THE MORPHOLOGY OF THE MYONEURAL JUNCTION AS INFLUENCED BY NEUROTOXIC DRUGS *

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Since Doyère[1] in 1840 first described motor end-plates in the striated muscles of insects, this structure has been subjected to continual investigation, marked by three periods during which the pace of investigation was accelerated. In the first period (1840 until about 1903), the names of Kühne, Krause, Ruffini, Ranvier, and Pacini dominated a literature which defined the normal histology of the myoneural junction.[2-4] Dale's[5] work in 1914, on the chemical mediation of nerve impulses at the synapse, provided impetus for a second concentration of studies on the problem, highlighted by the classic interpretations of Wilkinson[6] and the still controversial work of Kulchitsky,[7] Hunter,[8,9] and others who tried to systematize the structural variations of end-plates by identifying them with either efferent, afferent, autonomic, or somatic nerves, as well as with red and white muscle fibers. This phase of study was climaxed by Boeke's comprehensive review of the subject in 1932.[3] Interest then flagged until the 1940's when, because of the German threat during World War II to use anticholinesterase gases in chemical warfare, the end-plate was again subjected to intensive morphologic study and an attempt was made to correlate its structure with physiologic dysfunction.

Better understanding of the morphology *per se* has been achieved within the last 10 years mainly by the prodigious work of Couteaux, and, by the histochemical demonstration of cholinesterase by methods devised by Koelle,[10] Gomori,[11] and Barrnett and Seligman.[12] During this same period, however, a voluminous and influential literature was contributed by Carey[13-25] and his collaborators, which contained the remarkable assertions that the motor end-plate functioned like an exocrine gland, that it altered its shape under the influence of various drugs and stresses, and that the gold chloride staining method of Ranvier stained the secretion product of the end-plate, namely, acetylcholine.

Carey's work on the changing dimensions (ameboid motion) of end-plates under various physical and pharmacologic stresses was done by utilizing the gold chloride technique which stains the nerve arborization and which depends upon reduction of the gold salt to its metallic state. With this technique one can see the perinuclear granules of

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Kühne, while the periterminal networks, described as filamentous by Boeke,[8] using the Bielchowski stain, appear as solid oval masses at the end of the arborization. Reducing substances, some of which may be mobile, are responsible for the success of gold chloride as a staining agent.

The brilliant work that Couteaux[26] performed using Janus green B demonstrates that the muscular portion of the myoneural junction (his "subneural apparatus") has a definite form. A structure with identical shape and dimensions can be stained by Koelle's histochemical technique for cholinesterase (Fig. 1). Welsh and Zacks[27] have reported that Janus green B fails to stain the sole plate structure after the muscle has been incubated in diisopropyl-fluorophosphate. This supports the earlier conclusions by Couteaux and Nachmansohn[28] that most of the cholinesterase is situated in the muscular portion of the myoneural junction, specifically at the sole plate, and also suggests that at least part of the total cholinesterase at the junction is arranged in a definite morphologic pattern (Fig. 1).

In the end-plate stained with gold chloride, a non-staining zone (Fig. 2) can be seen around the arborization. After much study and reconstruction of the end-plate, we agree with Denz[29] that this zone is the "synaptic gutter" which partially surrounds the nerve ending filaments, and which is demonstrated as a positive image by Janus green B and Koelle's technique.

Recently, Couteaux and Taxi[30] have demonstrated that Koelle's histochemical technique used at a pH higher than that of 6 as prescribed by Koelle, will demonstrate the neural component. They believed this to be due to migration of the precipitated copper sulfide onto the nerve arborization; this occurred only in tissue that had been previously fixed in formalin. It is singular that formalin fixation confers some sensitivity on nerves for certain metallic impregnations: if silver nitrate, the agent of choice to demonstrate axons after formalin fixation, is substituted for gold chloride in the Ranvier technique in which tissues are fixed in citric acid, no portion of the nerve stains. The neural element is occasionally visible in tissues stained by Koelle's method without altering the pH of the incubating bath, or prefixing in formalin. Figure 3 shows the neural sheath of Henle outlined by copper sulfide. Whatever the cause for the change in staining specificity, however, the demonstration of the neural element by Koelle's stain supports Couteaux's original interpretation that the nerve arborization is superimposed on the cholinesterase "synaptic gutter" which is impressed onto the surface of the muscle fiber.

