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A HISTOCHEMICAL INVESTIGATION OF CHOLINESTERASES AT NEUROMUSCULAR JUNCTIONS IN MAMMALIAN AND AVIAN MUSCLE

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In 1953 Denz showed by biochemical and histochemical methods that acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) were apparently both present in the same motor end-plates of the rat diaphragm. However, Häggqvist (1960) has since reported that in some muscles of the rhesus monkey, AChE and BuChE occur separately in different end-plates; he found that AChE was localized in endings of the '*en plaque*' type and BuChE in endings of the '*en grappe*' type. This observation has obvious physiological implications, because the optimum substrate concentration for BuChE is higher than that for AChE (Mendel & Rudney, 1943); hence acetylcholine would tend to accumulate at endings from which AChE is absent. For this reason it seemed important to establish whether the two enzymes occur separately at neuromuscular junctions in other species. Some muscles from the rhesus monkey were examined for comparison. The activity of end-plate cholinesterase toward propionylthiocholine has also been tested.

There is a marked difference in form between the '*en plaque*' and the '*en grappe*' endings in some lower vertebrates, but the distinction is not always so clear in mammals (see Tiegs, 1953). For this reason these descriptions will not be used in this paper; instead, the term 'focal endings' will be used to denote those motor end-plates which occur singly (one per muscle fibre) and the term 'fine motor endings' will denote nerve terminals on muscle fibres with more than one motor ending.

METHODS

Muscles. The tissues studied were the tensor fasciae latae of the goat and guinea-pig, the gastrocnemius of the rabbit, the gastrocnemius and soleus of the rhesus monkey, the anterior and posterior latissimus dorsi of the domestic hen and the superior rectus oculi of all the above species. The goats and monkeys were killed with sodium pentobarbitone (Nembutal; Abbott Laboratories) and the other animals with ether; small pieces of muscle were removed immediately after death.

Histochemical method. In a few experiments fresh frozen sections were cut; but in most the tissues were fixed at 4° C for 4 hr in 10 % formalin in sodium sulphate solution (Na_2SO_4, 16·7 g/l.), then stored in 20 % alcohol at 4° C during the night and transferred to water for

1 hr before they were incubated. The total time in alcohol was usually less than 14 hr and in no case exceeded 20 hr.

In most experiments the staining method used was based on Lewis's (1961) modification of the Koelle (1950) technique. Pieces of fixed tissue were incubated for 1 or 2 hr at room temperature in an incubate at pH 5·5, in which the substrate concentration was 16 mM. The substrates used were acetylthiocholine (AcThCh) and butyrylthiocholine (BuThCh), both from Roche Products Ltd, and propionylthiocholine (PrThCh) kindly supplied by Dr Edith Heilbronn; all were obtained as the iodides. The iodine was precipitated with $CuSO_4$ during the preparation of the incubate. Di-isopropyl phosphorofluoridate (DFP) in concentrations of 10^{-7}, 10^{-6} or 10^{-5} M was used as inhibitor in some experiments. Tissues were soaked in acetate buffer (pH 6·5) containing the appropriate concentration of DFP for 30 min before incubation. DFP was also added to the incubate.

After incubation the tissues were placed for 6 hr in 10 % formalin in Na_2SO_4 with 0·5 % glacial acetic acid. They were then washed in distilled water and developed for 1 hr in a solution of sodium sulphide in 0·2 N acetic acid (1 g/45 ml.) pH 5·5. They were rinsed in several changes of water and finally stored in 20 % aqueous glycerol at 4° C. After 24 hr in glycerol, single fibres could be teased out and mounted in 'glychrogel' (Zwemer's).

In a few experiments fresh frozen sections 10–30 μ thick were mounted on slides and incubated in Lewis's incubate for periods of from 15 min to 2 hr. They were then rinsed in water and developed with Na_2S. In some other experiments fresh frozen and fixed frozen sections were treated with Koelle's (1950) incubate (substrate concentration 4 mM, pH 6·4) and developed with aqueous yellow ammonium sulphide (Koelle, 1950) or with alcoholic yellow ammonium sulphide (Bull, Lawes & Leonard, 1957).

