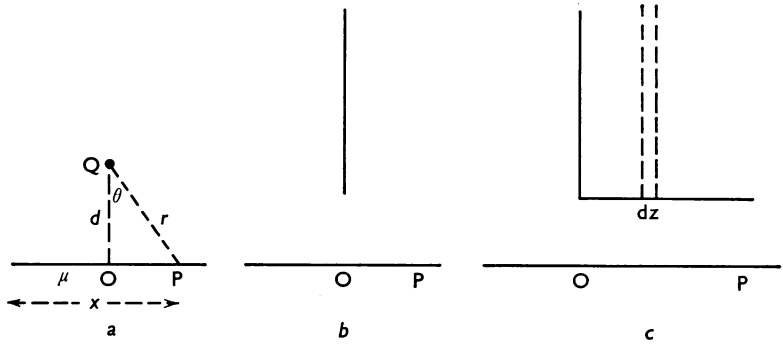
With the simplifying assumptions used by Bachmann & Salpeter (1965) the grain density in an autoradiogram produced by a tritium source in a thin layer of emulsion may be considered as proportional to the flux of radiation per unit area. If a *point source* is at Q the intensity in the emulsion at P which is at a distance r from the source (Text-fig. 8*a*) can be found by applying the inverse square law in the form $(\cos\theta)/r^2$ (e.g. Meyler & Sutton, 1958), where θ is the angle made by the direction of the rays and the normal QO to the surface. The angle term is necessary to allow for the effect of oblique incidence, and is met in photometry in the form of Lambert's cosine rule. If the points O and P on the x-axis are at distances μ and x from the origin then OP is $x-\mu$, and QO on the y-axis is d. Then the distribution of grain density $f_p(x)$ for a point source is

$$f_p(x) \propto \frac{\cos\theta}{r^2} \qquad (1)$$

and this can be expressed in different ways as follows:

$$f_p(x) \propto \frac{d}{r^3} \propto \frac{\cos^3\theta}{d^2}. \tag{2}$$

Equation (2) in the form $\cos^3\theta$ has been derived by Bachmann & Salpeter (1965). In some other treatments the intensity has been considered to be proportional to $1/r^2$, which is only applicable when the surface is normal to the direction of the radiation.



Text-fig. 8*a*. *Q* is a point source of radioactivity situated at a distance *d* from the nearest point O of a thin photographic emulsion. O and P are at a distance μ and x respectively from the origin. P is distant r from the source. The Figure may also be used to illustrate a line source at Q perpendicular to the plane of the paper and parallel to the plane of the emulsion. *b*, this represents a source forming a vertical sheet and extending in a direction perpendicular to the plane of the paper; P is a point in the emulsion. *c*, this represents a section through a semi-infinite half plane or straight edge with lower surface parallel to the plane of the emulsion. O is a point in the emulsion opposite the edge of the solid.

Text-fig. 8*a* may also be used to represent a *line source* parallel to the plane of the emulsion and distance *d* away, and perpendicular to the plane of the paper. It is shown in standard texts that the field produced by a current in a line source at a distance r is proportional to $1/r$. By an analogous argument it can be shown that the intensity produced by a line source of radiation is also proportional to $1/r$, in a direction normal to the radiation. Hence the intensity in the plane of the emulsion is proportional to $(\cos\theta)/r$, and the distribution function $f_1(x)$ for a line source is given by

$$f_1(x) \propto \frac{\cos\theta}{r}, \tag{3}$$

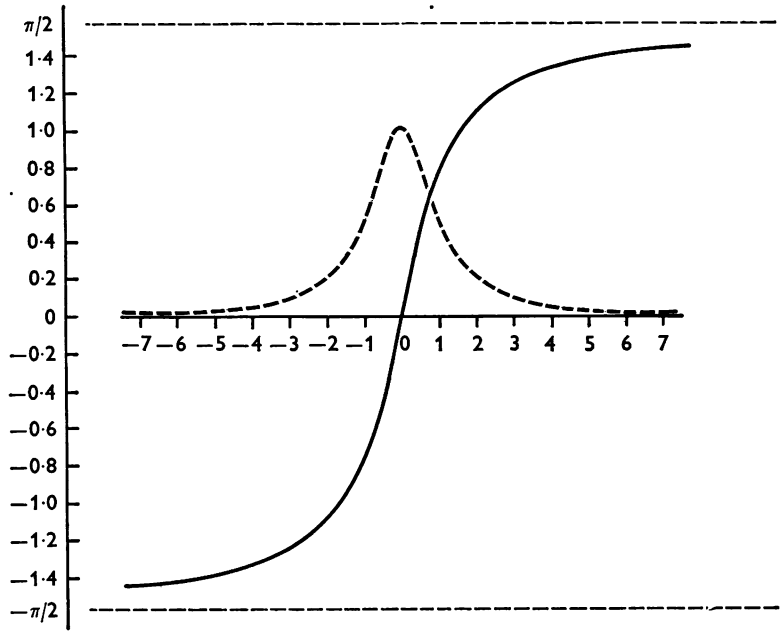
or

$$f_1(x) \propto \frac{\cos^2\theta}{d} \propto \frac{d}{r^2} \propto \frac{1}{d\left[1+\frac{(x-\mu)^2}{d^2}\right]}. \tag{4}$$