In the experiments to be described, rat muscles were subjected to various chemical influences; their dimensions, measured under both the gold and Koelle techniques, were compared to control values. It was considered that any evidence of ameboid motion of motor end-plates, as suggested by Carey, should be represented by dimensional changes of the synaptic gutter, as demonstrated by Koelle's technique.

Morphologic Methods

All motor end-plates were demonstrated by the Ranvier gold staining technique as described by Carey,[15] with certain modifications.

1. 5 per cent citric acid, 15 minutes
2. 1 per cent gold chloride, 25 minutes
3. Formic acid, 16 to 24 hours

Instead of small strips of muscle tissue, however, half of the costal cage was immersed in the citric acid solution at room temperature. The attached ribs help to splint the tissue, and to prevent the extreme contraction that occurs in muscle after death. In cases in which the musculus gracilis was sampled, the excised tissue was extended over lucite bridges to its resting length.

Excised parallel specimens were preserved over dry ice until submitted to the cholinesterase technique, when, prior to incubation, samples of muscle were thawed, then teased in saline solution. The solution used for incubation was made up according to the Koelle formula as given by Gomori.[11] Tissue from animals poisoned by anticholinesterases was incubated at 37° C. for 15 and 20 minutes; tissue from control animals and from those poisoned by drugs which have little or no anticholinesterase action was incubated for 10 minutes. Better definition of the image of the synaptic gutter at the sole plate is the ultimate result of decreasing the incubation time; when the enzyme activity is too vigorous, the image is often blurred by diffusion of the reaction end products.

Two series of experiments were performed on albino rats. The first was designed to test the effect on motor end-plates of various drugs injected intramuscularly, and the second to test the effect on motor end-plates of drugs given intravenously.

The drugs used were strychnine, hydrazine, diisopropyl fluorophosphate, tetraethylpyrophosphate, d-tubocurarine (intocostrin), and an anticholinesterase compound called ACL.

In the first experiment only specimens of m. intercostalis were examined, and in the second, samples of m. intercostalis and m. gracilis: the latter muscle was added because it was thought that the passive

movements of the chest cage during artificial respiration might influence the structure of the end-plates of the intercostal muscles. The experimental design, detailed in Table I, represents a total of 12 rats, one for each experimental group.

All doses are given in mg. per kg. or ml. per kg., except for those of ACL which are expressed as multiples of the estimated dose which will destroy 100 per cent of the subject animals (LD_{100}).

TABLE I
Experimental Design

	Experiment	Drug	Dose (per kg.)	Artificial respiration
Group A Drugs administered intramuscularly	1	Hydrazine	400 mg.	No
	2	Intocostrin	0.5 cc.	No
	3	Strychnine	2.7 mg.	No
	4	Strychnine and intocostrin	2.7 mg.; 0.5 cc.	Yes
	5	ACL	$LD_{100} \times 2.5$	No
	6	ACL	$LD_{100} \times 5.0$	Yes
	7	ACL and intocostrin	$LD_{100} \times 5$; 0.5 cc.	Yes
Group B Drugs administered intravenously (except intocostrin given intramuscularly)	1	Diisopropyl fluorophosphate	100 mg.	Yes
	2	Tetraethylpyrophosphate	105 mg.	Yes
	3	ACL	$LD_{100} \times 8$	Yes
	4	ACL	$LD_{100} \times 10$	Yes
	5	ACL and intocostrin	$LD_{100} \times 10$; 0.5 cc.	Yes

It should be noted also that, to minimize error from inter-animal variation, in experiment B_4 and B_5 the m. gracilis from one side was excised before the experiment, and the end-plates were then compared with those of the contralateral muscle excised after completion of the experiment.

Artificial respiration through a tracheal canula was used generally when anticholinesterase agents were administered because these drugs cause interference with normal respiratory function, and death due to anoxia would otherwise ensue. When respiratory death is so obviated, it is possible to give large amounts of the drugs. This accounts for the fact that in animals A_5, 6, and 7, and B_3, 4, and 5, many times the lethal dose could be given.

Intramuscular administration was accomplished by one injection or two at the most, and if the animal survived it was sacrificed with ether after 30 minutes. Intravenous injections were carried out by adminis-

tering small amounts of the drug every 5 minutes, through a catheter in the femoral vein, until death occurred.