RESULTS

In whole mounts treated by Lewis's method some cholinesterase activity could be demonstrated at nerve endings in all the muscles examined. Fresh or fixed sections treated by the same technique gave similar biochemical results, but morphological interpretation was not so easy as with whole mounts. In sections incubated in Koelle's medium staining was again obtained at neuromuscular junctions, but there was sometimes considerable diffusion of the stain. For these reasons all the pictures shown are taken from whole mounts treated by Lewis's method.

Mammalian muscles excluding superior rectus oculi. In all the mammalian muscles examined, with the exception of the superior rectus oculi, there was apparently only one (focal) motor nerve ending per fibre; the fibres of the muscle spindles were not examined. Plate 1 shows focal endings on fibres of the tensor fasciae latae of guinea-pig: *a, b* and *c* demonstrate the hydrolysis at the end-plate of acetyl-, propionyl- and butyrylthiocholine respectively. It was found consistently that the hydrolysis of BuThCh was less pronounced than that of AcThCh or PrThCh. Although it is difficult to illustrate this point convincingly with a photomicrograph it was always possible for an independent observer to distinguish between muscles incubated with BuThCh and those incubated with either of the other two substrates. Plate 1, *d–i* illustrates the effect of the inhibitor DFP on ChE activity. Hydrolysis of AcThCh and PrThCh was virtually unaffected by 10^{-6} M DFP (*d* and *e*) and a

concentration of 10^{-5} M was necessary to produce inhibition in the majority of endings (*g* and *h*). This is evidence that hydrolysis of both compounds was due predominantly to AChE. Activity towards BuThCh, on the other hand, was appreciably inhibited by 10^{-7} M DFP (*f*) and almost completely blocked by 10^{-6} M DFP (*i*); these results indicate that this substrate was hydrolysed by BuChE.

There is evidence therefore that both true- or AChE and pseudo- or BuChE are to be found in this type of end-plate, although the activity of the BuChE may be lower than that of AChE. The present method does not allow the successive demonstration of hydrolysis of the different substrates in one and the same end-plate (cf. the method of Klinar & Zupančič, 1962). However, the results give reasonable assurance that the two esterases are in fact present together in the same endings, since the number of endings staining with the three substrates was apparently equal. When, for example, adjacent muscle samples, each containing end-plates from the same bands, were incubated in the different substrates, the end-plate population shown up in any one sample was very similar to that in the neighbouring samples, irrespective of the substrate.

The shape of the endings stained for ChE in tensor fasciae latae of the goat and in gastrocnemius of the rabbit was slightly different from that in guinea-pig tensor fasciae latae, but in both these muscles the behaviour of the cholinesterases at the ending towards the substrates and inhibitors was exactly as described above.

The muscles taken from the rhesus monkey, the soleus and the gastrocnemius hydrolysed AcThCh and BuThCh equally; hydrolysis of PrThCh was not tested. Plate 2, *a* and *b*, shows that there is little, if any, difference in the activity towards BuThCh in the two muscles and that morphologically the endings are also very similar to one another.

Avian muscle, excluding superior rectus oculi. Plate 2, *c–e*, shows the nerve endings in posterior and anterior latissimus dorsi of the hen. In the posterior latissimus dorsi only one ending (focal) was present on each fibre but in the anterior latissimus dorsi fine motor endings occurred at frequent intervals along the length of the fibres (*e*). These findings are in agreement with those of Ginsborg & Mackay (1961). As can be seen from *c* the muscle fibres in posterior latissimus dorsi were of greater diameter and the endings were less compact than those in the mammalian muscles (cf. Pl. 1, *a*, *b* and *c*) but even so they resembled the latter endings in that the various branches joined to form a unified structure. The fine endings on anterior latissimus dorsi on the other hand were rather different (Pl. 2, *d* and *e*). They consisted of collections of small discrete structures, round or oval in shape, and no connecting structures were seen.

The cholinesterase in both types of ending hydrolysed AcThCh and

PrThCh, but it was not possible to demonstrate activity towards BuThCh in either posterior or anterior latissimus dorsi of this species. DFP affected the activity towards AcThCh and PrThCh equally. At a concentration of 10^{-6} M DFP caused some depression of the activity, and this was more marked than that produced by 10^{-6} M DFP in mammalian endings; at 10^{-5} M, there was complete inhibition. Since it has been established in this laboratory that AChE of the bird is more readily inhibited by DFP than is the mammalian enzyme, these results indicate that AcThCh and PrThCh are hydrolysed by AChE, and that BuChE is probably absent from these muscles or, if present, is in a comparatively low concentration.

