SUBMICROSCOPIC ORGANIZATION

OF THE POSTSYNAPTIC MEMBRANE

IN THE MYONEURAL JUNCTION

A Polarization Optical Study

BERTALAN CSILLIK, M.D.

From the Department of Anatomy, University Medical School, Szeged, Hungary. Dr. Csillik's present address is the Department of Pharmacology, Medical School, University of Pennsylvania, Philadelphia

ABSTRACT

Cross-striated muscles of frogs and rats were fixed in 3.3 per cent lead nitrate solution. Frozen sections 30 micra thick were mounted in different media and observed by polarization microscopy. The subneural apparatus of myoneural junctions exhibits a strong birefringence in these sections. Birefringence is exerted by a highly organized lipoprotein framework (postsynaptic material) which builds up the "organites" (junctional folds) of the postsynaptic membrane. Synaptic cholinesterase is closely associated with this material. Freezing and/or formalin fixation results in a destruction of the molecular organization of the postsynaptic material, but does not influence the synaptic enzyme activity. It is hypothesized from this study that the junctional folds (postsynaptic "organites") consist of regularly arranged, sheet-like lamellar micellae in the frog and of less regular, mainly radially arranged submicroscopic units in the rat. The micellar organization as revealed by polarization analysis is in good agreement with the electron microscopic findings reported in the literature. Intramicellar protein molecules of the resting postsynaptic membrane are arranged longitudinally, lipids transversely. Supramaximal stimulation or treatment with acetylcholine + eserine results in a disorganization of proteins and a re-arrangement of lipids. Denervation results in a rearrangement of lipids without any significant alterations of proteins. All these functional stresses influence only the molecular and not the micellar structure of the membrane. The function of the organized lipoprotein framework as an acetylcholine receptor is suggested.

INTRODUCTION

One of the most important advances in synaptology is the discovery of the fine structure of the postsynaptic membrane (PSM) in the myoneural junction. By means of supravital staining (9) and by histochemical methods (10, 14), Couteaux demonstrated that the junctional sarcolemma, i.e. the PSM of the myoneural junction,[1] consists of hundreds of small semicircular units ("organites") exerting strong acetylcholinesterase activity. The palisade-like sequence of "or-

[1] According to Couteaux's terminology, "sarcolemma" is identical with the muscle plasma mem-

ganites" forms a trough-like structure (the so called "subneural apparatus") under the axon terminal. Electron microscopic investigations (4, 5, 36, 38–45) revealed that the junctional sarcolemma is thrown into a number of more or less regular folds, and, furthermore, that the acetylcholinesterase activity is closely associated with this infolded membrane (28, 56).

As reported briefly in a previous publication (15), fixation of fresh muscle in a lead nitrate solution results in a strong birefringence of the subneural apparatus, pointing to a regular micellar and molecular organization of the postsynaptic membrane. The chemical basis of the specific affinity of the postsynaptic material for lead salts, i.e. the essence of the brightening effect of lead treatment, is at present unknown. As a matter of fact, lead permits the visualization of the birefringent material in postsynaptic structures only if applied to fresh muscle. Treatment with lead nitrate after formalin fixation and/or freezing is ineffective, suggesting that these manipulations disintegrate just those moieties of the postsynaptic substance where lead ions would combine under normal (supravital) conditions. It seems very plausible that under normal conditions lead combines with a specific physiological substance of the PSM and that the birefringence observed is due to a regular submicroscopic arrangement of this lead complex. The brightening effect of lead treatment might be, therefore, analogous to the anisotropic staining.[2]

brane plus basement membrane, not involving, however, the outer connective tissue sheath. In this paper this terminology will be adopted.
[2] It would be unreasonable to suppose that the birefringence observed after lead treatment is due to a simple lead nitrate precipitate, since this would

In the present paper an attempt will be made to correlate polarization optical characteristics of lead-treated myoneural junctions with data obtained by others in electron microscopic investigations. On the other hand, the effect of some physiological, pharmacological, and degenerative alterations as well as the relation of cholinesterase to the organized synaptic material of the PSM will be discussed.

MATERIAL AND METHODS

Investigations were performed on various muscles of *Rana esculenta* and albino rats (*Mus rattus*). Small pieces of fresh muscles were fixed in 3.3 per cent aqueous solution of $Pb(NO_3)_2$ for $\frac{1}{2}$ hour at room temperature. After fixation, the samples were washed in distilled water for 2 hours. Sections were prepared on the freezing microtome, mounted in various media, and examined under the polarization microscope (Zeiss Polmi A equipped with a Sénarmont mica plate compensator.)[3]

For a photographic recording, sections were mounted in glycerol, containing acetic acid in a 0.1 M final concentration. By virtue of the swelling property of acetic acid, birefringence of muscle fibres was abolished in these preparations, but resulted in a brilliant birefringence of synaptic structures with a pure background. (The effect of acetic acid is reversible; floating these sections in distilled

be too soluble to remain in the tissue during the histological procedure (freezing, sectioning, washing, etc.). In electron micrographs of lead-treated myoneural junctions (Lehrer, personal communication), no major needle-shaped crystals are to be seen, which could be responsible for the brightening effect. The reaction of lead nitrate with the postsynaptic material seems to be, therefore, on the molecular level.
[3] Furnished by the Hungarian Academy of Sciences.

FIGURE 1

Birefringence of a synaptic area in the thoracohumeral muscle of the frog. Lead-treated. Birefringence is confined to the "organites" (junctional folds) forming palisade-like rows of the subneural apparatus (arrows). Double lines indicate the borders of the muscle fibre. × 800.

FIGURE 2

The same microscopic field in the state of compensation.

