

CHOLINESTERASE IN DENERVATED END PLATES AND MUSCLE FIBRES

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ABSTRACT

Parallel studies were made of cholinesterase activities and localizations in denervated rat and rabbit gastrocnemius muscle. Koelle's histochemical reaction was used for demonstrating the localization of cholinesterases. Enzyme activities in whole sliced muscle were measured by electrometric titration. The Cartesian ampulla-diver technique was used for cholinesterase activity determinations in end plate regions or in small pieces of the muscle fibre itself. No changes in the activity of cholinesterases (ChE) were found in the whole denervated muscle which would account for its chemical supersensitivity. The ChE distribution pattern was changed so that the end plate region became less active in the denervated muscle than in the normal one. The decrease in ChE activity in the end plates seems to be largely compensated for by an increase of this enzyme elsewhere in the muscle. A possible connection between the spatial spread of cholinesterase activity and the enlargement of the acetylcholine-sensitive surface is discussed.

INTRODUCTION

Numerous attempts have been made to correlate the quantitative changes in the activity of the cholinesterases (ChE¹) of the denervated muscle with the increased sensitivity to acetylcholine (ACh).

Biochemical measurements of ChE performed on whole homogenized or sliced denervated muscle (5, 10, 13, 14, 19, 23, 24, 26, 27, 29, 35, 38), as well as on a single end plate region isolated from the denervated muscle (6, 7), do not offer a clear picture of the changes in ChE activity after denervation. Certain discrepancies between the results obtained by different authors might be partially ascribed to the fact that different animals,

different muscles, and different techniques have been employed.

Histochemical methods for ChE seem to yield more uniform results concerning changes of this enzyme in the end plate of the denervated muscle (3, 4, 11, 12, 21, 33, 34). All workers agree that there is a remarkable decrease in the ChE reaction in the end plates after denervation. There are some histochemical data available suggesting an increase in ChE activity in the denervated muscle fibre outside the end plate region (6, 19, 31, 38).

Recently, a group of authors has pointed out the parallelism between the supersensitivity of various normal and denervated muscles and their high specific activity of the so-called myosin ChE, which according to these authors is part of the contractile structure (19, 20, 38).

Since separate determinations of butyrylcholinesterase (BuChE) after muscle denervation are scanty, the present writers have studied this

¹ The abbreviation ChE is used when no distinction is made between aceto-cholinesterase and butyrylcholinesterase, so that it means in general the total cholinesterase activity when acetylcholine is used as substrate.

enzyme, using butyrylcholine (BuCh) and butyrylthiocholine (BuThCh) as substrates.

The aim of this work has been to investigate, in parallel experiments, on the same denervated muscle, the histochemical localization and the activity of cholinesterases in whole muscle and in isolated muscle fibres or end plate regions. The data obtained might throw light upon the question as to whether ChE plays a role in the supersensitivity of denervated muscle.

MATERIALS AND METHODS

Material

The experiments were performed on albino rats (120 to 150 gm) and rabbits (3000 to 4000 gm). Animals of both sexes were used.

With the animals under ether anaesthesia, the right sciatic nerve was cut close to the spinal cord and a length of the nerve, at least 1 cm, was removed. The animals were killed, from 1 to 13 weeks after denervation, by means of ether (rats) or by bleeding under ether anaesthesia (rabbits). Immediately after death the gastrocnemius muscles from the right (denervated) and the left (normal) leg were isolated, the latter muscle serving as the control.

For ChE activity measurements and for qualitative histochemistry, three or more animals were used at each time point after denervation. Each separated muscle was subjected to both tests. For the measurements of ChE activity in the whole denervated muscle, the muscles of 6 to 9 animals were sliced, mixed, and divided in aliquots for at least 3 parallel runs.

In rats as well as in rabbits, muscle atrophy became apparent approximately 2 weeks after denervation and reached its peak by the 5th to 6th week. The difference between the weight of the denervated and that of the normal muscle was taken into account in the calculation of the total ChE activity (Tables I and II, columns *b*).

Activity Measurements

The ChE activity of the whole sliced muscle was measured by continuous electrometric titration, using ACh or BuCh as substrate. The preparation of samples and other details of the procedure were described previously (9).

