PART II

NERVE

MORPHOLOGY IN MUSCLE AND NERVE PHYSIOLOGY

by

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As applied to biology, morphology embraces the study of the structure of cell and tissue constituents from gross and microscopic anatomy through the colloidal range and even to the molecular and atomic levels. With the introduction of electron microscopy it is now possible to visualize directly the structure of objects throughout the colloidal range. It is not unrealistic to expect that technical development will make possible direct visualization of such biologically important objects as the smaller protein molecules and possibly even the polypeptide chains. Simultaneously the theory and techniques of X-ray diffraction are also progressing. This method is already able to deal effectively with the analysis of the internal architecture of certain crystalline proteins; a major hurdle appears to be the development of suitable computing methods—a matter chiefly of technology and patience. Progress is also being made in the analysis of the less regularly constructed, but no less important biologically, fibrous proteins and complexes of proteins with lipids, nucleic acids and polysaccharides. This, too, is a matter of painstaking, patient development of techniques, experimental and theoretical.

Morphology is a science in its own right. The analysis of the detailed structure of the molecules and complexes which occur in tissues is largely the task of the physicist who, in turn, must depend upon the chemist to identify, isolate, purify and characterize the individual constituents. In the normal course, as physicists and chemists become interested in such substances, one may expect knowledge in this branch of crystallography slowly to unfold. Slowly because such complex, frequently imperfectly structured materials are not attractive to most crystallographers who are likely to regard them as "sick crystals", as one colleague expresses it. Actually, some of the most important protein crystals are far from "sick" structurally; upon the regularity of the internal structure of their molecules depend such fundamental vital properties as are manifested in the phenomena of immunology, genetics, and the ordered processes of growth and development. Relatively minute changes in the structure of certain protein molecules may make the organism sick (PAULING *et al.*[1], recently referred to sickle cell anemia as a "molecular disease"!). The great biological significance of structural studies has stimulated many physicists and chemists to devote their efforts to the problem. Hopefully their numbers will grow.

The detailed analysis of biomolecular structure is a long term task. The analysis starts with a rough characterization of the main structural features of a particular tissue entity. With the aid of the electron microscope the biologist relatively untrained in the discipline of crystallography can and must take an active in this phase. As the analysis

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becomes more detailed, eventually leading to the localization of the constituent atoms, the task becomes more that of the crystallographer. The physiologist and biochemist must make use of the information available at the moment in attempting to account for biological phenomena.

To what extent has structure analysis been of assistance in solving major physiological problems and what is the outlook for further advance in this field? In seeking a perspective regarding such a question a consideration of muscle contraction and nerve conduction may be instructive because of the contrast which these problems present in respect of inherent susceptibility to morphological investigation and to progress already accomplished. The following account is necessarily brief and attempts merely to indicate the trend of research in this field.

MUSCLE CONTRACTION

Contractility is particularly favourable for morphological study because it involves structural changes at all levels of observation. The voluminous literature of muscle histology, devoted largely to striated muscle, led to few important physiological clues. Perhaps the "reversal of striation"[2] on contraction was among the most suggestive. Even observations in polarized light were difficult to interpret. The positive form birefringence indicated that the fibrous proteins have widths small with respect to the wavelength of light. The relative isotropy of the *I* bands was long misinterpreted as indicating disorientation in these regions. MURALT AND EDSALL's demonstration of the positive birefringence of myosin focused attention on this protein as the contractile substance of muscle. ASTBURY's identification of myosin as the source of the wide-angle X-ray pattern of muscle, together with his hypothesis of intramolecular folding during contraction, helped to seek in myosin the substratum of contraction[3].

In the short time since electron microscopy has been applied to the problem, important advances have been made. The view that myosin is localized in the *A* bands, already discredited by quantitative considerations, was disproven by electron microscopy, which showed that the protein filaments extend as parallel bundles continuously through both *A* and *I* bands[4]. The relative isotropy of the *I* bands is therefore not due to disorientation. Recently the view has been taken that the isotropy results from the presence of negatively birefringent substances in the *I* bands which compensate the positive birefringence of the myosin; this material has been variously reported as nucleotides[5, 6], lipids[7] and phosphoproteins (*N* material)[8].