Methods of Evaluation

For the purpose of comparison of the motor end-plates of treated animals with those of untreated controls, at least twenty-four observations, each including three data, were made from each rat. Thus, the long and short diameters of the end-plates and the respective muscle diameters were measured from preparations separately stained by the gold and Koelle techniques. By this method, at least twenty-four sets of end-plate measurements were made for each muscle sample from a single animal. Measurements of the neural arborization from the gold-impregnated tissue, and subneural apparatus from the tissue stained by the Koelle technique were made usually from muscle samples contained on one to three slides. Moreover, many slides representing hundreds of end-plates which were not measured were examined for changes. End-plates to be measured could not be selected completely at random because those which stained indistinctly and those seen from side view had to be eliminated.

White rats weighing 150 to 225 gm. were used throughout the experiment, but despite this, the variation in size of end-plate from one animal to the other was very great. Part of this variation between animals is due to the fact that a direct correlation exists between end-plate diameters and muscle fiber diameters, so that the wider the muscle fiber the larger the end-plate.

Sixty observations from 5 control animals were sufficient to derive an equation which could be used to correct each end-plate measurement to an average muscle diameter, so that variation due to alteration in muscle diameter is cancelled out.

The general form of the equation is as follows:

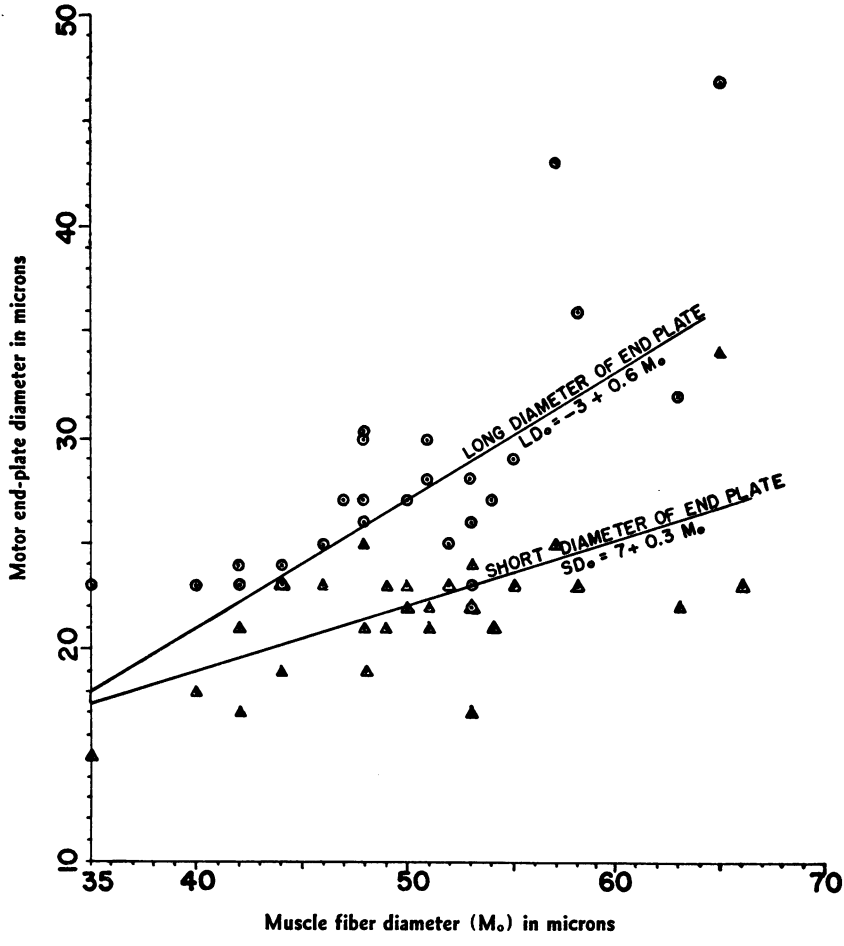
$$D = D_o - b\,(M_o - \overline{M}_c)$$

Where D is corrected end-plate diameter, D_o is the observed end-plate diameter, b is the regression coefficient, M_o the observed muscle diameter, and $\overline{M}_c$ is the average diameter of control muscles. The curves in Text-figure 1 show the correlation between long and short end-plate diameters and muscle fiber diameter in the intercostal muscle.