Mammalian and avian superior rectus oculi. In the superior rectus oculi of all the species examined both singly- and multiply-innervated muscle fibres were present. AcThCh, PrThCh and BuThCh were hydrolysed at both the focal and the fine endings, and DFP produced the same effect on ChE activity at the two sites. It appears therefore that the same types of cholinesterase are present in both sorts of ending. It should be emphasized here that in contrast to the posterior and anterior latissimus dorsi of the hen, in which hydrolysis of BuThCh could not be demonstrated, marked activity towards BuThCh was found in the superior rectus oculi of this species. Another point to note is that in the superior rectus oculi of all species the difference between the activity exhibited towards AcThCh and that towards BuThCh was less than in the other muscles of the same species previously described; in some experiments there appeared to be no difference at all (Pl. 3, *a* and *b*) and this was true of both fine and focal endings.

The morphology of the endings as revealed by the histochemical stain varied somewhat from species to species. In the goat the focal end-plates on the singly-innervated fibres were branched structures fairly compactly arranged; the fine endings were rather similar, though less compact (Pl. 3, *d* and *e*). They were sometimes smaller and sometimes larger than the focal end-plates and occurred along the fibre at intervals of approximately 100–300 μ (Pl. 3, *f*). The similarity in form between the focal and fine endings was also seen in the superior rectus oculi of the hen. In the rabbit, however, there were marked differences between the focal end-plates and the fine endings. The contrast between the two types of ending is shown in Pl. 3, *b* and *c*. Hess (1961*a*) showed similar differences in the two types of ending in the extraocular muscles of the guinea-pig; these were confirmed in the present work.

DISCUSSION

These experiments have shown that in all the mammalian species examined hydrolysis of both AcThCh and BuThCh can occur at neuro-

muscular junctions. The points at issue are whether these two substrates are hydrolysed by the same or different enzymes, and if the latter, whether these enzymes occur together in each end-plate or whether, as suggested by Häggqvist (1960), some endings have one enzyme and some another.

Previous workers (see Whittaker, 1951) have shown by biochemical methods that in a variety of species AChE, in contrast to BuChE, has comparatively little affinity for butyrylcholine compounds. For instance, Mounter & Whittaker (1950) have shown that the rates of hydrolysis of acetylcholine (ACh) and butyrylcholine (BuCh) by AChE in horse erythrocytes are in the ratio of 100:1·5. On the other hand in many species pseudocholinesterase hydrolyses BuCh more than 100 times faster than ACh (Myers, 1953). It is not possible to make any but a very rough quantitative assessment of enzyme activity in histochemical preparations; nevertheless the degree of hydrolysis of BuThCh at mammalian end-plates, particularly in the extraocular muscles, does suggest the presence of BuChE and so does the finding that activity towards BuThCh is considerably depressed by 10^{-7} M DFP while 10^{-6} M DFP has little effect on the activity towards AcThCh.

In contrast to the finding that in mammalian muscle BuThCh was hydrolysed at all endings, hydrolysis of BuThCh could not be demonstrated in either anterior or posterior latissimus dorsi of the hen. This does not agree with the findings of Hess (1961*b*) who reported that endings in both muscles were revealed when BuThCh was used as the substrate in a modified Koelle's medium. Since BuChE activity in endings in the superior rectus oculi of the hen could be demonstrated very clearly in the present work by Lewis's method, the discrepancy in these results cannot be due simply to differences in the method; at present there seems no obvious explanation.

It has been known for some time that true ChE of the hen has a greater affinity for propionylcholine than for acetylcholine (Myers, 1953). Recently, Blaber & Cuthbert (1962) showed that hen pseudocholinesterase is also unlike its mammalian counterpart and has properties intermediate between those of mammalian butyrylcholinesterase and avian acetylcholinesterase. It was because of such evidence that the affinity for PrThCh of ChE at end-plates in hen muscle was tested. Experiments showed that with this substrate endings in anterior and posterior latissimus dorsi and superior rectus oculi of the hen could be stained; they could also be stained in all the mammalian muscles which were tested for comparison. It is not clear, however, whether PrThCh was attacked by both AChE and BuChE. There was good evidence that AChE split this substrate, since it was hydrolysed in the presence of DFP in a concentration which would inhibit all BuChE activity but leave AChE still active. On the other hand, in the absence of

specific inhibitors of true ChE it was not possible to assess the degree of hydrolysis of PrThCh which might be attributable to BuChE alone. One might hope to see a quantitative difference after inhibiting BuChE, but as already explained it is not possible to make very accurate estimates of enzyme activity in histochemical preparations. Nevertheless, it would appear from these experiments that true cholinesterase at nerve endings in mammalian as well as in avian muscle has about an equal affinity for PrThCh and AcThCh. This result agrees with the findings of Nachmansohn & Rothenberg (1945) who reported that true ChE from various tissues had about an equal affinity for acetyl- and propionyl-choline.