In one set of experiments, pieces of muscle fibre and end plate regions were isolated, and ChE activities were measured microgasometrically by the ampulla diver method (7, 40). The reduced weight of some samples was determined by means of a sensitive Cartesian diver balance (8, 42).

To determine the ACh/BuCh splitting ratio of a normal or of a denervated end plate region of rat

gastrocnemius, measurements were performed microgasometrically on the same tissue sample and in the same ampulla diver for both substrates in succession. As soon as one measurement with one of the substrates was accomplished, the diver was opened (41) and emptied. The sample and the diver were thoroughly rinsed with the substrate to be used in the following run. Subsequently, the diver was charged, sealed, and manipulated according to the usual ampulla diver procedure.

The concentrations of the substrates were 3×10^{-3} M for ACh and 1×10^{-2} M for BuCh. In control runs, eserine to the concentration of 10^{-3} M was added to the reaction mixture.

Histochemical Procedure

Frozen, unfixed slices (10 to 80 μ thick) were used. The slices cut from the denervated and those from the normal muscle were treated in exactly the same manner.

Essentially, the simplified technique of Koelle was followed (17). The pH of the incubation medium was 6.2. The concentration of acetylthiocholine-iodide (AThCh) was 4.8×10^{-3} M and that of BuThCh was 10^{-2} M. The slices were exposed to the incubation medium for 30 or 60 minutes. The changes in ChE localization after denervation can be visualized best after a long incubation time. For comparison, the normal muscle was incubated for the same long period in spite of the fact that over-reaction obscures the fine structure of the end plate. The differentiation between ChE and ali-esterases was made by adding eserine, 10^{-4} M.

RESULTS

The changes in activity and in localization of ChE, which occur after denervation, were found to be different in the two animal species used in this work.

Rat Gastrocnemius Muscle

A. ChE ACTIVITY MEASUREMENTS

Table I shows the ChE activity of the whole sliced denervated muscle. Columns *a* present the ChE activities of 1 gm of denervated muscle expressed in per cent of the activity in 1 gm of normal muscle. In columns *b*, the atrophy (column *c*) of the denervated muscles was taken into account by multiplying the values of columns *a* by the values of column *c*. Thus, the values in columns *b* are relative measures of the changes in the enzyme activity of the whole muscle after denervation.

When ACh was the substrate, the activity

per gram of denervated muscle first decreased, then increased. The activity per whole muscle decreased steadily. With BuCh as the substrate, the ChE activity in denervated muscles increased both in concentration (columns *a*) and in total amount per degenerating muscle (columns *b*).

In experiments with isolated motor end plate regions, the splitting ratio between ACh and BuCh was determined about 4 weeks after denervation (Table II). As a consequence of the denervation the ACh/BuCh splitting ratio decreased, thus changing in favour of BuCh.

TABLE I
Denervated Rat Gastrocnemius Muscle
Splitting rates of ACh and BuCh at different times after denervation

Weeks after denervation	ChE activity (% of the normal muscle)				Ratio of weights denervated/normal muscle
	<i>a</i>		<i>b</i>		
	ACh	BuCh	ACh	BuCh	<i>c</i>
1	98	108	92	101	0.94
2	83	154	57	106	0.69
5	106		55		0.52
6		334		137	0.41
7	140	279	56	111	0.40

B. HISTOCHEMICAL RESULTS

On the 14th day after denervation the histochemical reaction of the motor end plates was clearly subnormal in extent, irrespective of whether AThCh (Figs. 1 *A* (control) and 1 *B* (denervated)) or BuThCh (Figs. 1 *C* (control) and 1 *D* (denervated)) was used as the substrate. The end plates were still detectable as late as 7 weeks after denervation. Simultaneously with the decrease in the staining reaction of the end plates, a copper sulfide precipitate appeared on the muscle fibres in the form of broken bands or twigs (Figs. 1 *B* and *D*). In all probability, the elements that stain are fragments of subterminal nerve endings and/or of terminal arborisations. The reaction was more pronounced with BuThCh than with AThCh. In the normal control muscle no such staining could be demonstrated (Figs. 1 *A* and *C*). The muscle fibres themselves, of normal or dener-

vated muscles, showed no histochemical ChE reaction under the conditions described above.