In contraction the protein filaments remain relatively straight and parallel, indicating that the contractile unit is thinner than the filaments (ca 150 Å). The distribution of the dense material in the *A* bands and on the *Z* membrane changes in agreement with the histological picture of reversal of striation.

Morphological studies were greatly stimulated by advances in our concepts of mechano-chemical coupling mediated by high-energy phosphate bonds and by the discovery by the Szeged group that myosin is composed of two proteins, a water-soluble myosin and actin, the actomyosin complex being sensitive to the action of adenosine-triphosphate (ATP). The general morphological features of the water-soluble myosin and the globular and fibrous actin were soon demonstrated with the electron microscope[9], together with the dissociating effect of ATP on the actomyosin threads[10].

Of great significance in the morphological approach to the contractile mechanism

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is the axial periodicity demonstrated both by small-angle X-ray diffraction[11] and by electron microscopy[4]. This period has a value of about 400 Å in uncontracted fibres and appears to be characteristic of muscle generally, for BEAR has observed it not only in vertebrate striated muscle but also in various invertebrate muscles which are generally regarded as being of the smooth type. In electron micrographs the filaments have a beaded appearance which gives rise to a fine banding of the myofibril, the distance between bands being about 400 Å. DRAPER AND HODGE[12] have shown the period very strikingly in electron micrographs of platinum-shadowed preparations. In their preliminary note they state that the axial period varies inversely with the degree of shortening of the muscle. Variations in the 400 Å period with fibre length were also noted by BENNETT[13] who believes to have shown that the filaments have a helical structure. If these points are satisfactorily documented and confirmed we shall have visual evidence of the contractile phenomenon at the near-molecular level.

Actually the relation between the 400 Å axial period demonstrated by X-ray diffraction and the pseudo-period of about the same value seen in electron micrographs is not clear. The largest meridional spacing observed in the X-ray patterns is about 27 Å which is an order of the larger period. If the situation is similar to that of paramyosin[14, 15] one might expect that the period which might be observable as cross bands in the electron microscope, would have a value of about 27 Å; the larger period of about 400 Å would be manifested as a geometric pattern of discontinuities within the bands. However, depending on the type of geometry of the intraperiod structure, discontinuities at a spacing larger than 27 Å may appear in electron micrographs. The solution of this problem will have to await a more detailed X-ray analysis and attainment of very considerably increased electron microscope resolution of the structure of the filaments.

ASTBURY, PERRY, REED, AND SPARK[16] have observed a spacing of 54 Å in fibrous actin. At large angles the pattern is not that of an alpha protein. This led the authors to the conclusion that the large-angle pattern of muscle is due to myosin while the small-angle pattern is due to actin; the full muscle pattern derives from actomyosin which exists as a complex in muscle. While this may prove to be the case, the diffraction evidence is not yet sufficiently detailed to require this conclusion.

The electron microscope investigation of contractility might be facilitated by examination of *in vitro* models such as the actomyosin-ATP system described by SZENT-GYÖRGYI[17]. This would be true if such systems permitted higher resolution than could be achieved in the myofibril and, particularly, if the essential properties of such a system faithfully portray those of muscle. Recently SZENT-GYÖRGYI[18] has found that muscles thoroughly extracted with 50% glycerol at low temperatures are capable of contraction when treated with ATP and produce the same tension as the intact muscle when maximally excited. Differences in the behaviour of this model as compared with intact muscle are attributed to the fact that the model may lack some of the proteins, lipids and other substances with which the actomyosin is normally associated in muscle. From studies of this model, as from the previous one of VARGA[19], the conclusion was reached that contractile substance is composed of functional units, "autones", and that contraction represents an all-or-none equilibrium reaction of these units; contraction and relaxation are two distinct allotropic states of the autones.