FIGURE 3

Birefringence of a synaptic area in the abdominal muscle of the frog. Lead-treated. Note the birefringence of myelinated nerve fibres (*M*) and that of the junctional folds (arrows). Double lines indicate the borders of the muscle fibre. × 550.
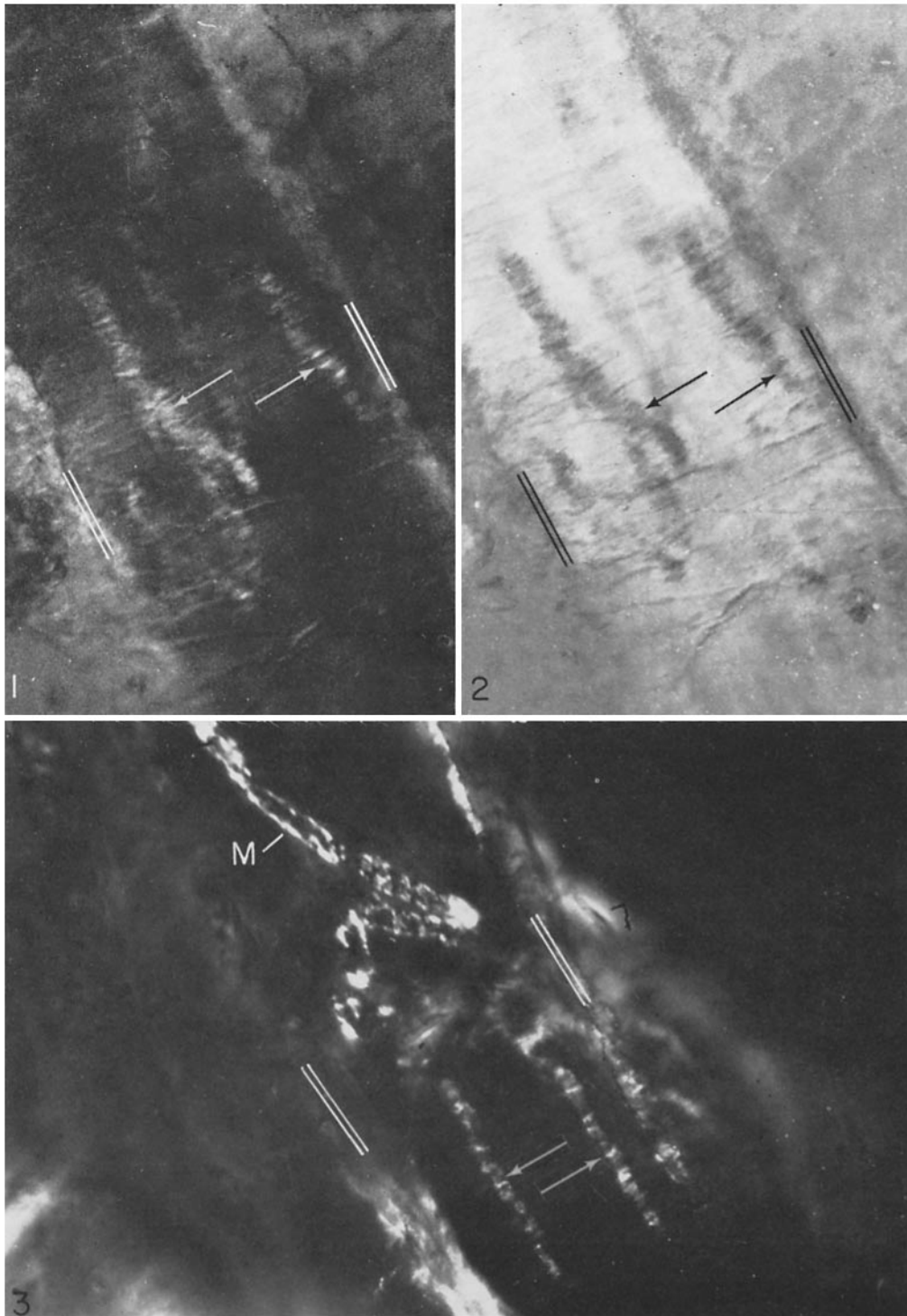
water results in a reappearance of background birefringence of muscle fibres.)

Imbibition experiments were performed on frozen sections of lead-treated material as follows:

Suitable sections were selected by inspection under a phase contrast microscope. A square area, not larger than 2 × 2 mm, containing characteristic subneural apparatuses, was cut out by means of a Graefe knife and glass needles. The topography of the subneural apparatuses in this square was recorded by a free-hand drawing or by photomicrography. The square was mounted successively in water ($n = 1.33$), in glycerol ($n = 1.456$), in various water-glycerol mixtures, in glue ($n = 1.52$), etc. A period of at least 2 hours was found necessary for a complete imbibition in the mounting medium. The optical retardation produced by several junctional folds (organites) was estimated in every single medium under high power (obj. × 40, oc. × 25). When the retardation values in 8 different media of various refractive indices were estimated, the values were plotted against the refractive indices of the media in a coordinate system. The graph obtained this way will be referred to below as an "imbibition curve." As a rule, after completion of an imbibition experiment the procedure was also repeated after extraction of lipids. For this purpose, the section was treated with warm acetone (56°C) for 6 hours. Some 285 imbibition experiments were carried out, including more than 2200 determinations of retardation values.

RESULTS

1. Submicroscopic Structure of the PSM in the Amphibian Myoneural Junction

a) NATIVE MUSCLES : On micro-dissection of native muscle fibers of the thoracohumeral muscle of the frog, a slight birefringence of the synaptic areas was observed. This soon disappeared, however, when a coverglass was placed upon the preparation. In fresh frozen sections or in formalin-fixed material, no double refraction of synaptic structures could be seen, in spite of the well pronounced birefringence of muscle fibres, myelin sheaths, etc.

b) MUSCLES FIXED IN A LEAD NITRATE SOLUTION : Fixation in an aqueous solution of $Pb(NO_3)_2$ resulted in a strong birefringence of postsynaptic structures; that of muscle fibres, myelin sheaths, etc. remained unaltered. The concentration of the lead solution, if employed within the limits of 3 to 10 per cent, had no importance. In order to rule out shrinkage, how-

ever, an isotonic (3.3 per cent) solution was employed.