Rabbit Gastrocnemius Muscle

A. ChE ACTIVITY MEASUREMENTS

In the experiments with whole sliced muscles, with ACh as the substrate, a slight decrease in the enzyme activity was noticed by the end of the 1st week after denervation (Fig. 3, curve *I*). At 2 weeks a new increase was apparent. It reached a maximum of 5 times the normal value at 6 weeks

TABLE II
Isolated End Plate Region of Denervated Rat Gastrocnemius Muscle
ACh/BuCh splitting velocity ratio at 4 weeks after denervation

	Muscle fibers isolated from:	
	Normal muscle	Denervated muscle
	4.5	1.2
	5.5	2.1
	8.8	
	5.0	3.0
		1.8
	7.5	3.2
	8.4	2.7
Mean ± s.e.	6.5 ± 0.75	2.3 ± 0.31
s.e. diff. =	±√0.75 ² + 0.31 ² = ±0.811	

The difference between the means ± standard error difference = 4.2 ± 0.811.

after denervation, and then began to decrease. While curve *I* compares with columns *a* in Table I (rat data), curve *II* compares to columns *b* in the same Table. This curve shows that the trend mentioned for curve *I* is refound also when correction is made for the developing atrophy of the denervated muscle.

Table III shows the activity of ChE of the whole denervated muscle and should be compared to Table I for the rat. However, developments were followed only during the phase of decreasing enzyme activities beginning at 6 weeks after denervation. ACh and BuCh activities follow the same course, only the latter are on a lower level than the former.

In the normal muscle, the enzyme activity, as measured by the Cartesian diver (8), using ACh

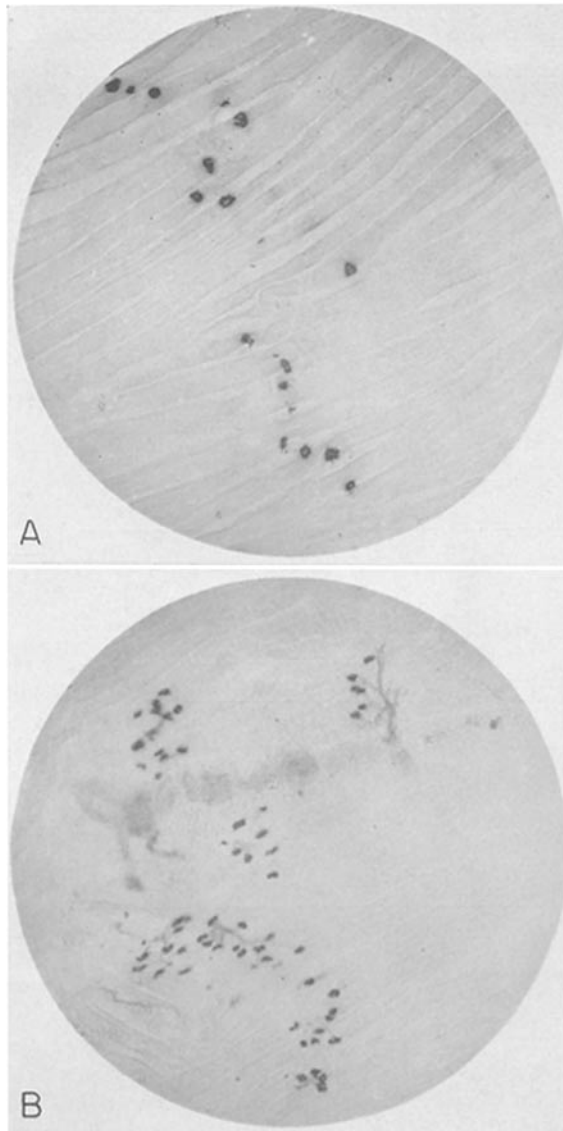


FIGURE 1 Photomicrographs of normal and denervated rat gastrocnemius muscle. $20\text{-}\mu$ sections. $\times 40$.