Unfortunately, as admitted by SZENT-GYÖRGYI[18] and as amplified by SANDOW[20] none of the partial systems and models thus far proposed fully displays the essential

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properties of muscle. So far as the morphological evidence is concerned, PERRY, REED, ASTBURY, AND SPARK[21] have shown by X-ray and electron microscope studies that the changes which occur when ATP is added to actomyosin is an intermolecular syneresis, the contraction occurring in a direction normal to that which characterizes muscle contraction. Moreover, there is no evidence from X-ray results for the existence of two distinct states of the "auxones". Upon contraction the large-angle pattern indicates a change from an alpha to a poorly defined, disoriented beta configuration. Efforts to obtain a characteristic small-angle pattern from contracted muscle have thus far met with failure. What little electron microscope evidence bears on this point suggests that the 400 Å axial period shows a continuous change in value with change in fibre length rather than two distinct states.

However valuable partial systems and models may be from the biochemical viewpoint, it is evident that, in the investigation of structural mechanism which is characteristic of muscle, final answers will be obtained by observation of nothing less complex than the muscle fibre itself.

There is no reason to doubt that the combination of X-ray diffraction and electron microscopy will be equal to the task of disclosing the molecular changes which occur in contraction. The small-angle X-ray analysis is particularly promising and may be expected in the near future to portray the main features of the lattice of BEAR's Type II protein. The more detailed structure at smaller separations, involving the configurations of polypeptide chains in relaxed and contracted muscle seems more difficult of unique solution unless more diffraction data can be obtained at large angles.

An electron microscope investigation of the extra-filamentous structures of the striated myofibril, including the materials concerned in the "reversal of striation", the *Z* membranes and the binding material which connects filaments to each other and to the sarcolemma laterally, offers much promise. However, primary interest attaches to the detailed structure within the filament and the changes of this structure with contraction. As compared with paramyosin the task of the electron microscopist will be considerably more exacting because of the smaller spacings involved. Obviously, at this level of size the most critical judgement of image quality and of optical artifacts will be required.

NERVE CONDUCTION

The problem of nerve conduction contrasts strikingly with that of muscle contraction as regards the contributions of morphology. This is due to the fact that the changes whicht occur in a nerve fibre when an impulse is conducted are far more subtle than those occurring during contraction and also to the fact that chemical characterization of nerve fibre constituents, particularly the proteins, is almost completely lacking. Until about the turn of the century the extensive histological literature emphasized primarily the neurofibrils which were regarded by many as the substratum of impulse conduction. In its most stimulating form this hypothesis visualized the interface between axoplasm and neurofibril as the locus of the electro-chemical changes which underlie impulse propagation[22]. BETHE'S[23] demonstration of a difference of stainability of neurofibrils under the anode and cathode of a polarizing current, due to the presence in axoplasm of a hypothetical "fibrillary acid", attracted little attention though the phenomenon seems quite genuine and has some renewed interest in the light of recent

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polarization experiments[24]. The ascendency of the membrane theory together with a growing distrust of structures which can be demonstrated only after fixation caused physiologists to lose interest in morphology as an immediate aid in studying the mechanism of impulse propagation. To many physiologists the nerve fibre became essentially a tube limited by a metastable interfacial film and containing a salt solution plus certain metabolizing substances capable, in some way, of maintaining the structural integrity of the fibre and of furnishing the energy needed for impulse propagation.

The conservative nature of the processes involved in the generation and propagation of the spike wave was demonstrated by studies of the thermal and oxidative changes. The excess oxygen consumption per impulse may be very small at low rates of stimulation[25] and, after treatment with azide, nerve is capable of conducting action waves of undiminished amplitude with no accompanying increase in oxygen consumption[26].

Currently there is renewed interest in the coupling of reactions of chemical metabolism with bioelectric processes. In addition to the much debated question of the role of acetylcholine[27–29] and of other "Erregungsstoffe"[30], suggestions have been offered linking particular chemical reactions with the polarization potential[31]. ATP-ase has also been invoked[32–34]. However, there is as yet no general agreement as to the role of such substances.

In the field of electrophysiology much progress has been made in the more accurate description of the electrical properties of the nerve fibre at rest and during activity. However, the present period is characterized by fundamental disagreement among the most competent investigators about the nature, origin and significance of the polarization and action potentials[35–37]. Characteristic also is the failure of the electrical studies to provide definitive clues as to the structure and chemical composition of the reacting system.