Equation (4) has been derived by Salpeter *et al.* (1969). Now if $(x-\mu)/d$ is z and distances are measured in terms of d so that d is considered to be unity then this is equivalent to a standardized Cauchy distribution

$$f(z) = \frac{1}{\pi}\frac{1}{1+z^2}. \tag{5}$$

This is the probability distribution obtained on the x-axis when θ in Text-fig. 8*a* is allowed to vary from $-\frac{1}{2}\pi$ to $\frac{1}{2}\pi$ so that the arm QP cuts the x-axis at a series of points (Aitken, 1957). This is shown by the dashed curve



Text-fig. 9. The dashed curve represents the standardized distribution of eqn. (5) and is produced by a line source (Text-fig. 8*a*). The distance along the x-axis at which the peak distribution falls to half is taken as unity. The curved line represents the integrated distribution of eqn. (6), and is produced by a straight edge (Text-fig. 8*c*).

in Text-fig. 9 which is flatter than a Gauss distribution. If the distance along the x-axis required to reduce the peak density to half is λ then the expression may also be standardized by moving the origin to O and measuring distances in terms of the spread parameter λ.

Text-fig. 8*b* shows a *vertical sheet* made up of a number of line sources each parallel to the plane of the emulsion and perpendicular to the plane of the paper, stacked one on top of each other. The β-particles produced

by tritium travel a limited distance, and the intensity at O which is closest to the sheet would depend on the energies of the particles. Each line source would produce a distribution similar to that of eqn. (5). It can be shown that the sum of a number of variates of this form would also have a Cauchy distribution (Thomasian, 1969), and the resultant density would resemble the dashed curve in Text-fig. 9.

Salpeter *et al.* (1969) have measured the distribution produced by a thin vertical sheet of radioactive material 0·05 μ thick and they found that the grain density was similar to that given by eqn. (5). In the case of thick emulsions the total grain density may be considered as made up of several layers each with a Cauchy distribution and by a similar argument the total density would still be of the form given by eqn. (5).

Text-fig. 8*c* shows a section through a *semi-infinite half plane* or straight edge, which may be considered as made up of many vertical sheets, each perpendicular to the plane of the emulsion and stacked one behind the other and forming a solid whose lower face is parallel to the plane of the emulsion. If distances are measured from O, which is opposite the edge, and standardized as above then dz may be used to represent the width of one of the sheet sources. The intensity at some point P in the emulsion due to this source is proportional to dz and also to $f(z)$ which is given in eqn. (5), and the distribution produced by a semi-infinite half-plane when expressed in standardized units is obtained by integrating from minus infinity to z:

$$f_E(z) = \frac{1}{\pi}\int_{-\infty}^{z} \frac{dz}{1+z^2} = \frac{1}{2}+\frac{1}{\pi}\arctan z. \qquad (6)$$

This is the integral of a Cauchy distribution and is given by the continuous curve in Text-fig. 9.

As the reference point P is moved nearer to the edge the grain density falls, and the distribution in the emulsion also extends beyond the point which is opposite the edge of the solid. Similar distribution have been obtained experimentally by Hill (1962), and in Text-fig. 5 the results have been fitted to a curve which resembles that shown in Text-fig. 9.

The grain density opposite the edge falls to 50 % of the maximum value, and from the properties of the integrated Cauchy curve the interval which corresponds to 25 and 75 % of the maximal value (Text-fig. 5) gives twice the resolution defined as the distance from the edge of the source at which the grain density falls to half (Appleton, 1966; Nadler, 1951). This is also the resolution expressed as the half-width of the bell-shaped curve which describes the response of the film to a line source (Text-fig. 9), and it is also the distance from a line source within which half the developed grains would be found, as used by Salpeter *et al.* (1969). The value in practice

comes close to 0·9 μ in the present experiments with thick sections, and this is consistent with the mean path for β-particles from tritium (Eidinoff, Fitzgerald, Simmel & Knoll, 1951). The resolution obtained by these operational definitions differs somewhat with different shapes of source (Salpeter *et al.* 1969), and the value used here is close to but not identical with the predicted half-width of the bell-shaped curve which describes the density produced by a point source, used by Doniach & Pelc (1950).

