INVESTIGATIONS ON THE FINE STRUCTURE OF STRIATED MUSCLE FIBER

DR. EMILIO VERATTI

Assistant in Histology, Laboratory of General Pathology &

Histology, Professor C. Golgi, Director. University of Pavia

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TRANSLATED BY

Carlo Bruni, M.D.

H. Stanley Bennett, M.D.

Don deKoven, A.B.

MUSCLE TISSUE has been the object of unremitting research since the initial era of histologic studies, the end of the 17th Century, when Hooke (58) and Loeuwenhoek (73) applied the recently invented microscope to the study of muscles, through to the end of the 18th Century, with the work of Fontana (32) and Proschaska (94), and right up to our own times. Scanning the history of science, we see how keen has been the animus to investigate the structure of contractile elements in the hope of discovering the secret mechanism of the most important phenomenon of animal life—movement!

Studies on muscle tissue by anatomists and physiologists throughout the years number in the hundreds. These studies have been conducted with diverse methods—each more perfect than the last, as technology became enriched with instruments, more precise methods of observation and measurement, and new contrivances capable of revealing the most minute structural details—and conducted with diverse objectives, as general notions on animal organization were modified. These studies represent many generations' steadfast pursuit of the solution to a great problem in biology.

And still we are far from reaching the goal. From the physiologic point of view, none of the numerous theories devised to explain contraction has attained the aim of bringing the phenomenon into conformity with the general laws of physics and chemistry (Bowmann, 10, 11; Amici, 1; Brücke, 13; Krause, 67; Merkel, 82, 83; Engelmann, 25–27, 29, 30; Verworn, 118; Ranvier, 101; Geddes Patrick, 37; MacDougal, 21, 22; Neuman, 90; Marshall, 77; Müller, 87; Rutherford, 111, 112). Moreover, with the advance of research, the majority of these theories have proved to be in disagreement with anatomical data and to have been based on inaccurate observation. Agreement is lacking from the anatomical viewpoint not only on an infinity of specific questions, but even on the matter of the elementary structure of the contractile substance. In fact, even if the majority of anatomists now admit, especially as a result of researches in embryology and comparative anatomy in the lower animals, that the essential constituents of muscle fiber may be depicted as primitive contractile fibrils, there remains no dearth of authoritative investigators either in past epochs or in recent times who oppose this doctrine, affirming that the contractile part does not have a fibrillar form in the living elements, and that the filaments isolable from muscle fibers with chemical reagents are artificial products (Remak, 102; Haeckel, 48; Leydig, 71; Margo, 75, 76; Münk, 88; Kühne, 68, 69; Carnoy, 18; Melland, 81; Marshall, 77, 78; Van Gehuchten, 38–40; Ramon y Cajal, 98).

An explanation of the clear disproportion between the great mass of effort spent in studying the contractile substance and the scarcity of results obtained must be sought on one hand in the considerable difficulties histological analysis

of muscles presents and, on the other, in the lack (until recent times) of technical procedures that might permit mechanical or optical isolation of the constituents of the muscle fiber and render them accessible to observations without altering their form or constitution.

Besides ordinary difficulties inherent in the study of the fine organization of all tissues whose structure is at once complicated and easily alterable, muscle tissue presents quite peculiar difficulties linked with the optical properties of the parts that make up its elements. It will suffice to point out that small variations in the position of the objective focus with respect to the plane of the object being observed produce variations in the image, such that those parts that appear lighter at a given focal position appear darker when the objective is lowered. The result is an inversion of both the longitudinal striation (positive and negative images of Rollett, Retzius, etc.) and transverse striation. If one can speak of light and dark striations in connection with this inversion, it is only because of a convention which considers as basic the image at the lowered focus (Heidenhain, 54). According to Schäfer (114), a comparable variation of the image obtains in modifying the direction of the light beam reflected from the microscope mirror to illuminate the preparation. It is well known that several authors were so struck by the importance of refraction and reflection phenomena in the formation of the microscopic image of muscle fibers that some were led to deny that the striation is an expression of a particular internal structure of the elements, attributing the occurrence of the light and dark striations to an effect of light reflection on the numerous diversely-curved features of the surface of the fiber (Haycraft, 50, 51), while others granted that the striation owes its appearance in fresh fiber either solely to total reflection of light rays at the transverse membrane that these authors maintain exists in conjunction with the line of Amici (Heppner, 55; Ramon, 98), or to the confluence of the luminous areas which, in accordance with the laws of light reflection, are formed around the granules of the first order transverse reticulum (Melland, 81; Van Gehuchten, 38). Moreover, variations in the image due simply to the modality of reflection of the light rays were regarded as an expression of structural diversity and as evidence of displacements of the constituent parts of the fiber in different stages of functional activity (*vide* criticisms put forward to Merkel's theory on contraction). The effect of these unfavorable conditions is felt especially in observing living muscles in transparent animals such as the larvae of *Corethra plumicornis*, *Cypris*, *Cyclops*, etc. (Wagener, 119; Laulanié, 70; Van Gehuchten, 38), muscles in the fresh state (Exner, 31), or fixed but unstained muscles.