The appalling ignorance about the chemical composition, particularly of the proteins, of peripheral nerve may in part be due to the unattractiveness of investigating a tissue in which the structure of interest is presumably a paucimolecular layer of uncertain location. Amino acid analyses have been made on the socalled "neurokeratin" but the location of this protein is uncertain. Originally the term was applied to the protein of the myelin sheath. However, BLOCK[38] concluded that it is more probably located in the axis cylinder and may be the protein of which the neurofibrils are composed. A pseudo-nucleoprotein was isolated from the axons of the giant fibres of the squid and from lobster nerves[39]. Since this complex seems to occur in the central nervous system as well as in peripheral nerve it was considered characteristic of nerve and was termed "neuronin". Its possible relation to neurofibrils is not known. The chemical characterization of this entity is at best very sketchy, but it can at least be definitely localized in the axon. Chemical investigations are now being carried on by J. FOLCH and his collaborators on the proteins and lipids of the brain. Already a liponucleoprotein and several other proteins have been isolated and partially characterized[40]. Though it is impossible at present to say whether these proteins are located in the perikarion, the axon or in extrafibrillar material, it may be possible, once the pure constituents are thoroughly characterized, to devise methods by which their presence in the components of peripheral nerve may be demonstrated.

In view of the situation as outlined above, it is perhaps not surprising that morphological studies have thus far contributed relatively little to an understanding of

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impulse propagation in nerve. To gain a perspective as to the promise of further investigations at or near the molecular level it will be useful to consider what information of this sort is now at hand. The discussion will be centered around the two chief components of the fibre, the axon (myelin) sheath and the axon (axis cylinder).

THE AXON SHEATH

The general features of the molecular architecture of the myelin sheath have been deduced from polarized light and X-ray diffraction studies[41]. Essentially the sheath consists of lipid-protein layers about 180 Å thick wrapped concentrically about the axon. The lipid phases exist as smectic mesomorphic double layers of mixed lipids, the paraffin chains being oriented normal to the planes of the layers, *i.e.*, radially in the sheath. The protein component is intercalated between double layers of lipids in thin sheets estimated to have an over-all thickness of 25–30 Å per period. This is presumably the protein which, on fixation, gives rise to the neurokeratin network. In view of our ignorance of the properties of this protein it is impossible to say anything about its configuration in the very thin layers in the sheath. When nerve is dried the thickness of the layers is reduced by about 25 Å and a considerable fraction of the sheath lipids is extravasated from the organized structure to form separate lipid phases. In the skeleton of the original structure which remains it seems probable that a fraction of the lipid molecules is firmly bonded to the thin protein layers and that this linkage maintains the structure in the dried sheath. The nature of this linkage can only be surmised though one may suspect that the acid groups of the cephalin molecules may be involved.

Thus far electron microscopy has contributed little to our knowledge of sheath structure though advances in this direction may be expected when sectioning methods are applied. From osmic acid fixed nerves disintegrated with sonic oscillations, SJÖSTRAND (*unpublished*) has observed fragments of very thin layers which may have been derived from the myelin sheath. He had previously demonstrated with the electron microscope that the outer limbs of the retinal rods consist of stacks of thin discs[42, 43]. This is in agreement with the polarized light analysis which indicated that, like those of the myelin sheath, the thin layers contain lipid and protein components oriented perpendicular and parallel, respectively, to the planes of the layers. It has been suggested[44] on very inadequate grounds, that the protein of the rod outer limbs may be a type of "neurokeratin". DE ROBERTIS and the writer have also observed thin layers in preparations from fragmented myelinated nerves. Curiously the fragmented layers frequently show characteristic angular cleavage. If the layers actually derive from the sheath this type of cleavage is unexpected since the sheath has thus far been considered to be uniaxial with optic axes normal to the layers. Measurements of the thickness of the layered fragments may help determine their origin since the over-all thickness of the sheath layers is known from X-ray data.