The results of all those experiments, in which the three substrates have been used with or without DFP, suggest that both true- or acetylcholine-sterase and pseudo- or butyrylcholinesterase do occur in end-plates. It remains to be established whether they are present together or separately; unfortunately it is difficult to get direct evidence on this point. Klinar & Zupančič (1962), who studied this problem in cat muscles, used a method in which they first incubated tissue with AcThCh and a reversible inhibitor of BuChE and photographed the result. After the sections had been well washed they re-incubated them with BuThCh and an inhibitor of AChE and re-photographed the same field as before. In other experiments they reversed the order of their incubations. In every experiment they found that while the second photograph of a pair showed more intense staining than the first, none showed any additional end-plates. They interpreted this as meaning that each end-plate examined contained both enzymes. While this may in fact be true, the method does not provide unequivocal support for this conclusion. The authors do not specify if the staining was developed between the incubations but it would appear from the pictures that this was so. The possibility remains therefore that enzyme which was not active under the conditions of the first incubation might have become inhibited by the development process and would not therefore have been shown up by the second incubation. The greater depth of staining observed after the second incubation could result from further chemical development of the products of the first incubation.

The present experiments do not demonstrate directly the simultaneous presence of the two enzymes in individual end-plates. Nevertheless, the observation that in samples of the same mammalian muscle there was no obvious difference in the number of end-plates stained whether AcThCh or BuThCh was used as substrate does suggest that the majority of endings contain both cholinesterases. Even though the results can provide only indirect evidence for the simultaneous occurrence of the two enzymes in mammalian endings it is clearly established that AChE and BuChE are found in both focal and fine (multiple) nerve endings. There is no evidence

that in the species studied focal (*en plaque*) endings contain only AChE and fine (*en grappe*) endings contain only BuChE. The possibility remains that although both enzymes may be present together, the first may be physiologically more important in one sort of ending and the second in another. There may even be some factor which is independent of end-plate morphology which determines the presence and relative importance of the two enzymes. Thus, in the hen, neither the focal end-plates in posterior latissimus dorsi, nor the fine endings in anterior latissimus dorsi, apparently contain BuChE. On the other hand, in several species, including the hen, BuThCh showed up focal and fine endings on superior rectus oculi equally clearly and in both sorts of ending the reaction to BuThCh was, as far as could be judged, as strong as to AcThCh.

Since completing this work my attention has been drawn to the experiments of Pecot-Dechavassine (1962) on cholinesterases in fish, amphibian and mammalian muscles. Much of her work is concerned with tissue homogenates, but her histochemical findings in the baboon are in agreement with the histochemical results from mammalian muscles reported in the present study.

SUMMARY

1. The cholinesterases present at neuromuscular junctions have been studied histochemically in muscles from the goat, guinea-pig, rabbit, rhesus monkey and domestic hen.

2. In mammalian muscle acetylcholinesterase and butyrylcholinesterase were both present in the single, focal end-plates and in the fine endings on fibres with more than one ending.

3. In the domestic hen acetylcholinesterase was found in all endings examined. Butyrylcholinesterase was present in both focal and fine endings in superior rectus oculi, but it could not be demonstrated in either posterior or anterior latissimus dorsi.

4. Hydrolysis of propionylthiocholine occurred at endings in all muscles examined from the goat, guinea-pig, rabbit and hen.

5. It is suggested that acetylcholinesterase and butyrylcholinesterase are probably present together in most endings; there is no evidence to support the view that one morphological type of ending contains one enzyme and the second type of ending another.