Birefringence of synaptic areas is confined to lamellar units 3 to 8 micra in size, arranged in palisade-like rows, apparently identical with the "organites" of the subneural apparatus as described by Couteaux (9–11, 14) and by others (17). Optical phenomena of the sarcolemma (crumplings, etc.) can often be traced directly into some of these birefringent units (Figs. 1 and 2). The intimate correlation of birefringent rows with myelinated nerve fibres is in most cases conspicuous (Figs. 3 to 7). Compare birefringent "organites" (junctional folds) of the subneural apparatus (Fig. 8) with those demonstrated by means of the Koelle-Gerebtzoff (25, 22) acetylcholinesterase reaction (Fig. 9) under high power.

The birefringence of the junctional folds is positive with respect to their longitudinal axis.[4] The degree of retardation was about 15 to 18 mμ in specimens mounted in glue (gum arabic, $n = 1.52$). Imbibition curves plotted from individual organites have a convex form, not declining to zero even at the minimum point (Fig. 10, Curve A). According to Schmidt (52) and other investigators (21, 55), this type of imbibition curve characterizes structures possessing both form- and intrinsic-birefringence.

Partial extraction of lipids with warm acetone (6 hours at 56°C) resulted in an enhancement of the retardation. Imbibition curves plotted from lipid-extracted specimens showed a form similar to that of curves obtained before acetone-treatment, differing only in higher values (Fig. 10, Curve B). The increase of the intrinsic birefringence after removal of lipids suggests that the arrangement of lipids in the folds is similar to that of other membranous structures transverse to the non-lipid constituents (21, 45).

In order to ascertain whether these "non-lipid constituents" represent proteins or polysaccharides, a periodic acid–Schiff staining has been performed on sections fixed in formalin or in lead nitrate. In formalin-fixed sections, structures closely resembling the subneural apparatus could be seen which are probably identical with the "brush of Kühne." After denervation, however,

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[4] Longitudinal axis of an "organite" (junctional fold) is normal to the course of the synaptic gutter, in a plane tangential to the surface of the muscle fibre; see the three parallel lines on Fig. 8.
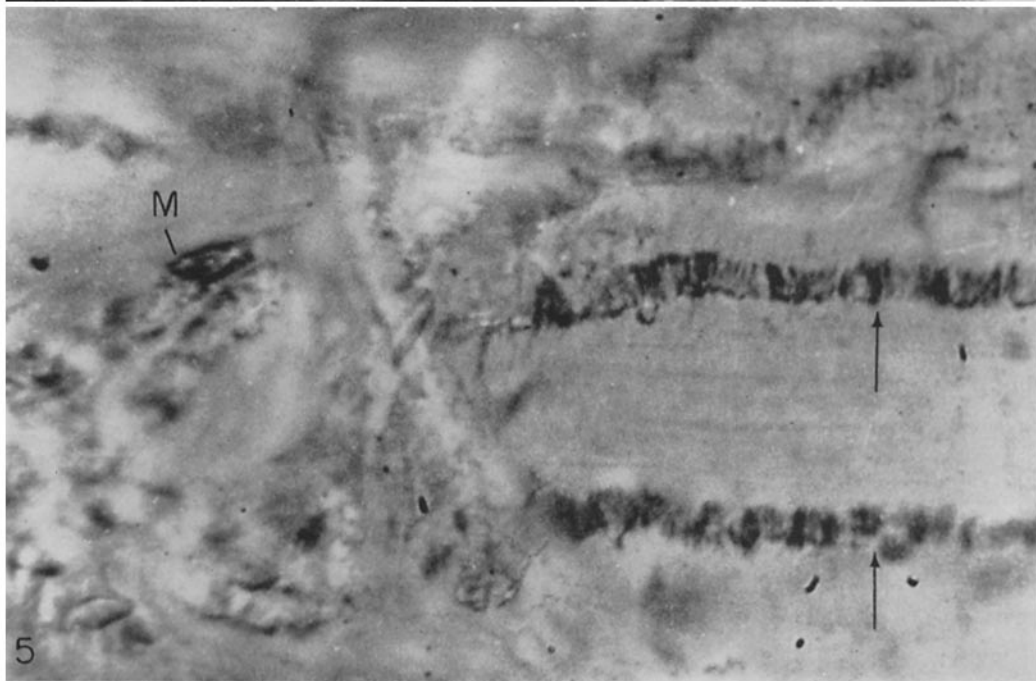
FIGURE 4

Detail of the same synaptic area as that shown in Fig. 3 under high power. M = myelinated nerve fibre; arrows point to the rows of junctional folds. $\times$ 1200.

FIGURE 5

The same microscopic field in the state of compensation.

these structures disappeared in 5 to 8 days, indicating their dependence, at least in part, on presynaptic structure and/or function. It seems, obvious therefore, that the non-lipid constituent of the PSM is mainly protein.

Accordingly, junctional folds of the amphibian PSM seem to be built up of protein micellae carrying lipid molecules arranged transversely with respect to the protein particles. On the basis of the positiveness of the form-birefringence, these micellae must be situated parallel to the longitudinal axis of the folds. Imbibition experiments do not furnish any direct information on the shape of micellar units. Indirect evidence, however, suggests that they are lamellar. A rodlet form-birefringence would fail to show up in the depths of the synaptic gutter, where the rodlets would be parallel to the beam of polarized light. On the other hand, one often sees folds leaning somewhat to the side, exerting no or only very slight double refraction, a fact which also suggests that the organites consist of sheet-like lamellar units.[5] The plausible arrangement of micellar units in amphibian PSM is sketched in Fig. 11.

c) LEAD TREATMENT: This is essential for revealing the birefringence of the PSM. Treatment of fresh samples with warm acetone *without* a preceding lead fixation does not reveal any birefringence of the PSM.

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[5] In our first experiments carried out with a quite simple polarization microscope, these structural details could not have been studied sufficiently. The micellar units were designated, therefore, tentatively as rodlet-like ones (15).