Results

All agents used produced varying degrees of muscle spasm, usually initial clonus which entered a tonic phase before paralysis or death

occurred. Hydrazine exerted the most dramatic effect, stimulating great perpetual activity so that the animal became extremely agitated, performing wild uncontrolled twists and turns, consummated by leaps 2 to 3 feet in the air, and other gyrations requiring strength and agility much beyond the ability of the normal rat. The animals given strych-



Text-fig. 1. Correlation of motor end-plate diameters with muscle fiber diameters in rat intercostal muscle.

nine developed tonic rigidity of all muscles, including the intercostal group, with death preceded by opisthotonus. Animals treated with anticholinesterase agents generally developed trembling and fibrillary twitches, which were succeeded by clonic and tonic spasticity as the dose increased, often with resultant loss of motor function. The heart rate slowed initially, and subsequent acceleration signalled the onset

of anoxia. Terminal arrhythmias were common. Tenacious bronchiolar secretions often complicated the efforts at artificial respiration, so that the pressure of the respirator had continually to be augmented as the dose was increased. Despite this measure, however, some animals suffocated because of intrapulmonary obstruction. When anoxia was not directly implicated, the cause of death was unknown. Animals given

TABLE II

The Effect of Various Drugs on the Dimensions of the Motor End-Plate

	Intercostal muscle*						Gracilis muscle†					
	Gold stain			Koelle stain			Gold stain			Koelle stain		
Experimental group	No. of observations	Long diameter	Short diameter	No. of observations	Long diameter	Short diameter	No. of observations‡	Long diameter	Short diameter	No. of observations	Long diameter	Short diameter
Control§	60 (a)	30±7	22±1	72 (b)	25±3	24±2	48 (c)	48±5	29±4	60 (d)	36±4	29±2
A1	12	21	16	45	30	23						
A2	12	25	20	45	28	22						
A3	12	26	22	37	26	24						
A4	12	30	22	45	24	23						
A5	12	30	21	45	25	23						
A6	12	31	18	25	28	24						
A7	12	27	18	46	29	23						
B1	12	27	19				12	45	27	46	27	23
B2	12	33	23				12	55	30	45	51	34
B3	12	33	20	12	30	24	12	63	24	47	37	34
B4	12	25	21				12/12	43/43	26/26			
B5	12	28	20				12/12	48/51	30/30			

* All end-plate diameters corrected for average muscle diameter of 50 μ.

† All end-plate diameters corrected for average muscle diameter of 75 μ.

‡ Treatments B4 and B5 are divided into pretreatment measurements of one gracilis and post-treatment measurements of the contralateral gracilis.

§ Expressed as mean value ± the standard deviation. (a) 5 animals; (b) 6 animals; (c) 4 animals; (d) 5 animals.

intocostrin twitched, and often had abortive convulsive movements which persisted for a few seconds before the paralytic phase commenced.

In all experiments the generalized effects of the drugs were allowed to persist for at least 30 minutes, during which time marked muscular activity was observed. Table II shows the results of this study. After all end-plate diameters are corrected for variation in muscle fiber diameter, their distribution appears to be random and indicates no significant differences between experimental groups. In no instance are there consistent changes. When there appears to be a change from control values in end-plate dimension, such as in the gold-stained

arborization of animals treated with hydrazine (Table II), the Koelle preparation of adjacent end-plates shows change in the opposite direction. Significant in this respect is the lack of difference between pretreatment and post-treatment samples of the m. gracilis taken from opposite sides of the same animal in experiments B_4 and B_5. Certainly no qualitative differences between treated and control animals could be determined by microscopic examination of multiple muscle preparations.

Injections of intocostrin, sufficient to paralyze the animals completely, had no effect on end-plates when this drug was used alone or in conjunction with other drugs. It should be noted, however, that in this experiment the average diameter of the intercostal muscle fibers ($50\ \mu$) differed markedly from that of the m. gracilis ($75\ \mu$), and that often the structure as well as the size of the end-plates in the respective muscles varied when stained by the gold technique (Figs. 4 and 6). This difference in structure did not manifest itself in specimens stained by the Koelle technique, although the difference in size was apparent.

Furthermore, no distinct change in aurophilia was produced which could possibly be related to drug action. The cystic bulges at the termination of the efferent neuron, which were described by Carey as being concerned with the "jet-pump" action of the nerve, were never observed.

Discussion

The influence of Carey's work has been greater among workers in such fields as pharmacology and physiology[31,32] rather than in pathology. An illusory attitude about the specificity and precision of histochemical techniques, and a lack of recognition of the capriciousness of metallic impregnation techniques, may have led many to accept Carey's net conclusions without reserve.