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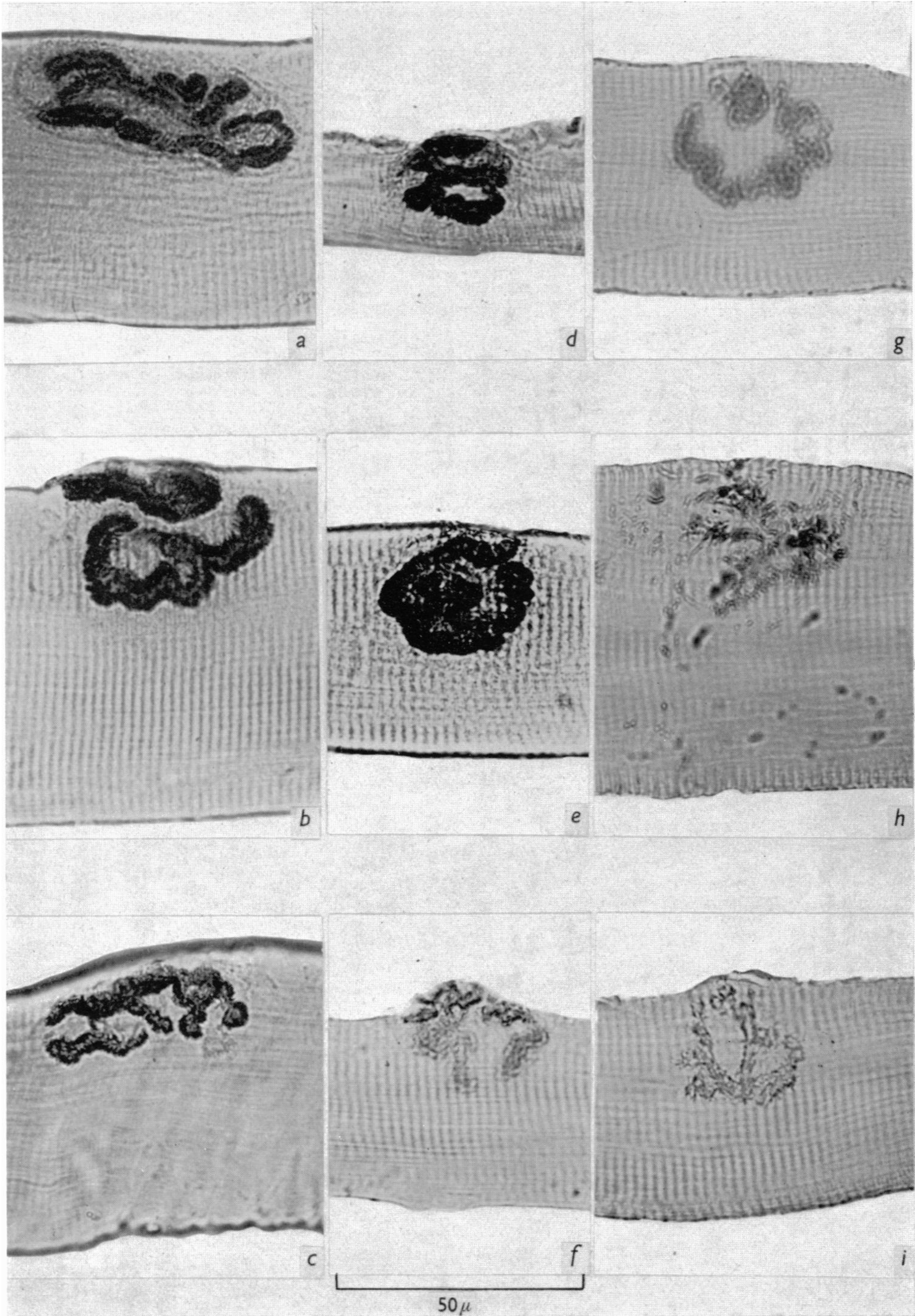