REFERENCES

AITKEN, A. C. (1957). *Statistical Mathematics*, 8th ed., pp. 70–71. Edinburgh: Oliver and Boyd.

APPLETON, T. C. (1964). Autoradiography of soluble labelled compounds. *Jl R. microsc. Soc.* **83**, 277–281.

APPLETON, T. C. (1966). Resolution in soluble-compound autoradiography. *J. Histochem. Cytochem.* **14**, 414–420.

BACHMANN, L. & SALPETER, M. M. (1965). Autoradiography with the electron microscope. *Lab. Invest.* **14**, 1041–1053.

BARKA, T. & ANDERSON, P. J. (1963). *Histochemistry: Theory, Practice and Bibliography*, p. 261. New York: Harper & Roe.

BOYD, G. A. (1955). *Autoradiography in Biology and Medicine*, pp. 13–15. New York: Academic Press.

BROWN, D. A., HOFFMAN, P. C. & ROTH, L. J. (1969). ^{3}H-nicotine in cat superior cervical and nodose ganglia after close-arterial injection *in vivo*. *Br. J. Pharmac. Chemother.* **35**, 406–417.

BURNS, B. D. & PATON, W. D. M. (1951). Depolarization of the motor end-plate by decamethonium and succinylcholine. *J. Physiol.* **115**, 41–73.

COOKSON, J. C. & PATON, W. D. M. (1969). Mechanisms of neuromuscular block. *Anaesthesia* **24**, 395–416.

CREESE, R. & ENGLAND, J. M. (1970). Decamethonium in depolarized muscle and the effects of tubocurarine. *J. Physiol.* **210**, 345–361.

CREESE, R., ENGLAND, J. M. & TAYLOR, D. B. (1969). Method of localizing end-plates in unstained frozen muscle. *J. Physiol.* **200**, 7–8*P*.

CREESE, R. & MACLAGAN, JENNIFER (1967). Autoradiography of decamethonium in rat muscle. *Nature, Lond.* **215**, 988–989.

CREESE, R. & TAYLOR, D. B. (1967). Entry of labelled carbachol in brain slices of the rat and the action of D-tubocurarine and strychnine. *J. Pharmac. exp. Ther.* **157**, 406–419.

CREESE, R., TAYLOR, D. B. & TILTON, B. (1963). The influence of curare on the uptake and release of a neuromuscular blocking agent labeled with radioactive iodine. *J. Pharmac. exp. Ther.* **139**, 8–17.

DEL CASTILLO, J. & KATZ, B. (1955). On the localization of acetylcholine receptors. *J. Physiol.* **128**, 157–181.

DEL CASTILLO, J. & KATZ, B. (1957). A comparison of acetylcholine and stable depolarizing agents. *Proc. R. Soc.* B **146**, 362–368.

DONIACH, I. & PELC, S. R. (1950). Autoradiograph technique. *Br. J. Radiol.* **23**, 184–192.

EIDINOFF, M. L., FITZGERALD, P. J., SIMMEL, E. B. & KNOLL, J. E. (1951). Intracellular localization of compounds labeled with tritium, H^3, by radioautography. *Proc. Soc. exp. Biol. Med.* **77**, 225–229.

ENGLAND, J. M. (1970). The localization of end-plates in unstained muscle. *J. Anat.* **106**, 311–321.

HILL, D. K. (1962). Resolving power with tritium autoradiographs. *Nature, Lond.* **194**, 831–832.

HILL, D. K. (1964). The space accessible to albumin within the striated muscle fibres of the toad. *J. Physiol.* **175**, 275–294.

JENDEN, D. J. (1955). The effect of drugs upon neuromuscular transmission in the isolated guinea pig diaphragm. *J. Pharmac. exp. Ther.* **114**, 398–408.

JENDEN, D. J., KAMIJO, K. & TAYLOR, D. B. (1951). The action of decamethonium (C 10) on the isolated rabbit lumbrical muscle. *J. Pharmac. exp. Ther.* **103**, 348.

JENDEN, D. B., KAMIJO, K. & TAYLOR, D. B. (1954). The action of decamethonium on the isolated rabbit lumbrical muscle. *J. Pharmac. exp. Ther.* **111**, 229–240.

KATZ, B. & MILEDI, R. (1964). Further observations on the distribution of acetylcholine-reactive sites in skeletal muscle. *J. Physiol.* **170**, 379–388.