A, normal, substrate AThCh, incubation time 30 minutes. The histochemical reaction is localized on the end plates.

B, 28 days after nerve section, substrate AThCh, incubation time 30 minutes. The histochemical reaction of the end plates is subnormal in extent; outside the end plates a deposit of copper sulfide in the form of broken bands or twigs can be seen.

as the substrate, was localized in the end plate, while the muscle fibre itself was found to be inactive under the same experimental conditions (Brzin, unpublished). On the other hand, as shown in Table IV, ChE activity was traced along the whole length of the denervated muscle fibre (at 6 weeks after denervation). The activity per unit reduced weight varied from sample to sample without relation to the presence or absence of end plates. Clearly, the enzyme is unevenly distributed when different muscle fibres are compared. The data do not yet permit statements

concerning the finer pattern of distribution along the single fibre. The important fact remains that samples of denervated muscle fibres carrying end plates showed no higher ChE activity than those devoid of end plates.

B. HISTOCHEMICAL RESULTS

The extent of the histochemical reaction of the end plate ChE of rabbit muscle was clearly lowered at 1 to 2 weeks after denervation, irrespective of whether AThCh or BuThCh was used as substrate. After 4 weeks, the picture seen in Fig. 2

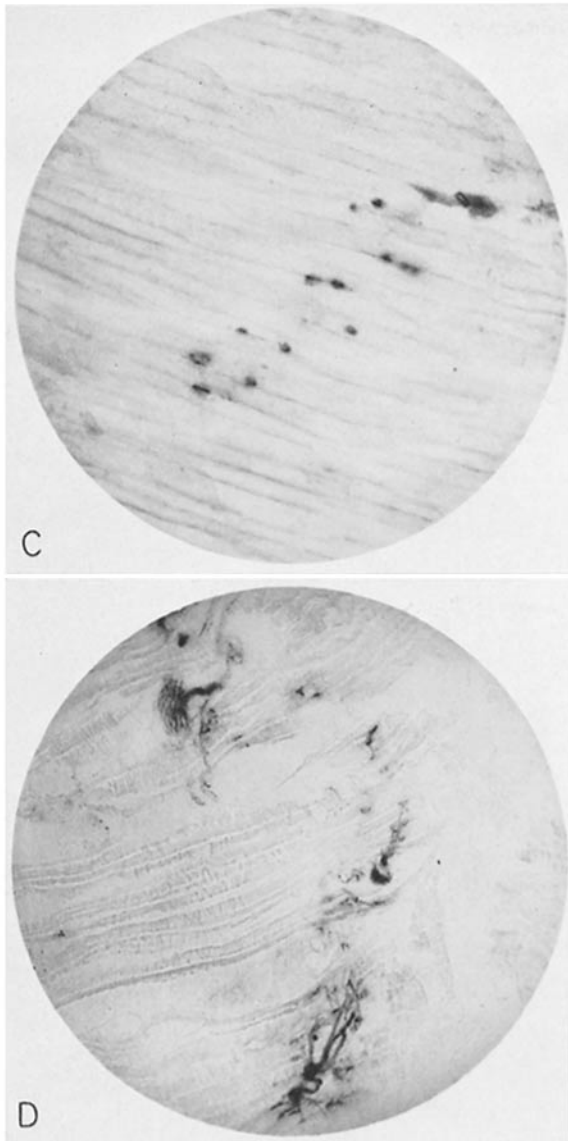


FIGURE 1 (*cont.*)

C, normal, substrate BuThCh, incubation time 60 minutes. The histochemical reaction is localized on the end plates.

D, 14 days after nerve section, substrate BuThCh, incubation time 60 minutes. Only traces of end plates can be seen; the deposit of copper sulfide in the form of twigs is clearly visible.

A and *B* is obtained. However, when by the end of the 13th week (AThCh) and by the end of the 7th week (BuThCh) after denervation the experiments were discontinued, ChE activity could still be traced. As demonstrated in Fig. 2 *B*, at 4 weeks after the denervation there is pronounced ChE activity outside the end plates. The muscle fibres were unequally stained with a deposit of copper sulfide of varying intensity. This phenomenon became clearly visible about 2 weeks after the denervation, and it grew more and more pronounced within the following period of obser-

vation. There was a quantitative difference between the reactions in which AThCh was the substrate and those in which BuThCh was used as the substrate, the reaction being prompter and more intensive in the former.