The following criticisms of Carey's work might be justified:

Normal variation is not defined but is so large that chance alone could have accounted for his results. Although very large numbers of animals were used, representing many thousands of observed end-plates, no statistical evaluation of the results is presented other than unconvincing changes in percentages. The change in diameter of the end-plates as a function of treatment is emphasized, yet there is no mention of the intramuscular, intermuscular, and inter-animal variation in end-plate size, which can be only partially compensated by correcting for differences in muscle diameter.

The end-plate dimensions, especially the longer one, can be correlated with the diameter of the muscle fiber it innervates. From the curve in Text-figure 1, the end-plate diameter can be predicted roughly from any given muscle fiber diameter. Carey does not take this into consideration, so that if the muscle bundles in treated and

control animals happen by chance to have fibers of different diameters, the end-plate diameters will reflect this change.

Although Carey does mention that various muscle groups are used, it is not stated whether they were examined as a homogeneous entity, or whether the different groups were matched for pre-experimental and post-experimental comparison.

In favor of Carey's hypothesis several facts can be presented:

There is precedent in the literature for some of his conclusions.[33-35] The experiments he performed were always reproducible in his hands and in those of his assistants.

Despite the lack of statistical evaluation ot the results, the changes reported in the large number of animals certainly seem to represent a trend.

Denz[29] emphatically controverts Carey's conclusions, and his statement is worthy of quotation in its entirety:

"The acceptance of the view that toxic agents can produce characteristic changes in myoneural junctions has usually been based on one or other of Carey's papers and not on a critical assessment of the available evidence, which, taken as a whole, does not support either the hypothesis that morphological lesions have been produced by a variety of toxic agents, or the ancillary suggestion that the great variability of myoneural junctions in normal animals is the consequence of their varying physiological states."

Denz[29] failed to observe morphologic alterations to anticholinesterase agents in rat muscle when these structures were studied by three different methods—gold, silver, and methylene blue. The present paper consequently adds a fourth to this group, the Koelle technique, with which no motor end-plate alterations can be observed, either with anticholinesterase agents, d-tubocurarine (intocostrin), or central nervous system stimulants, such as strychnine and hydrazine.

Miura[33] described changes in the motor end-plates of lizards, but not of frogs, after these animals were subjected to chronic curare poisoning. Herzen and Odier[34] (1904) recorded a change in the frog paralyzed with curare, but the end-plates they described may be the *"terminaisons en grappe"* which Wilkinson[6] cited as a normal finding, and perhaps an immature form of end-plate.

Woollard[35] was taken sharply to task by Denz[29] for his description of lesions of motor end-plates in beriberi and in inanition; Rogers, Pappenheimer, and Goettsch[36] failed to find alterations in the silver-stained end-plates of guinea-pigs which had developed muscular dystrophy ascribed to vitamin E deficiency. Dublin, Bede, and Brown,[37] using Carey's technique, found lesions in the gold-stained end-plates in specimens of muscle taken for biopsy from patients with poliomyelitis, but at least one end-plate (their Figure 8) is no different than one of the many normal variants that can be seen. Chor,[38] who

obtained similar results with silver stains, believed that results with the gold chloride technique were too variable to be reliable.

The gold stain, as do all stains, represents a histochemical reaction. That we are unable to define the substance or substances which react with the stain does not mitigate this fact.

As seen in Figures 1 and 2, the Koelle technique and the gold technique do not outline the same anatomical structure, and generally that which is unstained by the gold is brought out with Koelle's technique and *vice versa*. Although nuclear staining, so common in the Koelle and even in alkaline phosphatase techniques (both of which depend finally upon deposition of metallic compounds), is thought to be an artifact,[10,11,39] it is interesting to note that even with respect to nuclei, the Koelle and gold techniques stain different structures. Figure 5 shows the perinuclear staining by the gold method. Similar condensations of gold at the myoneural junction, the so-called granules of Kühne, are probably related to the nuclei of the sole just as the aurophilic granules and nuclei are related in Figure 5.

Although we never have been able to correlate changes in the amount of perinuclear staining with changes in physiologic status of muscle, these condensations represent either an increased concentration of some metabolite around the nuclei or, perhaps, precipitations of uncharged colloidal particles by substances of opposite charge.