2. Submicroscopic Structure of the PSM in the Mammalian Myoneural Junction

The best polarization patterns were observed in longitudinal sections obtained from the diaphragm of the rat. This muscle is thin enough to be completely impregnated with the lead nitrate solution. In these preparations, subneural apparatuses of the same size and form as those visualised by cholinesterase technics can be seen, exerting a brilliant birefringence (Figs. 12 and 13). As contrasted to the subneural apparatuses of the frog, in mammalian junctions only the edges of the junctional folds show birefringence, while their central part, forming the bottom of the synaptic gutter, is optically inactive. (In sections "stained" for cholinesterase also the bottom of the gutter exhibits enzymic activity).

Imbibition experiments (performed on the edges of junctional folds) resulted in curves similar to those obtained in the frog, showing both form and intrinsic birefringence (Fig. 16, Curve A); after treatment with warm acetone the curves run higher than before (Curve B).

The failure of birefringence in the central part of junctional folds most probably indicates that the micellar organization of this part is different from that of the edges. As a matter of fact, a geometrical arrangement of radially situated, regular rodlet-like (cylindrical) micellar units would yield such a polarization pattern. The real organization, as it may be concluded from Andersson-Cedergren's reconstructions (2), is somewhat more complicated, but not incompati-

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FIGURE 6

Birefringence of a synaptic area in the thoracohumeral muscle of the frog. M = myelinated nerve fibres. Arrows point to rows of junctional folds of the subneural apparatus. Lead-treated. $\times$ 600.

FIGURE 7

The same microscopic field in the state of compensation.

FIGURE 8

Enlarged detail of the birefringence pattern of several organites in a lead-treated specimen. Three parallel lines amongst the junctional folds ("organites") indicate their longitudinal axis referred to in imbibition experiments. $\times$ 2500.

FIGURE 9

Cholinesterase activity of the junctional folds ("organites") of the subneural apparatus. The identity of these enzyme-active structures with the birefringent ones in Fig. 8 is conspicuous. Formalin fixation, Koelle-Gerebtzoff acetylthiocholine method. $\times$ 2500.
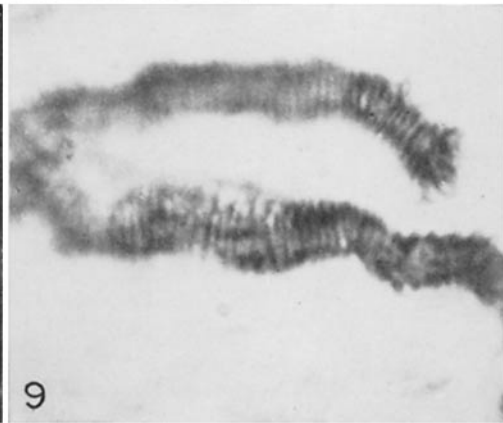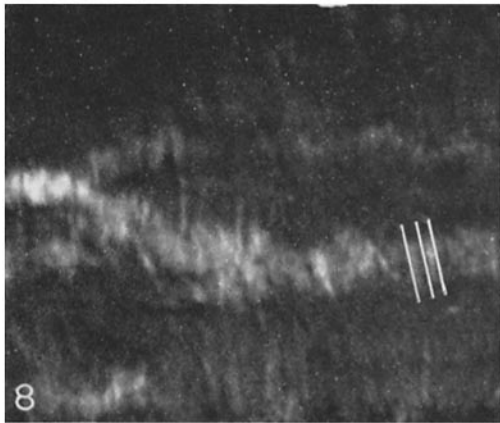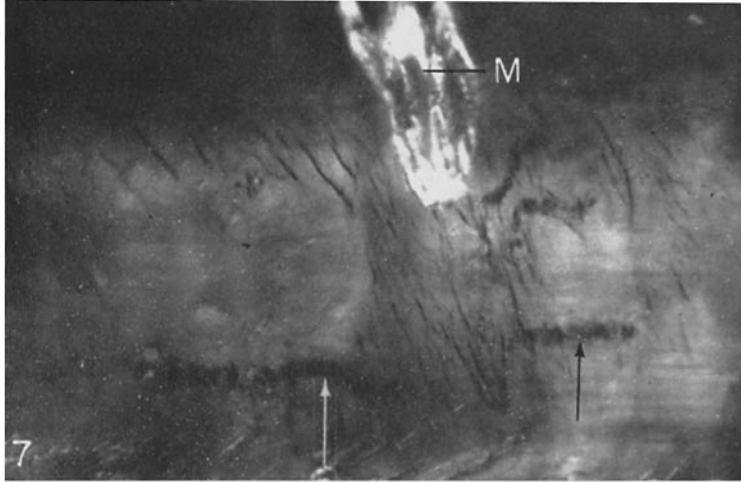
ble with the polarization optical observation (see Discussion).

The intrinsic birefringence observed in all the myoneural junctions tested suggests that the micellar units are themselves also birefringent. Positiveness of the rest-birefringence and its increase after lipid extraction indicates that non-lipid components run parallel with the micellar units, while lipids are arranged transversely.
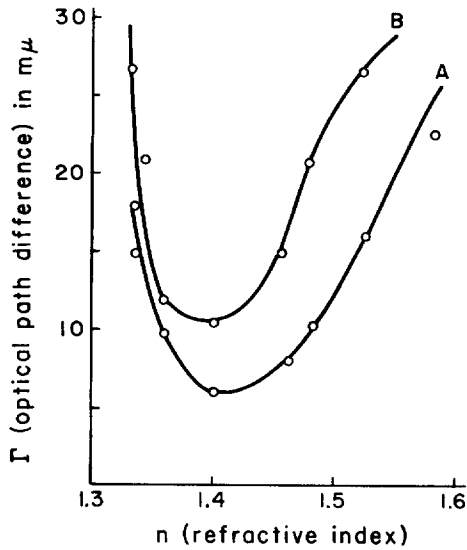

Figure 10

Imbibition curves of a junctional fold of the frog's thoracohumeral muscle. *A*, before acetone treatment; *B*, after acetone treatment.