In order to prove that the histochemical reaction along the muscle fibre was not an artefact due to diffusion of thiocholine, the areas carrying end plates and those without them were separated by cutting, and they were then stained on the same slide. The sample showed essentially the same distribution pattern of ChE activity as was ob-

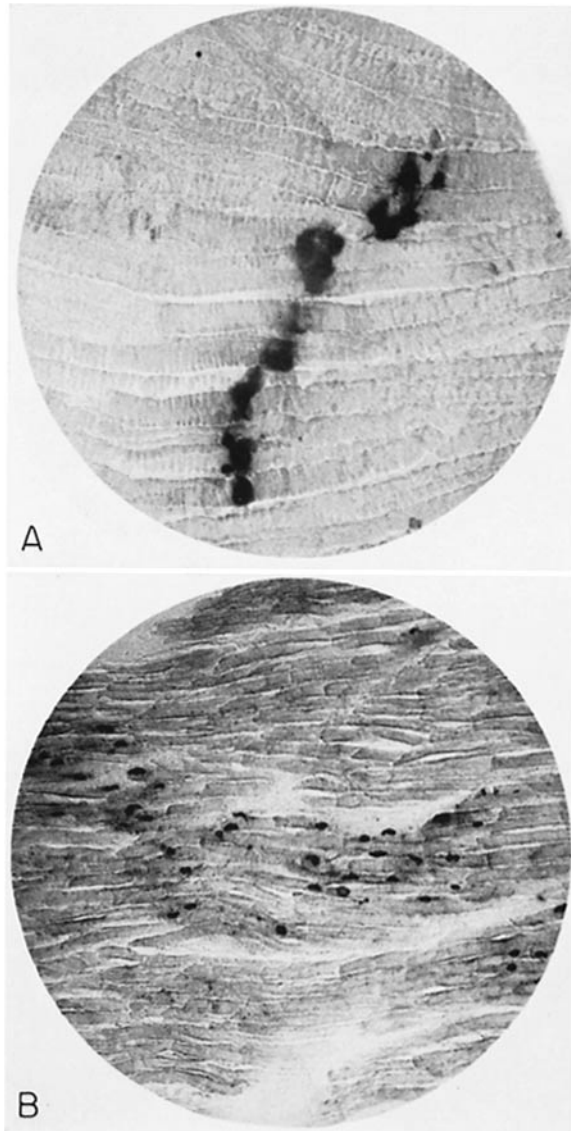


FIGURE 2 Photomicrographs of normal and denervated rabbit gastrocnemius muscle. 20- μ sections. $\times 40$.

A, normal, substrate AThCh, incubation time 60 minutes. There is a pronounced over-reaction and diffusion of reaction product from end plates to adjacent areas owing to prolonged incubation time; the muscle fibres themselves show no histochemical reaction.

B, 28 days after nerve section, substrate AThCh, incubation time 60 minutes. The histochemical reaction of the end plates is clearly subnormal in extent; the atrophied muscle fibres are unequally stained with a deposit of copper sulfide.

served when the histological section was not cut in two. This agrees with the quantitative measurements shown in Table IV. In the normal adult gastrocnemius muscle no ChE activity could be histochemically demonstrated outside the end plates.

DISCUSSION

The data contained in the literature referred to in the Introduction and the results of the present work show that the chemical supersensitivity of a denervated muscle is not necessarily accompanied

by a decrease in the total ChE activity. Supersensitivity develops even when there is a considerable rise in the ChE activity. The quantitative measurements demonstrate that the ChE activity (substrate: ACh) in the whole gastrocnemius muscle may either decrease or increase after denervation, depending on the animal species used. On the other hand, the ChE activity when measured with BuCh as substrate seems to be slightly increased in the rat muscle and it is definitely increased in the rabbit muscle.