Slight insight into some of the mechanisms involved in metallic impregnation phenomena has been gained through the recent work of Board,[40] who demonstrated the ability of specific substances in tissue to reduce the silver ion in a photographic plate, and we have demonstrated that gold chloride emulsions will react to unfixed tissues placed in contact with them so that a "picture" of the tissue slice will result. Of further interest in this respect is the fact that silver nitrate, when this compound is substituted for gold chloride in the Ranvier technique, is reduced by the muscle but does not deposit on the nerve or the end-plate. This suggests that the reactions in the nerve, end-plate, and muscle may be due to different reducing substances.

If the reduction of gold is dependent upon reducing systems in the muscle and nerve, it is possible that in conditions of extreme muscular fatigue or long-standing paralysis alterations in the reducing potential of nerve and muscle tissue may occur. Changes, such as those described by Carey, then would depict changes in the chemical composition and reactivity of these tissues rather than anatomical alterations.

Figure 7 shows a muscle preparation which was overstained by allowing it to remain in gold chloride for 80 minutes. The gold is

clumped in coarse masses and fails to demonstrate fine structure. A similar change is found after pretreatment of the muscle with acetone for 30 minutes either at 4° C. or at room temperature, before submitting it to the gold technique (Figs. 8 and 9). Some of the changes, described by Carey, resemble the coarse gold deposition that can be achieved either by overstaining or by pretreatment of the tissue with acetone. Conceivably the technique in Carey's hands was sufficiently critical so that the least change in muscle chemistry could be correlated with the tinctorial effects achieved with gold. We have not been able to achieve such delicate balance of the technique in this laboratory.

Changes in muscle response to Koelle's technique as a result of preimmersion of the muscle in acetone at −15° C. were recently reported in the Russian literature.[41] A picture similar to our Figure 9 is shown. Since Koelle's demonstration of cholinesterase is dependent upon a metallic precipitation, it is possible that the pretreatment of muscle by various chemicals could cause changes, the final result of which would be to alter the size of the copper sulfide particles so that unusual sites of deposition would occur. Thus, hypothetically, staining variation might be contingent upon biochemical change secondary to physiologic alteration in the tissue.

Although the theoretic basis of Carey's conclusions, namely, that lesions of the myoneural junction as demonstrated by the gold stain do occur and reflect biochemical change, may well be valid, application of this technique as a biochemical or precise morphologic indicator cannot be justified until there has been more penetrating investigation of the dynamics and physics of the metallic colloids and their interaction with body tissues.

SUMMARY

Morphologic alterations were not observed in the motor end-plates of animals subjected to various neurotoxic drugs. Variation in size and structure of motor end-plates as demonstrated by the gold chloride technique is so large in untreated animals that its use as a diagnostic aid is implausible.

Statistical analyses were performed by I. A. De Armon.

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[*Illustrations follow*]

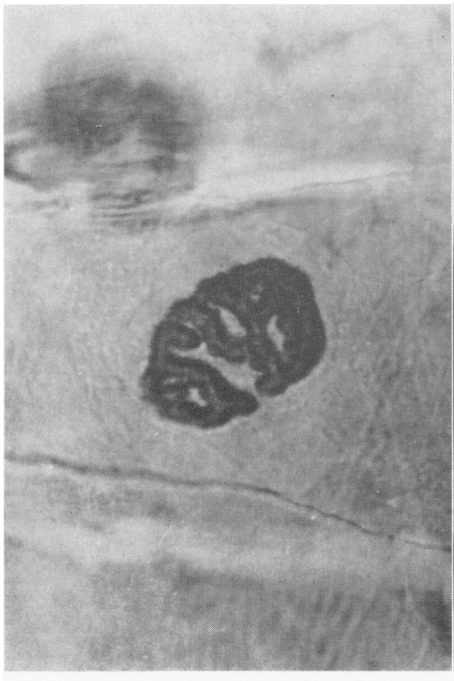
LEGENDS FOR FIGURES

FIG. 1. Subneural apparatus, or synaptic gutter. Koelle method for demonstration of cholinesterase. × 700.

FIG. 2. Axonal arborization of end-plate demonstrated by gold chloride method. × 700.

FIG. 3. Subneural apparatus and outline of neural sheath of Henle. Koelle method for demonstration of cholinesterase. × 700.

FIG. 4. Large intercostal muscle fiber and neural arborization. Gold chloride method. × 700.