LÜTHI, U. & WASER, P. G. (1965). Verteilung und Metabolismus von ^{14}C-Decamethonium in Katzen. *Archs int. Pharmacodyn. Thér.* **156**, 319–347.

MEYLER, D. S. & SUTTON, O. G. (1958). *A Compendium of Mathematics and Physics*, p. 319. London: English Universities Press.

MILEDI, R. (1962). Induction of receptors. In *Enzymes and Drug Action* (C.I.B.A. Foundation Symposium), ed. MONGAR, J. L. & DE REUCK, A. V. S., pp. 220–235. London: Churchill.

NADLER, N. J. (1951). Some theoretical aspects of radioautography. *Can. J. med. Sci.* **29**, 182–194.

OJA, H. K., OJA, S. S. & HASAN, J. (1966). Calibration of stripping film autoradiography in sections of rat liver labelled with tritium. *Expl cell Res.* **45**, 1–10.

PATON, W. D. M. (1962). In *Curare and Curare-like Agents* (C.I.B.A. Foundation Study Group No. 12), ed. DE REUCK, A. V. S., p. 33. London: Churchill.

PATON, W. D. M. & RANG, H. P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. R. Soc.* B **163**, 1–44.

PATON, W. D. M. & ZAIMIS, E. J. (1949). The pharmacological actions of polymethylene bistrimethylammonium salts. *Br. J. Pharmac. Chemother.* **4**, 381–400.

PEACHEY, L. D. (1965). The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *J. cell Biol.* **25**, 209–231.

PEARSE, A. G. (1953). *Histochemistry, Theoretical and Applied*, pp. 410–411. London: Churchill.

ROGERS, A. W. (1967). *Techniques of Autoradiography*, pp. 86, 101–115, 137–160, 228–239. Amsterdam: Elsevier.

SALPETER, M. M., BACHMANN, L. & SALPETER, E. E. (1969). Resolution in electron microscope radioautography. *J. cell Biol.* **41**, 1–20.

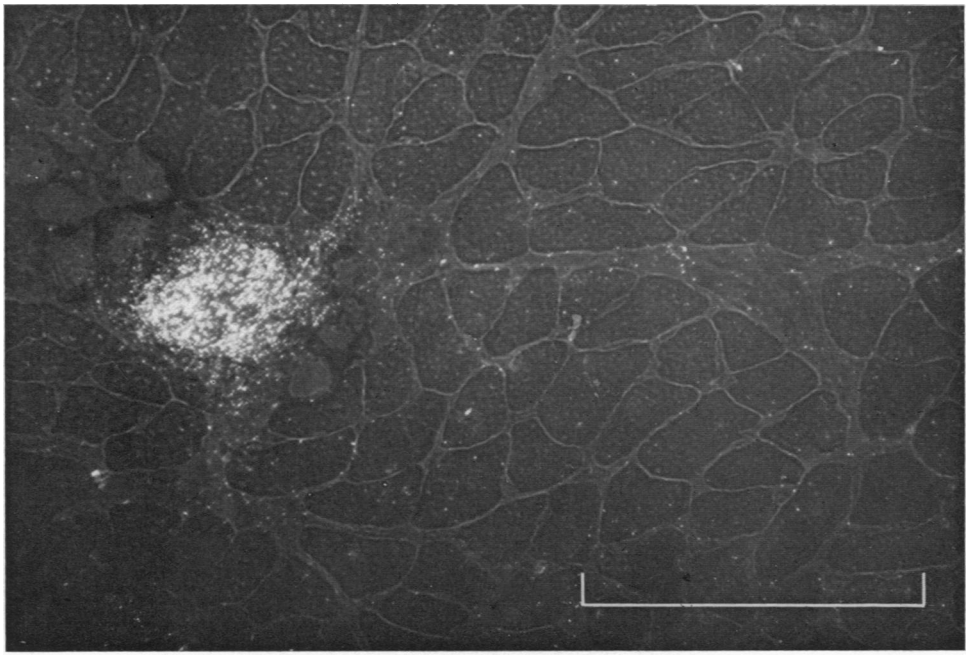
SCHIAFFINO, S. & MARGRETH, A. (1969). Coordinated development of the sarcoplasmic reticulum and T system during postnatal differentiation of rat skeletal muscle. *J. cell Biol.* **41**, 855–875.

TAYLOR, D. B., CREESE, R., NEDERGAARD, D. A. & CASE, R. (1965). Labelled depolarizing drugs in normal and denervated muscle. *Nature, Lond.* **208**, 901–902.