3. The Effect of a Supramaximal Stimulation on the Submicroscopic Organization of the PSM

The effects of stimulation were tested on both amphibian and mammalian muscles. In frogs, experiments were carried out on the gastrocnemius muscle (6 animals). Anesthesia was induced by an ip injection of urethane. The left sciatic nerve was exposed and stimulated, by means of silver electrodes, with tetanizing stimuli furnished by a square wave generator (50 Hz). After a period of 60 to 120 minutes the frogs were sacrificed by decapitation. Small pieces of both gastrocnemii were prepared for a polarization analysis (fixation in a 3.3 per cent lead nitrate solution, washed in distilled water, frozen sections, mounted in different media, etc). Experiments were performed on

the deep plantar musculature of four rats, as described above in frog muscle.

Under the polarization microscope no differences between control and stimulated material could be seen. However, imbibition experiments carried out before (Curve *A*) and after extraction of lipids (Curve *B*) revealed characteristic alterations (Figs. 17 and 18). In striking contrast to the controls, in stimulated PSM, the extraction of lipids resulted in a diminution of the retardation; the curves *B* decline to zero in amphibia and decrease to 3 to 4 mμ in mammals. Imbibition curves of this type characterize structures possessing a form-birefringence without or with a minimal intrinsic-birefringence.

The alterations observed indicate that supramaximal stimulation does not influence the *micellar* organization of the PSM since form-birefringence remained unaltered. In the intra-micellar (molecular) organization, however, considerable changes take place. Absence (in the frog) or diminution (in rat) of the intrinsic birefringence in acetone-treated specimens indicates that polypeptide chains arranged regularly under normal conditions became more or less irregular as a consequence of the synaptic activity. On the other hand, the arrangement of lipids seemed to be altered. A probable interpretation of these alterations will be presented in the Discussion.

4. The Effect of Several Pharmacological Agents on the Submicroscopic Structure of the PSM

In order to find an explanation for the alterations evoked by supramaximal stimulation, the action of several chemical agents has also been studied. Small pieces of the thoracohumeral muscle of the frog were incubated in various solutions at room temperature, or the muscle was infiltrated with the solution *in vivo*. After 10 minutes the muscles were fixed in a 3.3 per cent lead nitrate solution and prepared for polarization analysis as described above.

a) THE EFFECT OF ACETYLCHOLINE (4 frogs; 2 supravital and 2 *in vivo* experiments). Imbibition curves of junctional folds of both control and acetylcholine-treated muscles (10^{-3} M) showed an identical pattern. Extraction of lipids resulted in an increase in the strength of the positive birefringence.

b) THE EFFECT OF ACETYLCHOLINE AND ESERINE (5 frogs; 3 supravital and 2 *in vivo* experiments). Imbibition curves of muscles treated with a mixture of acetylcholine (10^{-3} M and eserine 10^{-4} M) resemble those of supramaximally stimulated ones.

c) THE EFFECT OF ESERINE ALONE (2 frogs; 1 supravital and 1 *in vivo* experiment). Eserine (10^{-4} M) failed to produce any changes in the polarization optical properties of the PSM.

though these investigations revealed in general the structural changes induced by denervation (see Discussion), it seemed reasonable to reinvestigate this problem with special reference to molecular alterations.

Experiments were performed on the deep plantar musculature of 20 rats. The left sciatic nerve was transected under sterile conditions. Every 5 days two animals were killed by decapitation, and their plantar muscles were fixed
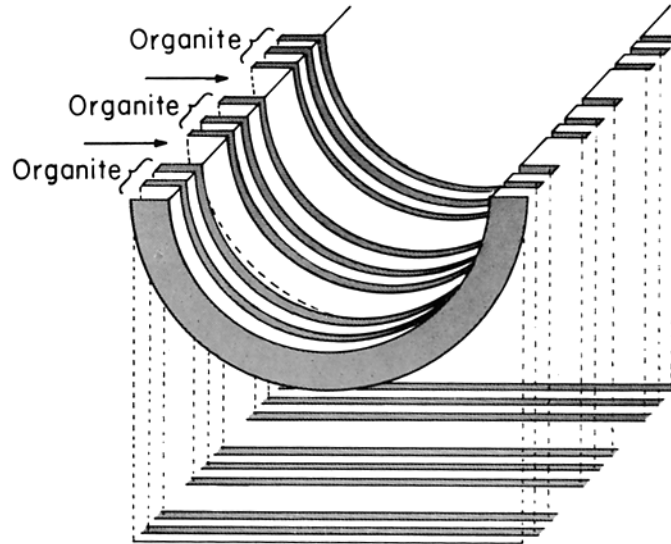

FIGURE 11

Perspective (above) and projection (below) sketches of the postsynaptic membrane in the frog's myoneural junction as concluded from its polarization optical properties. "Organites" (junctional folds) consist of sheet-like lamellar micellae. The interspace between organites is indicated by an arrow.

d) THE EFFECT OF NEOSTIGMINE (3 frogs, 2 supravital and 1 *in vivo* experiment). In striking contrast to eserine, neostigmine induced characteristic alterations of the PSM resembling stimulation or treatment with acetylcholine + eserine. The only difference was that extraction of lipids resulted in a less pronounced decrease of birefringence (Fig. 19).

5. The Effect of Denervation on the Submicroscopic Structure of the PSM

The structure of subneural apparatuses in denervated muscles has been investigated by a number of authors using histochemical methods as well as electron microscopy (8, 26, 37, 48, 53; 5, 40). Al-

for $\frac{1}{2}$ hour in a 3.3 per cent lead nitrate solution and prepared for polarization analysis. In order to inhibit nerve regeneration, transection of the sciatic nerve was repeated on the 10th and 21st day.

In accordance with the results of preceding histochemical investigations (48), the subneural apparatuses exhibited signs of hypersegmentation (in the first 2 weeks) and a gradual fragmentation (from the 3rd week on). Because of the briefness of the period of investigation (50 days), no *granularization* of the subneural apparatuses could be observed.