The ACh/BuCh splitting ratio of the end plate

region of a rat gastrocnemius tetanic muscle fibre, measured by microgasometry, changes after denervation in favour of BuCh (Table IV). The ratio approaches the values found for a normally innervated tonic muscle fibre from the same muscle (unpublished data). Whether or not there is a causal connection between the tonic character of a muscle and its relatively high BuChE content is at present obscure. The fact remains that BuChE is widely distributed in animal tissues according to a pattern which suggests correlation between this enzyme and a number of functional properties of the contractile tissues (30). This

region of the gastrocnemius muscle exhibits 30 to 50 per cent of the normal ChE activity. In the rabbit, at 5 to 6 weeks after denervation, the end plates cannot easily be demonstrated by histochemistry. Still, the corresponding figure seems to be even higher than in the rat and mouse (*cf.* Table IV). This apparent discrepancy between histochemistry and microgasometry might be explained by the suggestion that, in all cases, after denervation the decrease of the ChE activity in the end plate is compensated for, only to different degrees, by an increase in ChE activity of the surrounding muscle fibre which always

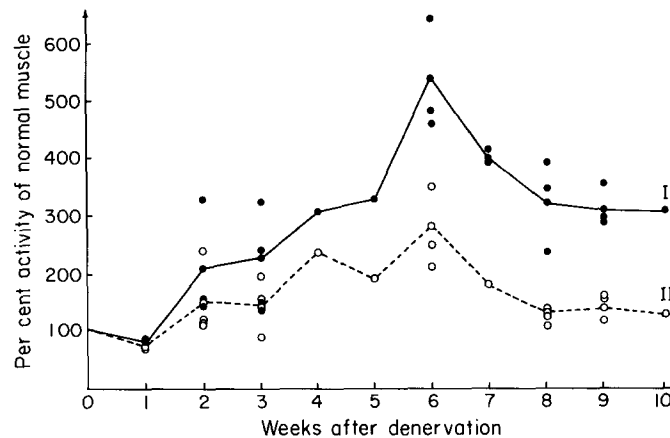


FIGURE 3 Changes in cholinesterase activity of denervated rabbit gastrocnemius muscle at different times after denervation.

I, activity per gram muscle.

II, relative activity per muscle.

localization, together with the kinetic properties of the enzyme and its inhibition by specific drugs, suggests that the active groups of BuChE are chemically similar to (18, 37), or that BuChE is even identical with (25), Langley's ACh receptor (22) of certain tissues. Even then, the role of BuChE in the denervated muscle remains obscure.

It is commonly believed that the ChE activity of a normal gastrocnemius muscle is mostly localized in the end plates and that this activity represents an important contribution to the enzyme activity of the whole muscle. It seems significant, therefore, that there is always a decrease in the ChE activity of the end plates after denervation, regardless of a decrease or increase in the muscle as a whole.

At 5 to 7 weeks after denervation, when in the rat and in the mouse the end plate can hardly be histochemically visualized, the isolated end plate

adheres to our end plate preparations as they are used for microgasometry. Whereas in the denervated rabbit muscle increased activity of ChE could be demonstrated in the myofibre by the histochemical reaction (Fig. 2), this is not quite so for the rat muscle. In this case, too, there is a compensatory extrajunctional increase in ChE activity after denervation, but apparently the increase is confined to nerve endings which ramify outside the end plate and on the surface of the fibre (Fig. 1 *D*). In the case of the rat, the question remains as to whether, in fact, all the extrajunctional ChE (substrates ACh and BuCh) is localized to the suggested nerve endings, or whether it will be necessary to first assume, then demonstrate, ChE in the muscle fibre proper. Improved histochemical, or microgasometric and isolation, techniques may then be required.

The spatial changes in enzyme distribution

after denervation rather than the total ChE activity of the denervated muscle might have a bearing on the muscle's chemical supersensitivity. High sensitivity to ACh and tonic muscular characteristics often seem to be accompanied by increased ChE activity on, or in, the muscle fibre outside the neuromuscular junction. Such is the case not only in the denervated muscle (in which the nerve connection is degenerated) but also in the foetal muscle (in which connection to the nerve has not yet been established (15)).