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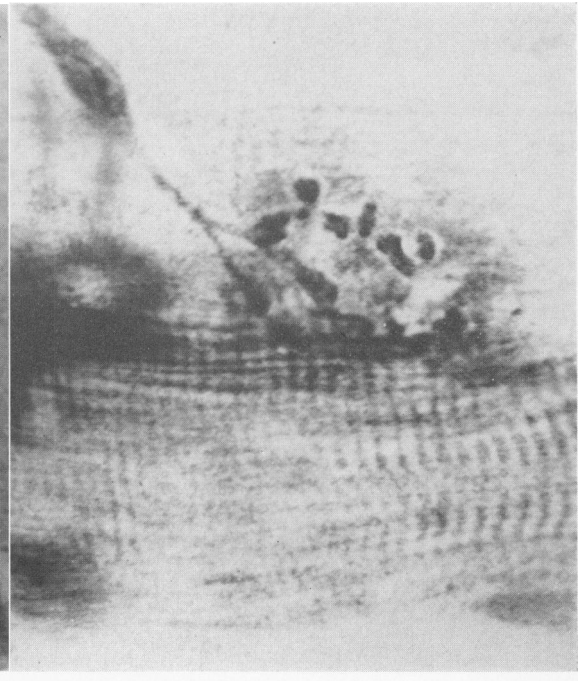

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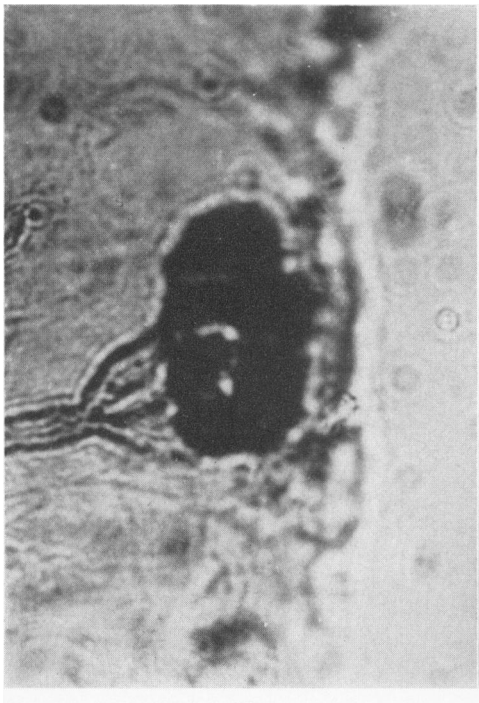

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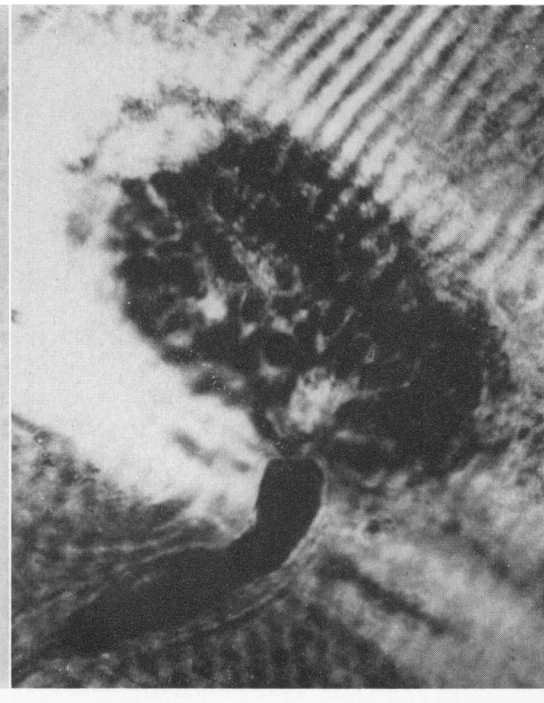


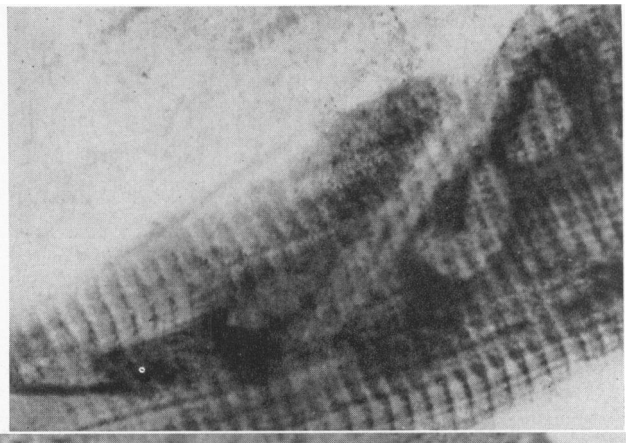
4

FIG. 5. Perinuclear deposition of gold in muscle fiber. Gold chloride method. × 700.