The X-ray and polarized light results concern only the highly organized lipid-protein substance of the sheath. Determination of the detailed structure of the various other sheath components which have been observed histologically must await electron microscope study in thin sections. Among these structures are the boundaries of the sheath at the incisures, the Golgi funnels and spirals of Rezzonico, the axolemma membrane, the Schwann cell and the outer fibrous investiments. The structure at the node will be particularly interesting because the limiting envelope of the fibre at this point

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has especial physiological significance. Technical difficulties make it hard to study this surface structure with polarized light.

From polarized light studies it has been suggested that all nerve fibres may possess a lipid-protein sheath having the same type of architecture as that of the myelin sheath[45]. Such a sheath has been demonstrated in several types of invertebrate fibres though the investigation has not yet been extended to the so-called naked fibres such as the Remak fibres. In the limiting case the naked fibre may possess a surface structure no more complex than the plasma membrane itself. The polarized light method is probably sufficiently sensitive to detect molecular orientation in such paucimolecular layers. However, the bearing of such data on the problem of impulse propagation would still remain to be shown.

No direct connection between sheath ultrastructure and physiological properties has been demonstrated, although a correlation has been pointed out between sheath birefringence, *e.g.*, essentially lipid concentration, and velocity of impulse propagation[45]. This correlation is at best only rough when applied to the fibres of a particular type of nerve but seem more suggestive when fibres of widely different types of nerves are considered. For several types of vertebrate and invertebrate fibres having approximately equal conduction velocities, TAYLOR[46] found that the product of fibre diameter and sheath birefringence is roughly constant.

THE AXON

The most interesting structures in the axon are, of course, the neurofibrils. Only in exceptional cases can these objects be observed in the fresh fibres, the chief lore of the literature being concerned with fixed and stained preparations. The neurofibrils may approach the limit of microscopic resolution in fixed and stained preparations. Hence it is readily understandable that, if they pre-exist in the fresh axon, they may not be visible, particularly if refractive index relations are unfavourable. In the dark field microscope ETTISCH AND JOCHIMS[47] observed no structure in the fresh axon though very fine collagen fibrils of the connective tissue were clearly visible, indicating a fundamental difference in the two types of fibres. After treatment with reagents such as $CaCl_2$ or fixatives, neurofibrils immediately appear. Apparently only slight colloidal alterations suffice to make them visible. It was concluded by PETERFI[22] that the fresh axon is a rodlet sol capable, under very slight chemical provocation, of forming a fibrous system. He suggested that the mutual interaction of the elongated micelles may be intimately associated with impulse propagation.

Electrical studies have failed to indicate any direct role of axoplasm except as a passive conductor of current. An electrode may be inserted into the axon of the squid giant fibre without blocking conduction. But if the inner surface of the cell membrane is injured conduction ceases[48, 49]. However, CURTIS AND COLE'S[49] statement that "This makes it seem rather unlikely that there is an internal structure in the axon which takes a prominent part in the active mechanism of propagation" must be accepted with caution since there is no evidence that the manipulation mentioned disrupted any axonic structures which might be present as it did the membrane structure.

Changes in the colloidal organization of the axon with activity have been sought, but thus far the experimental techniques have been very crude. It has been claimed that the fibre exhibits changes in contour with electrical polarization, swelling at the

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anode and flattening at the cathode[22]. More recently FLAIG[50] believed to have shown that the viscosity and turgor of the axoplasm of the squid giant fibre is considerably increased during activity. He suggested that excitation increases the viscosity by shifting the sol-gel equilibrium. If FLAIG's results are confirmed, careful investigation of the light scattering by the axon might be warranted. The existence of elongate particles in the fresh axon is demonstrated by the positive birefringence which, though weak, is measureable in large axons such as in the squid giant fibre. Semi-quantitative analysis of the positive form birefringence indicated that though the oriented fibrous structures occupy a small portion of the axon volume, they must have a considerable degree of regularity of internal structure, for their intrinsic birefringence is comparable with that of myosin or collagen fibres[51].