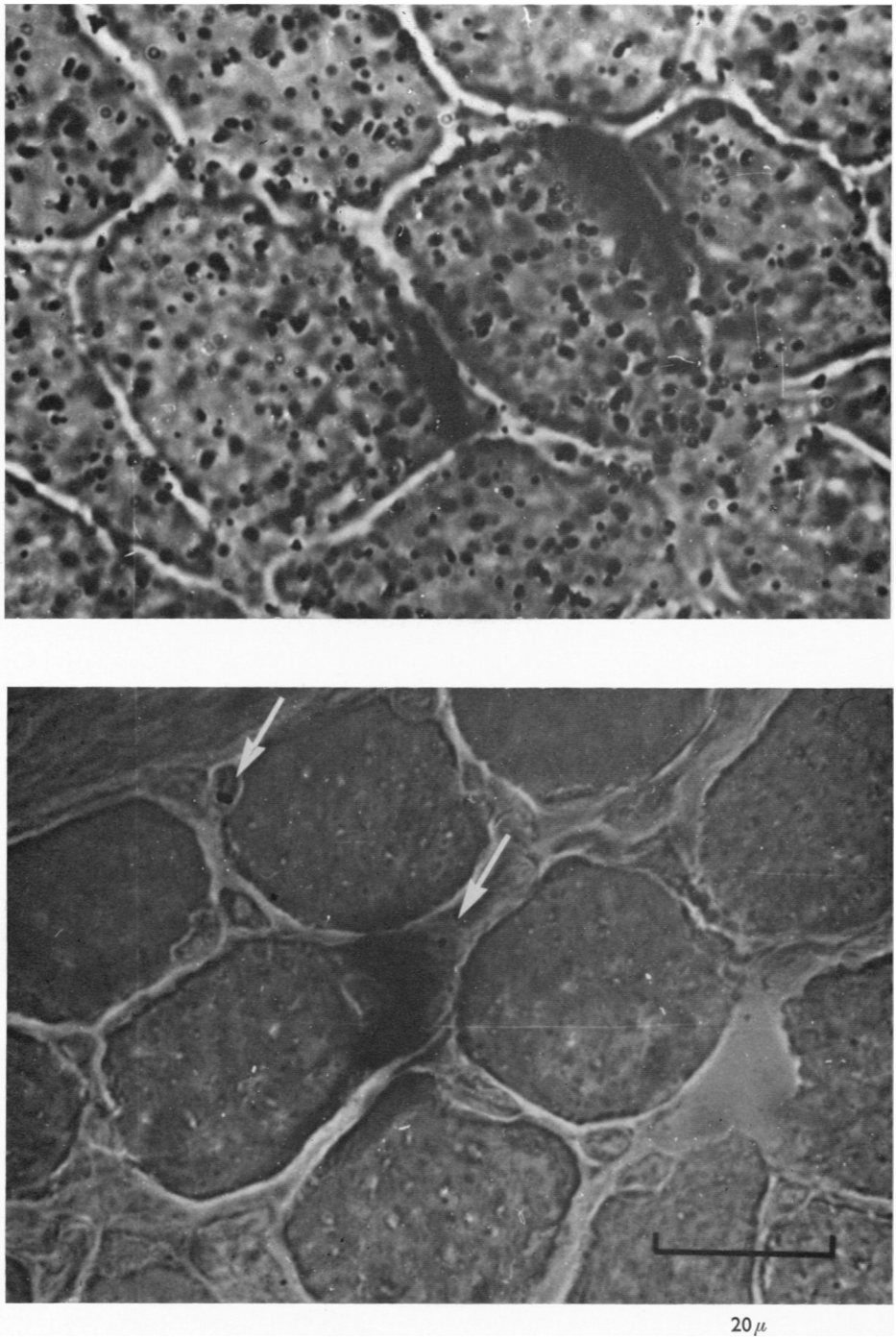
TAYLOR, D. B., DIXON, W. J., CREESE, R. & CASE, R. (1967). Difiusion of decamethonium in the rat. *Nature, Lond.* **215**, 989.