The subneural apparatuses exhibited a very strong birefringence after the 10th postoperative

day. This conspicuous optical effect culminated 40 days after denervation. On the surface of the muscle fibres a birefringent zone could often be observed in these preparations. The polarization microscopic patterns characterizing normal and denervated subneural apparatuses of the deep plantar musculature are indicated on Figs. 14 and 15.

Imbibition experiments performed on several junctional folds revealed well defined structural changes. The alterations described below refer to muscles 40 days after denervation. Similar alterations, although of a smaller extent, could also be observed after the 10th postoperative day.

As can be seen on the graph (Fig. 20, Curve A), the imbibition curves of the denervated samples are localized considerably higher than the normal ones. The steepness of these curves is somewhat less than that of the corresponding control. Partial extraction of lipids with warm acetone resulted in striking contrast to the controls, in a decrease of birefringence indicating major molecular alterations (Fig. 20, Curve B). The less pronounced steepness of the curves may be considered to be a sign of a shortening of the micellar units.

DISCUSSION

According to these investigations, after a special histological fixation a regular micellar and molecular organization of the PSM could be demonstrated by means of polarization microscopy. The molecular structure of the PSM showed characteristic alterations after physiological, pharmacological, and pathological influences. The special lead-reactive postsynaptic material,

of which the PSM is composed, seems to be a lipoprotein. This assumption is supported by the fact that acetone treatment induces alterations in the birefringence of the membrane, and, furthermore, by the fact that freezing and/or formalin fixation have a destructive effect. These manipulations especially disorganize lipoproteins (21, 29, 30). Recent investigations in our laboratory showed that mammalian subneural apparatuses can be visualised by means of a slightly modified Sudan-B staining (Gajó and Kálmán, unpublished).

An important question concerns the relation of the birefringent postsynaptic structures to the subneural apparatuses "stained" by cholinesterase techniques. The identity of these patterns in light microscopic dimensions is conspicuous. It should be stressed, however, that cholinesterase activity can be demonstrated even after fixation and/or freezing (10, 11, 22, 23, 25, 54), while lead coupling is completely prevented by these manipulations (49, 50). To interpret lead treatment simply as a "visualization of cholinesterase" would, therefore, be unsound.

Whether or not the localization of the lead-reactive postsynaptic material and that of the enzyme are identical in submicroscopic dimensions also seems to be a problem which can be answered by electron microscopy only. A combination of histochemical methods with electron microscopic techniques leads to the conclusion that the enzyme is located in the primary and secondary synaptic clefts (28, 56). The electron microscopic localization of the lead-reactive substance is, on the other hand, problematic.

FIGURE 12

Birefringence of a subneural apparatus in the rat's diaphragm. Birefringence is confined to the edges of the folds (arrow). Lead-treated. X 1000.
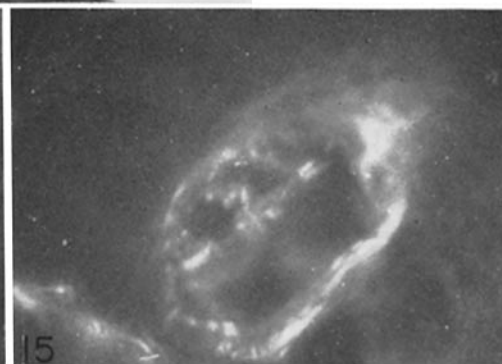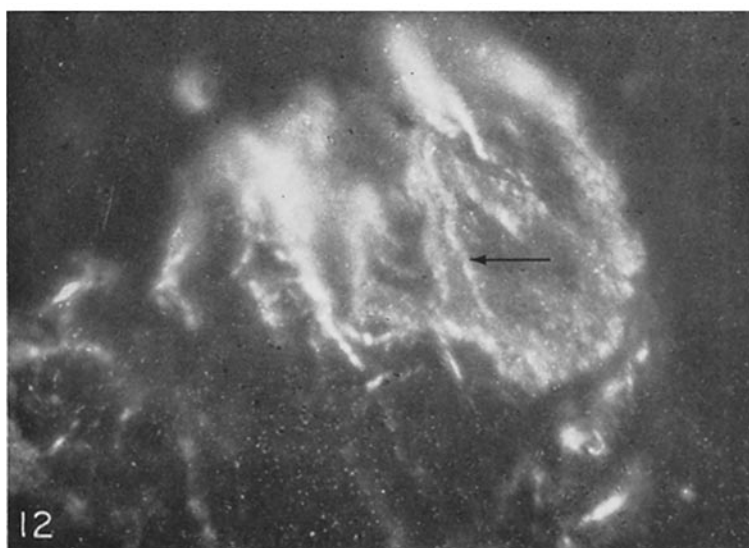
FIGURE 13

Cholinesterase activity of a subneural apparatus in the rat's gastrocnemius. Identity of this structure with the birefringent one in Fig. 12 is conspicuous. Formalin fixation, Koelle-Gerebtzoff acetylthiocholine method. X 1000.

FIGURE 14

Birefringence of a subneural apparatus in the rat's deep plantar muscle. Lead-treated. X 1000.

FIGURE 15

Birefringence of a subneural apparatus in the rat's deep plantar muscle 40 days after denervation. Enhancement of double refraction is conspicuous. Lead-treated. X 1000.
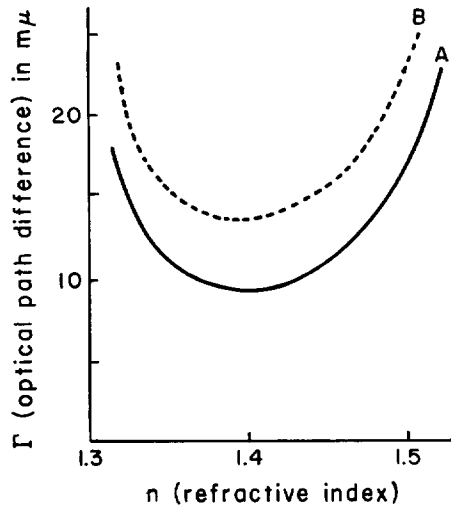
FIGURE 16

Imbibition curves of a junctional fold of the rat's diaphragm. *A*, before acetone treatment; *B*, after acetone treatment.