It is known that tonic muscles are more sensitive to ACh than are tetanic muscles. At the same time, in the rat the end plate region of a tonic (red)

TABLE III
Denervated Rabbit Gastrocnemius Muscle

Changes in splitting rates of ACh and BuCh at various times following denervation

Weeks after denervation	ChE activity (% of the normal muscle)				Ratio of weights denervated/normal muscle	
	a		b			c
	ACh	BuCh	ACh	BuCh		
6	690	460	379	253	0.55	
7	410	370	184	166	0.45	
8	387	255	166	110	0.43	
9	290	437	145	219	0.50	
10	312	310	131	130	0.42	

fibre shows a lower ChE activity than does the end plate region isolated from a tetanic (white) fibre (8). However, the ChE activity of the whole tonic muscle is higher than that of the tetanic muscle (10, 20). Therefore, in the two types of muscles a different distribution of these enzymes on the end plate and on the muscle fibre is suggested. Outside the neuromuscular junction a tonic muscle fibre must be richer in ChE than is a tetanic muscle fibre. This agrees with the early observation (10, 16) that high ACh sensitivity of a normal or denervated muscle is correlated with high ChE activity and high ACh content; furthermore, it lines up with the observation that high ACh sensitivity is accompanied by a considerable increase of the area over which each muscle fibre is sensitive to ACh. In the light of these findings, Ginezinsky (16) postulated that the receptor for

ACh should be in the form of a muscle fibre particle which embodies both ACh and ChE. Ginezinsky's results concerning the spreading of the sensitive area after denervation have recently been confirmed by more refined procedures (1, 36). Miledi (28) holds that, in the light of this evidence, the supersensitivity phenomenon can be satisfactorily explained by the expansion of the sensitive area. He furthermore suggests that other denervation phenomena, including also changes in ChE concentration, can, at the most, play a minor part. However, it is now difficult to disregard the evidence that the spread of the chemically sensitive area in a denervated muscle tends to be paralleled by a spread in ChE activity. The evidence presented permits the view that the receptory sites for ACh and the enzymatic capacities for the splitting

TABLE IV
Denervated and Normal Rabbit Gastrocnemius Muscle

Cholinesterase activity of isolated muscle fibres on, or outside, the end plate region (6 weeks after denervation)

Animal no.	Number of end plates	Reduced weight of the sample $\mu\text{g} \cdot 10^{-2}$	ChE activity		
			$\mu\text{l} \text{CO}_2 \cdot 10^{-3}/\text{hr}$	$\mu\text{l} \text{CO}_2/\mu\text{g RW}$	
Denervated muscle	1	0	62	31.8	0.051
		0	43	14.6	0.034
		2	22	12.7	0.058
	2	3	18	16.4	0.091
		0	41	8.4	0.020
		0	71	8.4	0.041
		1	12	3.5	0.029
	3	3	24	14.3	0.059
		0		10.2*	
		1		8.4*	
		2		32.8*	
	Normal muscle	1			
3		1	42.3†		
3		1	68.5†		
	3	1	38.2†		

* Sample size visually estimated about 20 to 40 $\mu\text{g}/10^{-2}$ RW (reduced weight).

† Sample size was not estimated. The ChE activity of the normal fibre was found to be negligible when compared to the ChE activity in the end plate.

of this substance are integrated in the same functional (particulate or molecular) unit (16, 32, 39), or even that the sites for the two activities are identical (18, 43).

Against a simple causal connection between the expansion over the denervated muscle fibre of ChE activity and the supersensitivity to ACh is the fact that the two phenomena seem to appear at different times after denervation. The latter phenomenon precedes the former (38). However, this topic calls for much more detailed studies of the progression of the spreads from the neuromuscular junction, both of the hypersensitivity to ACh and of the activities of the enzymes which split ACh.

The observed manifold and complex behavior of muscle ChE after denervation, as well as under

some special conditions, calls for a thorough reassessment of their role. This attractive problem has gained interest from a stimulating hypothesis by Barnett and Palade (2) according to which ChE in the M bands of the muscle fibre is part of a mechanism controlling the conduction of stimuli within the muscle fibre.

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