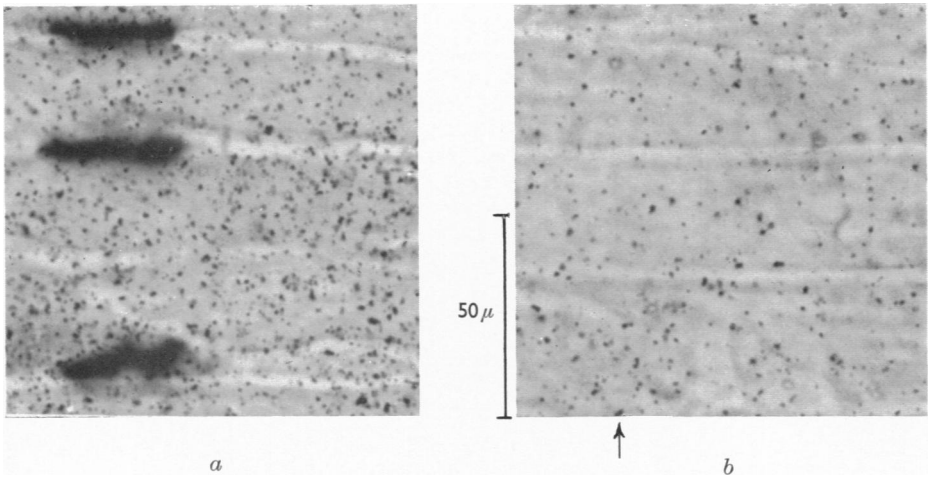
TAYLOR, D. B. & NEDERGAARD, O. A. (1965). Relation between structure and action of quaternary ammonium neuromuscular blocking agents. *Physiol. Rev.* **45**, 523–554.

THESLEFF, S. (1955). The effects of acetylcholine, decamethonium and succinylcholine in neuromuscular transmission in the rat. *Acta physiol. scand.* **34**, 386–392.

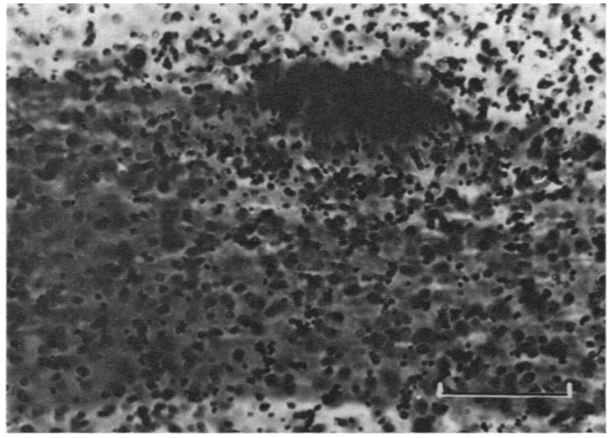


(*Facing p.* 384)