FIG. 6. Axonal arborization of end-plate in posterior gracilis muscle. Gold method. × 700.

FIG. 7. Nerve and muscle sample overstained by gold chloride method. End-plates are light, or fail to stain. × 240.

5



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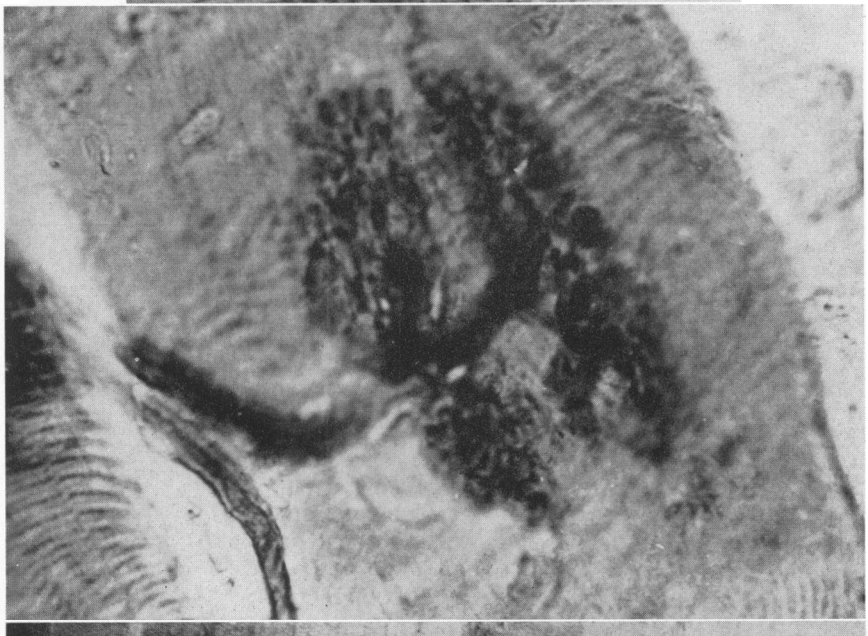

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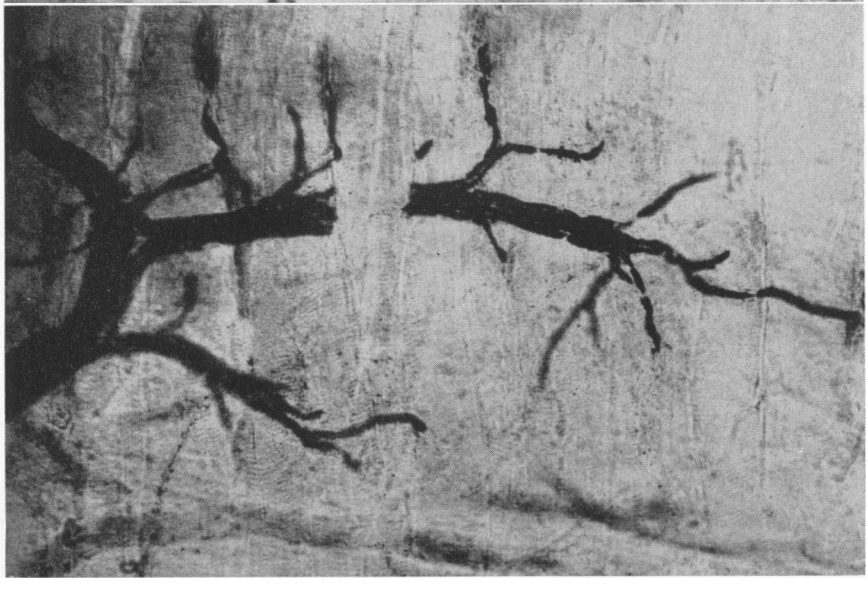

FIG. 8. Nerve and muscle sample stained by gold chloride technique after initial immersion in acetone at room temperature for 30 minutes. There is intensification of the nerve tree and disappearance of end-plates. × 240.

FIG. 9. Clumping of gold in tissue first incubated in acetone at room temperature for 30 minutes. × 240.

8

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