FIGURE 18

Imbibition curves of a junctional fold of the rat's deep plantar muscle after prolonged stimulation (tetanizing stimuli 50 Hz for 120 minutes). *A*, before acetone treatment; *B*, after acetone treatment.
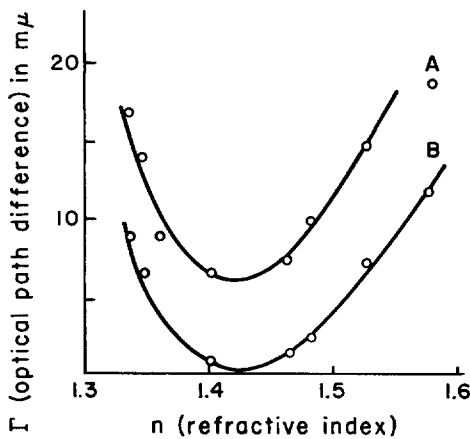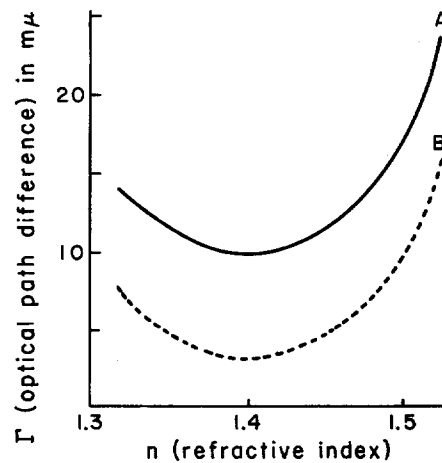

FIGURE 17

Imbibition curves of a junctional fold of the frog's gastrocnemius after prolonged stimulation (tetanizing stimuli 50 Hz for 120 minutes). *A*, before acetone treatment; *B*, after acetone treatment.
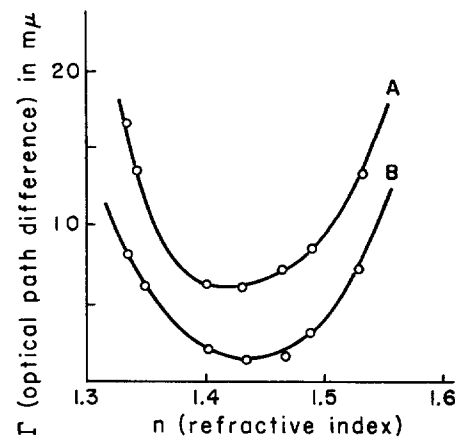

FIGURE 19

Imbibition curves of a junctional fold of the frog's thoracohumeral muscle after *in vivo* treatment with prostigmine. *A*, before acetone treatment; *B*, after acetone treatment.

According to Zacks and Blumberg (56, 57), in muscles treated with lead nitrate solution the lead precipitate is located "primarily in the sarcoplasmic columns of the subsynaptic apparatus." It should be pointed out, however, that in these electron micrographs the density of the sarcoplasmic columns is very poor; lead-treated specimens can hardly be distinguished from the sections treated with osmium tetroxide alone. It seems

plausible, therefore, that these micrographs do not show the actual localization of the lead-reactive substance. It seems more likely that these sections were obtained from the part of the muscle that was not impregnated satisfactorily by the lead salt. On the other hand, Lehrer found, in ultrathin sections obtained from lead-treated material, a heavy electron opaque precipitate in all membranous structures, especially in the folded mem-

branes of the subneural apparatus (personal communication). This suggests that the lead-reactive substance is localized in the PSM itself. The enzyme may be closely associated with this substance or, alternatively, the enzymic activity may be exerted by this substance.

In the following discussion, an attempt is made to correlate the main structural characteristics of amphibian and mammalian PSM revealed by polarization microscopy with the data obtained by electron microscopy.

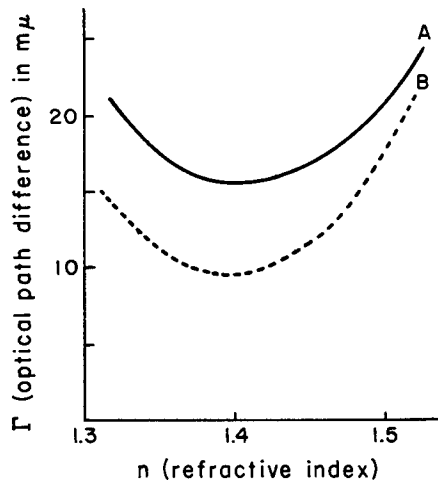1. Electron micrographs of *amphibian* myoneural junctions were published by Reger (41), Robert-

FIGURE 20

Imbibition curves of a junctional fold of the rat's deep plantar muscle 40 days after denervation. *A*, before acetone treatment; *B*, after acetone treatment.

son (44), Birks, Huxley, and Katz (4), and by Birks, Katz, and Miledi (5). In cross-section the PSM has a semicircular form without, however, the regular radial projections characterizing mammalian and reptilian junctions. In longitudinal sections (4) multiple branching invaginations may be seen; their periodicity (0.7 micron) is in good agreement with the periodicity of the "organites" observed in light-microscopic histochemical preparations (17). It seems obvious, therefore, that such a branching unit (a junctional fold) is identical with a light microscopic "organite." The invaginations themselves might correspond to the sheet-like lamellar micellae postulated on the basis of polarization microscopy. On the basis of the close submicroscopic relation

of lead-reactive substance to cholinesterase, it is obvious that the junctional folds exert cholinesterase activity (in histochemical preparations) *and* birefringence (in lead-treated specimens).