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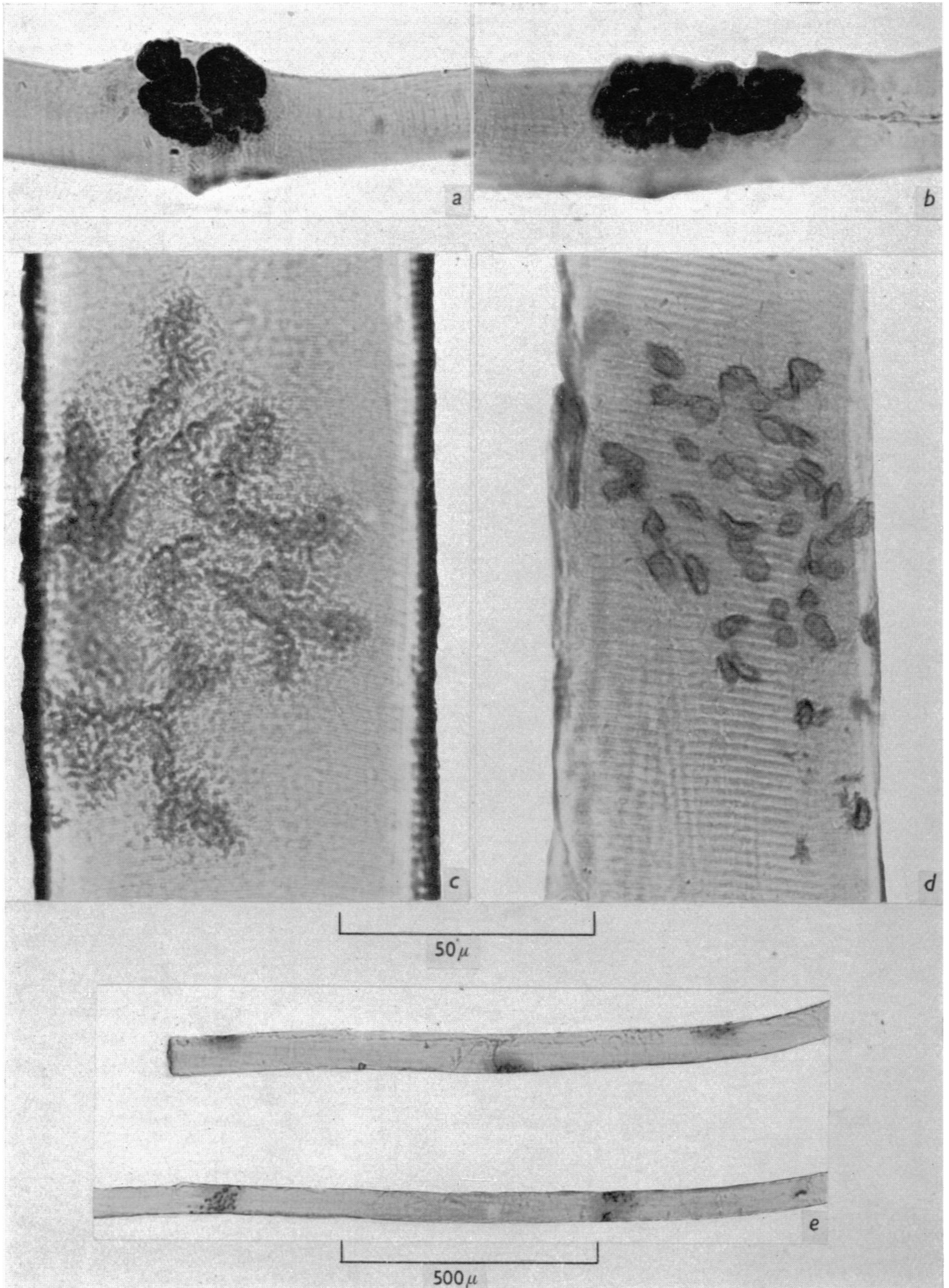