No change in molecular orientation in the axoplasm of squid giant fibres during activity could be detected by polarization optical means[52]. Using a sensitive photoelectric method capable of recording small changes in birefringence without appreciable time lag, it was concluded that if any change occurred it was less than 0.2% of the initial birefringence for the spike process and less than 0.08% for the slow recovery processes. Unless more sensitive methods yield positive results it may be concluded that impulse propagation is associated with little if any change in orientation of the elongate particles of the axon.

From electron microscope studies, RICHARDS, STEINBACH, AND ANDERSON[53] described contorted fibrils composed of kinked elongate particles in axoplasm extruded from squid giant fibres. They suggested that these structures may form the basis of neurofibrils. DE ROBERTIS AND SCHMITT[54] observed characteristically double-edged fibrils in electron micrographs of material obtained by sonic fragmentation of frozen sections of formalin fixed nerves of various types. Such structures had never before been observed. For descriptive purpose the fibrils were tentatively called "neurotubules". The dense material at the edges is for the most part removed by washing with water. It is not yet clear to what extent this dense material is associated with the fibrils in the natural state and to what extent it may have been deposited upon them during the preparative procedure.

After staining with phosphotungstic acid or shadowing with heavy metal the fibrils have a cross-striated appearance. The axial period averages about 650 Å and detailed intraperiod structure has been observed. Since this period is similar to that of collagen[55] and since nerve fibres are closely invested with connective tissue the possibility that neurotubules may be collagen fibres invested with dense materials of undetermined origin was carefully considered. The fragmentation technique employed makes it difficult to determine the location of the neurotubules in the nerve fibre. All the evidence was consistent with the view that they are of axonic origin. Important in the reasoning was the fact that typical double-edged fibrils were not observed in preparations of nerves which had been allowed to undergo degeneration *in vivo* (WALLERIAN) or *in vitro*[56]. However, in recent experiments on late degeneration, results at variance with those previously described were obtained. Preparations from nerves degenerated for as long as three weeks were not devoid of double-edged fibrils but contained them in considerable abundance. The reason for this discrepancy is not clear. However, in view of the importance of the degeneration changes to the argument that fibrils are of axonic origin, the entire matter is being reinvestigated. Speculation as to the possible role of the neurotubules in nerve function would be premature at this time.

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Recent experiments suggesting that axoplasm may be continuously moving peripherally from the cell body in the normal neuron[57, 58] have stimulated renewed interest in the colloidal properties of the axon as they concern trophic phenomena. It seems probable that application of the thin sectioning technique may prove valuable in studying axon structure with the electron microscope and that such studies may throw light on the physical basis of trophic processes.

The axons of fresh fibres offer little promise for X-ray diffraction studies because of their high water content. It was estimated that the axon proteins of the squid giant fibre account for only 3 or 4% of the wet weight of the fibre[51]. Dried frog, lobster and crab nerves show equatorial diffractions at about 11 Å. It is probable that these diffractions arise from connective tissue because alcohol-dehydrated axons isolated from squid giant fibres showed only two disoriented rings at about 4.7 and 10 Å, characteristic of denatured protein[59]. These patterns are similar to those obtained from fibres spun from axis cylinder protein. These X-ray investigations of axon structure were not exhaustive and, in view of current electron microscope results, warrant further careful study.

From the above account it is clear that the problem of structure analysis in nerve is a formidable one. It is particularly challenging because of the high sensitivity of the colloidal organization to physical or chemical manipulation and because the chemical reactions underlying the physiological process are completely unknown.

There can be little doubt that X-ray and electron microscope techniques, if sufficiently acutely applied, are capable of penetrating to or near the molecular level in nerve as has already been accomplished in the case of contractile tissue. Hardly more than a beginning has been made thus far. Progress with the morphological problem would be greatly accelerated if the chemical properties of the nerve proteins were known. The biochemical problem is itself quite formidable bur there is no reason to doubt that it would yield if subjected to a concerted attack by modern methods of isolation and characterization. The bioelectric aspects have attracted the best efforts of many competent investigators and their analysis is still proceeding. The time has come for an equally concentrated attack upon the morphological, biochemical and enzymological aspects. Only thus may we expect to make significant progress with a problem as complex as that of nerve function.

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