2. Electron microscopic cross-sections of *mammalian* (and reptilian) myoneural junctions show a more complicated arrangement. This pattern, described for the first time by Palade (36), Reger (38), and by Robertson (42), consists essentially of radially arranged invaginations of the sarcolemma. The folded appearance of the PSM, as demonstrated in serial sections and reconstructions by Andersson-Cedergren (2), is due to profiles of flattened pouches, arranged more or less regularly in radial direction, as seen especially in her series C. It is reasonable to assume that the birefringence observed at the edges of the folds is due to the transversal orientation of the pouches, while at the base of the synaptic gutter the birefringence is reduced, for the pouches acquire here a parallel orientation with respect to the beam of polarized light. With some correction, therefore, the folds of the mammalian PSM may be looked upon as radially arranged submicroscopic units, producing optical phenomena like cylindrical micellae.

3. Stimulation resulting in characteristic electron microscopic alterations of the presynaptic protoplasm (18) fails to produce any changes in the electron microscopic structure of the PSM (5). Accordingly, no micellar alterations (no alterations in the form-birefringence) could be observed after prolonged stimulation by means of polarization optics. At the same time, however, important alterations of the molecular organization (alterations in the intrinsic birefringence) took place after stimulation, which, of course, might not be detected by electron microscopy.

These molecular alterations consist essentially in a disorganization of proteins and some rearrangement of lipids. The normal state seems to be characterized by expanded protein particles.[6] After stimulation, these protein particles show a random distribution. It seems plausible that these events are due to a summation of the minute alterations evoked successively by some 100,000

[6] According to the classical concept (52), the intrinsic birefringence is due to the regular arrangement of polypeptide chains. In his more recent papers, however, Frey-Wyssling claims (21) that it is due to the arrangement of pearl-chains of minute globular protein particles.

stimuli. It may be assumed, therefore, that similar (but short-lasting) alterations also occur at the transmission of every single impulse. One might speculate that such a rearrangement of the molecular structure of the PSM might give rise to more active ionic fluxes through the membrane (33), in accordance with the Hodgkin-Katz theory (19).

4. Structural alterations similar to those evoked by supramaximal stimulation have also been induced by means of administration of acetylcholine, if the synaptic acetylcholinesterase had been inhibited. A very simple reason for the deformation of protein molecules may be their temporary coupling with the ester, if one assumes that this coupling requires a structural alteration of the protein, as suggested by Nachmansohn (34). The lead-reactive substance of the PSM seems therefore to be identical with the "acetylcholine receptor" postulated by Langley (27) as early as in 1907.[7]

The difference between the action of eserine and neostigmine, respectively, (if administered without acetylcholine) is conspicuous. This may be due to the fact that eserine, a tertiary amine, blocks conduction without depolarization, while neostigmine, a quaternary amine, produces conduction blockade with a simultaneous depolarization as demonstrated by Altamirano and his colleagues (1). In this respect, we failed to observe any structural alterations after the administration of d-tubocurarine, which, although a quaternary amine, does not produce depolarization of the membrane (34).

5. The effects of denervation on pre- and postsynaptic structures have been studied by numerous authors. As Cajal (7) showed in his classical investigations, presynaptic nerve fibres degenerate rapidly, while postsynaptic structures survive denervation for a considerable period of time; both facts could be confirmed also by electron microscopic (5, 40) and histochemical studies (8, 26, 37, 47, 48, 53). In the course of our previous experiments carried out by means of histochemical methods (48), it was found that the cholinesterase activity of the subneural apparatuses decreases very slowly after nerve section; the fragments of the enzyme-active junctions could be detected even 6 months after denervation. The degenerative

[7] A chemical analysis of the lead-binding capacity of Ehrenpreis' receptor-protein (20) might be of particular interest.

changes of the apparatuses consisted of the following phases: hypersegmentation (in the 1st month); fragmentation (up to the 4th month); and granularization. As shown by several authors (5, 40), these light-microscopic alterations of the subneural apparatus are not associated with any considerable changes in the electron microscopic structure of the PSM.

Accordingly, the polarization optical pattern of the micellar structure does not exhibit any major alterations after denervation. In molecular dimensions, however, a striking rearrangement takes place. This is evident from the fact that birefringence of denervated subneural apparatuses is significantly stronger than that of controls, and from the fact, furthermore, that extraction of lipids results in a decrease of retardation in denervated specimens, as contrasted with the enhancement observed in control muscles. The conspicuous strong birefringence of denervated junctions may be a consequence of molecular alterations in the structure of the postsynaptic substances, making lipids less resistant to warm acetone treatment. As shown by Barnes and Beutner (3), acetylcholine and related compounds are capable of producing electrical potentials on water-lipid surfaces. It seems as though molecular alterations evoked by denervation might alter the effect of acetylcholine to the PSM. Thus, the well known "denervation hypersensitivity" might be a consequence of such a molecular rearrangement, which does not preclude at all the possibility that the diminution of cholinesterase activity (12, 13, 6, 47, 48, 51, 53) and the appearance of extrajunctional receptor loci (32) also play a role in the development of acetylcholine hypersensitivity after motor nerve section.

Finally, some remarks should be made on the localization of periodic acid–Schiff positive material in the myoneural junction. Presence of this material in motor end plates has already been described by Noel (35) and, more recently, by Zacks and Blumberg (57). Noel claims that this material is an element of the "manchon peritéloneuritique," identical with Couteaux's subneural apparatus. In view of the rapid disappearance of periodic acid–Schiff positive material after denervation, it seems obvious that the "manchon" and the "subneural apparatus" are two distinct structures—the former a presynaptic, the latter a postsynaptic one.

Summarizing, the conclusion seems justified that the PSM of the myoneural junction consists

of a regularly arranged, lead-reactive lipoprotein material. This structure is organized to a high extent (in both micellar and molecular dimensions) and, at the same time, is considerably labile, both properties characterizing irritable tissues. The estrolytic activity of the PSM is closely related to this organized structure. Further investigations are in progress to establish whether such a structural organization is present in other types of synapses.

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