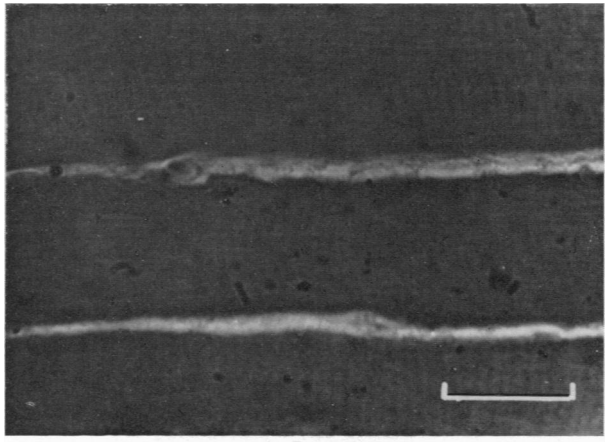

a
b
20 μ

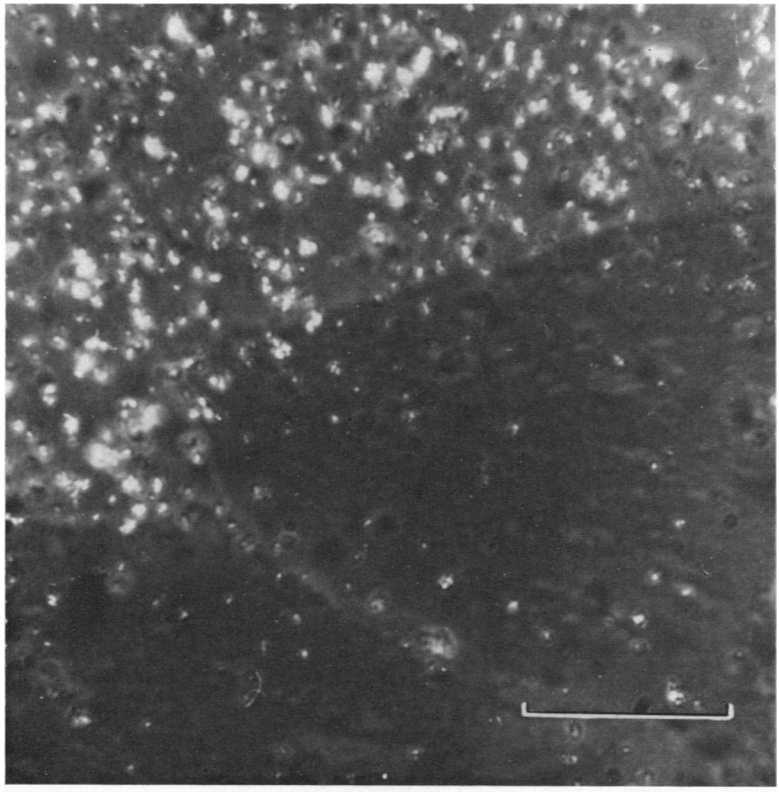

50 μ
a
b

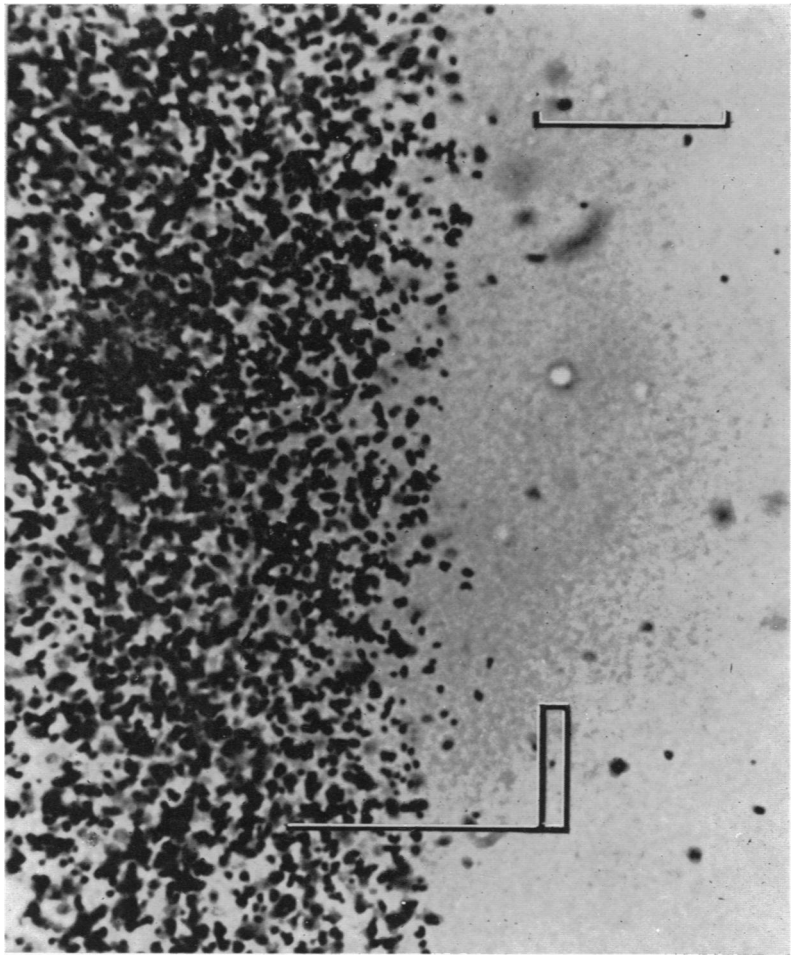
a

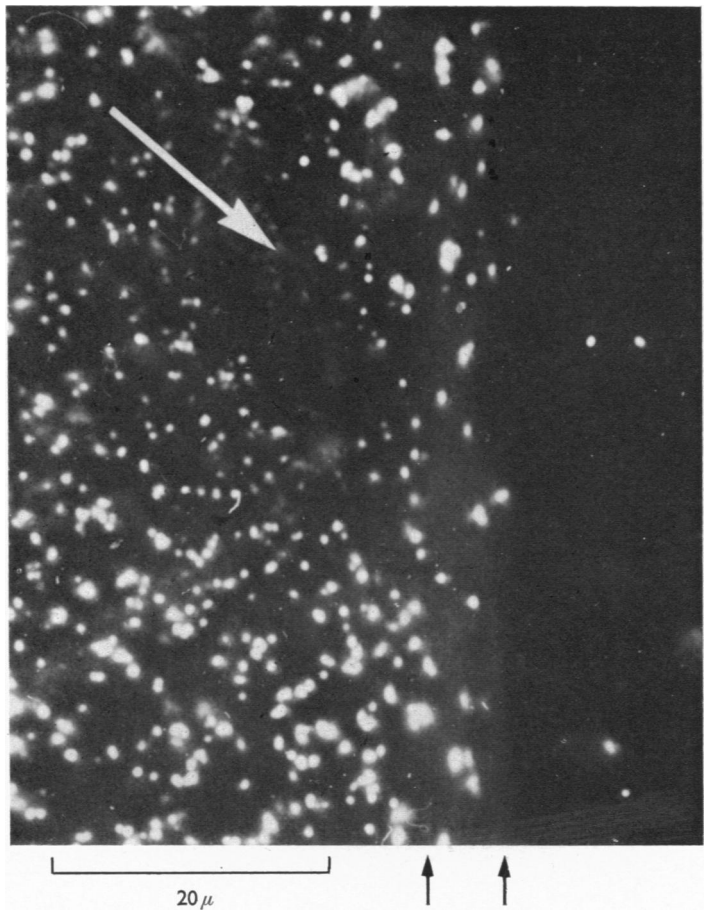


b




20 μ




20 μ

THOMASIAN, A. J. (1969). *The Structure of Probability Theory with Applications*, pp. 387–390, 472–473. New York: McGraw-Hill.

WASER, P. G. (1960). The cholinergic receptor. *J. Pharm. Pharmac.* **12**, 577–594.

WASER, P. G. 1966). Autoradiographic investigations of cholinergic and other receptors in the motor endplate. In *Advances in Drug Research*, ed. HARPER, N. J. & SIMMONDS, A. B., vol. 3, pp. 81–120. London: Academic Press.

WAUD, D. R. (1968). Pharmacological receptors. *Pharmac. Rev.* **20**, 49–88.

EXPLANATION OF PLATES

PLATE 1

Autoradiogram prepared from transverse section of rat diaphragm muscle removed and frozen 30 sec after I.V. injection of tritiated decamethonium dichloride (1·64 mg/kg). The autoradiogram was photographed at low magnification under incident light, dark-field illumination so that the silver grains appear as white specks. The grains are concentrated over the lumen of the main artery of the muscle, and only appear infrequently over the adjacent veins, interspaces and muscle cells. Calibration 100 μ.

PLATE 2

Autoradiogram from sections of diaphragm muscles cut transversely through the region containing the band of end-plates, and photographed in transmitted light so that the silver grains appear as black dots. *a*, muscle removed and frozen 10 min after I.V. injection of tritiated decamethonium dichloride (1·64 mg/kg). The position of two end-plates is shown by the dark azo-dye stain of the esterase. There is a high grain density over the fibres. The grains are not all in the same plane and some are out of focus. *b*, control autoradiogram prepared from a muscle which had received no radioactive drug, processed together with radioactive section. One fibre shows stained esterase. Calibration 20 μ.

PLATE 3