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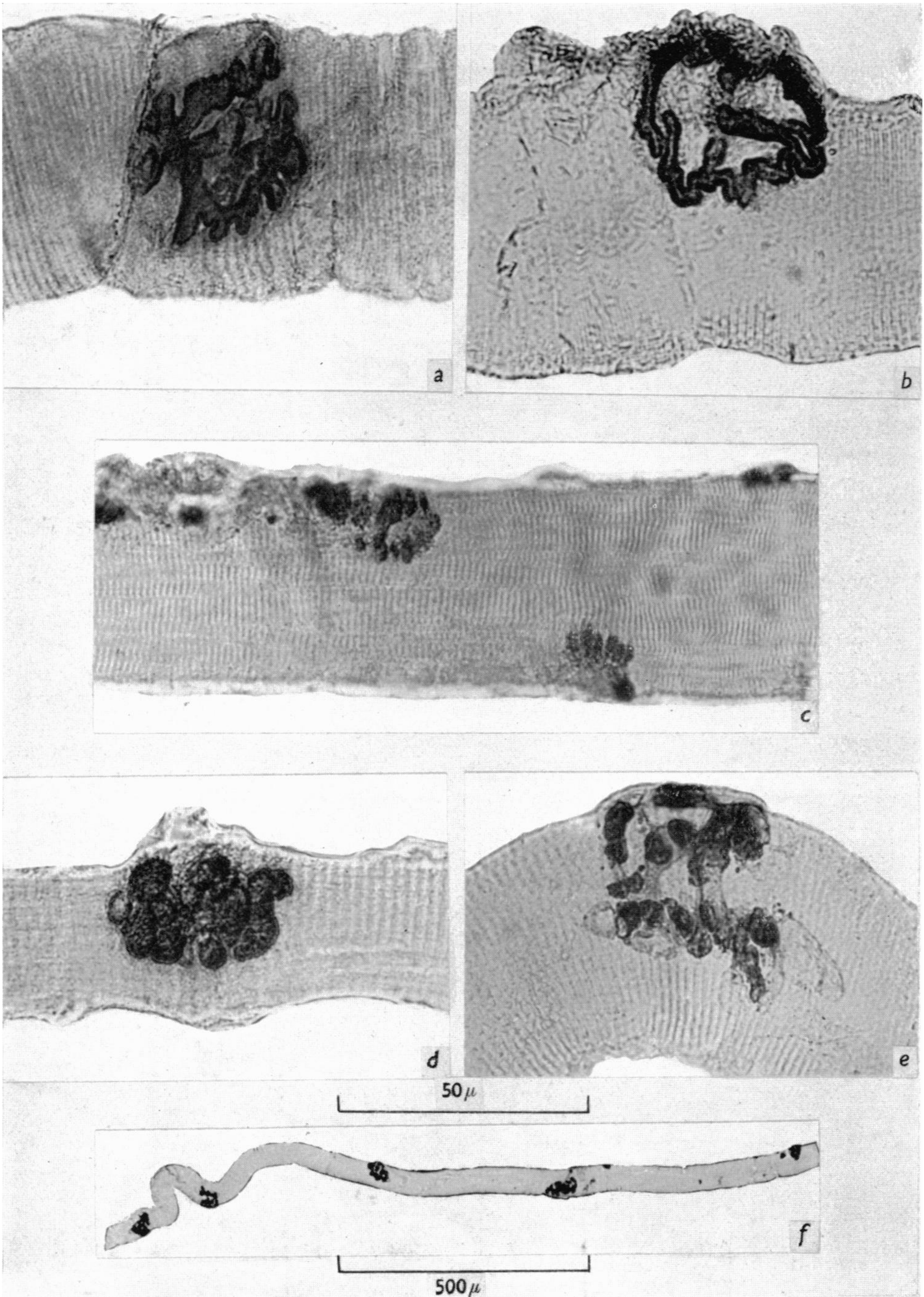

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EXPLANATION OF PLATES

PLATE 1

Guinea-pig tensor fasciae latae. Whole mounts of muscle fibres.

a, *b*, *c*. Staining of end-plates following incubation with AcThCh, PrThCh and BuThCh respectively. *d*, *g*. Staining of end-plates following incubation with AcThCh after treatment with 10^{-6} M DFP and 10^{-5} M DFP respectively. *e*, *h*. Staining of end-plates following incubation with PrThCh after treatment with 10^{-6} M DFP and 10^{-5} M DFP respectively. *f*, *i*. Staining of end-plates following incubation with BuThCh after treatment with 10^{-7} M DFP and 10^{-6} M DFP respectively.

PLATE 2

a, *b*. Monkey soleus and gastrocnemius respectively. Staining of end-plates following incubation with BuThCh. *c*, *d*. Hen posterior and anterior latissimus dorsi respectively. Staining of endings following incubation with PrThCh. *e*. Distribution of fine endings on hen anterior latissimus dorsi. AcThCh as substrate.

PLATE 3

a, *b*. Rabbit superior rectus oculi, focal endings. Staining of end-plates following incubation with AcThCh and BuThCh respectively. *e*. Rabbit superior rectus oculi, fine endings· Staining of endings following incubation with BuThCh. Note: magnification as in *a*, and *b*. *d*, *e*. Goat superior rectus oculi, focal and fine endings respectively. Staining of endings following incubation with PrThCh. *f*. Goat superior rectus oculi, fine endings; PrThCh as substrate. Note: low-power magnification.