Autoradiogram prepared from longitudinal section of peroneus muscle removed and frozen 2 hr after I.V. injection of tritiated decamethonium dichloride (0·82 mg/kg). On the left-hand side (*a*) the position of the end-plates is shown by the dark azo-dye stain of the esterase. There is a high grain density. Right-hand side (*b*) shows the same fibres at a distance 600 μ from the end-plates, as indicated by the arrow. The grain density is much reduced. Vertical calibration 50 μ.

PLATE 4

a, Autoradiogram prepared from longitudinal section of extensor digitorum longus muscle removed 10 sec after retrograde I.A. injection of tritiated decamethonium dichloride (0·55 mg/kg). The esterase at the end-plate has been stained by the azo-dye method. There is a high grain density over the fibre and over the interspaces. *b*, control autoradiogram from untreated muscle. Calibration 20 μ.

PLATE 5

Autoradiogram prepared from tibialis anterior removed 30 sec after retrograde I.A. injection of tritiated decamethonium dichloride (0·55 mg/kg). The cells shown have been sectioned at a distance of several mm from the end-plates. In this region there is a high grain density over the interspaces but little drug has entered the fibres. Incident light and dark-field illumination as in Pl. 1. Calibration 20 μ.

PLATE 6

Autoradiogram prepared from a diaphragm muscle which had been dippled in solution containing tritiated decamethonium dichloride. The grains are over the layer of frozen ice which adhered to the surface of the muscle, and the peritoneum is situated on the extreme left-hand side of the field. The black rectangle shows the size of the area from which grain counts were obtained, and the horizontal line indicates the orientation of the areas used for the series of counts. Upper calibration 20 μ.

PLATE 7

Autoradiogram prepared from transverse section of a diaphragm removed and frozen 90 min after I.V. injection of tritiated decamethonium dichloride (1·64 mg/kg). The white arrow shows the position of the esterase stain at the end-plate of a cell adjacent to the peritoneal border of the diaphragm. The grains are photographed in incident light so that the cell margins and other details are not displayed. The grain density is high over the muscle cells but falls rapidly at the edge of the tissue. The two arrows at the bottom edge indicate the position of the peritoneum. Calibration 20 μ.