A second series of obstacles in the study of the fine organization of muscle arise from contractility. Everyone involved in this work knows how difficult it

is to obtain muscle fibers fixed in extension or in perfect relaxation. It is known, in fact, that most often, at the instant the fixative comes in contact with the muscle fiber, a modification occurs which leads to a shortening of the fiber and which, at least from the morphologic point of view, is identical with contraction. This modification induced by reagents takes place also in the muscles of animals poisoned with substances capable of diminishing or extinguishing direct excitability of muscle (quinine in very high doses, veratrin). Nor is this problem overcome by the widely used method of bringing the fixative into contact with the muscle while the latter is mechanically held in extension, because, as the studies of Ranvier (101), Renaut (103), and others show, the appearance of a muscle fiber differs according to whether it is fixed during relaxation or while under the action of a stimulant capable of producing contraction even though shortening of the fiber does not ensue because it is prevented mechanically. Also, to judge with precision whether a given fragment of fiber is in extension, in complete contraction, or in one of the intermediate stages (or in which of these), is not exempt from difficulties, even granting that one resorts to measurement of the distance between the striations (especially between two successive lines of Amici (Z)), taking as a basis of comparison the results of determinations by Engelmann (28) and others who have occupied themselves with these patient micrometric researches.

One of the things that strikes us most forcibly (when, to alert ourselves to the current state of our knowledge of the structure of muscle elements, we undertake a study of the vast literature on the subject) is the disproportion between the complexity of questions authors have set out to resolve and the imperfection of methods they have employed. A small group of methods, each with slight modifications of details, constitutes the base of the great majority of studies. Against the very broad variety of materials is matched uniformity of technique: observation of fresh material in ordinary light, polarized light, or with the aid of the polarizing spectrum; observation on muscles fixed usually in alcohol or in chromic solution; staining with dyes of vegetable origin or with aniline dyes; the gold chloride method applied on fresh tissue or on tissue previously fixed in alcohol; several attempts at [enzymatic] digestion; maceration in various liquids; ... and there, sketched in general lines, are the narrow limits of the muscle techniques. With these methods, observation must deal with the fiber *in toto*, isolated either by laceration or crushing, and it cannot but cause surprise to see what little use has been made in muscle fiber studies of thin sections—a method modern histology has found to be required for rapid progress. In view of the poverty of technology in this field, it seems strange that until now only a few isolated attempts have been made to apply to striated muscle fiber those methods that in other fields have given results of major importance by virtue, especially,

of their common capacity to expose single elements of tissues or single parts of these elements—by selective staining. I refer to the black reaction of Golgi, to Apáthy's gold impregnation methods applied to thin sections of fixed tissues, and to Heidenhain's iron hematoxylin stain also applied to thin sections. With Golgi's method—we have so far only the works of Fusari which, perhaps because of their overly condensed exposition and the lack of figures, have not attained the wide attention they deserved, and several studies by Ramon y Cajal in which, as I will show, the author has not given an exact interpretation to his extremely interesting results. (Heidenhain, in his most diligent critical study on the structure of contractile substance, cites in his bibliography only one of Fusari's three reports and notes that he was not able to read the work in the original.) With Apáthy's methods—the studies on worms, published some years ago (2–6), which have contributed so much toward a solid foundation to the theory of the fibrillar structure of muscle fiber, were not extended methodically into other classes of animals. With Heidenhain's method—only very recently has the hematoxylin stain been applied by its author and by others, in particular to the study of the muscle elements of the myocardium (Heidenhain, 53, 54; Hoyer, 59, 60), and in certain embryologic researches (Godlewski, 41, etc.).

I have undertaken a series of methodical investigations on striated muscle fibers using the three methods noted above and have compared the results. For control and orientation purposes, I have not neglected the other methods more widely used up to the present (see above). I believe I have arrived at certain conclusions not entirely without interest, especially insofar as the fine structure of the sarcoplasm is concerned.

I do not believe it would be proper for me to summarize all research on muscle fiber before beginning an exposition of my own results. I am, first of all, unequal to the task, in view of the large number of publications and the difficulty of finding one's position in the midst of the disparate and contradictory opinions of the various authors on almost every point. Moreover, such an effort is not indispensable since we have in Heidenhain's work a scrupulously diligent and recent critical review of the structure of contractile substance. As the occasion presents itself, I will point to those of the earlier works with which the facts I have been able to expose have closest connection, or which concern questions that I too, from the nature of my studies, have been led to consider. At the outset, then, I will review the works of Fusari and Ramon y Cajal because, in a certain sense, they represent the starting point of my studies.

In a very general way we can assume that muscle fiber in the vertebrates derives embryologically from elements of epithelial nature belonging to the inner layer (medial wall of the primitive segments). It seems certain that, while in some cases each fiber derives from a single cell in which the nucleus proliferates

rapidly without subsequent division of the protoplasm (Barden Russel, 8), in other cases it derives instead from a complex of cells which fuse with each other in the first moments of development (Maurer, 80). In the protoplasm of the elements of the primitive muscle epithelium (by a process the exact mechanism of which is unknown—as is true of all processes of this kind), there is a differentiation of fibrils oriented in the same direction as that in which the future muscle will exert its traction. The fibrils rapidly increase in number and orient themselves and form groups in the protoplasm from which they derive, according to different laws in different animals and in different classes of muscles in the same animal.

When the formation of fibrils is completed, there remains in the primitive protoplasm a more or less abundant residuum occupying the spaces between the fibrils and sometimes forming a continuous layer around them (when the fibrils are grouped in the center), and other times forming a compact mass in the central part of the fiber (when the fibrils are arranged around the periphery). At the same time, at the periphery of the protoplasm there is differentiation of a surrounding membrane having the morphological quality of a cuticle, and deriving phylogenetically from the basement membrane of the muscle epithelium (Maurer, 80)—the sarcolemma.[1] In the higher animals, some nuclei undergo a process of regression so that only those remain which are situated at the periphery in contact with the sarcolemma.

In the fully developed muscle fiber we shall therefore distinguish the sarcoplasm, the nuclei, the primitive contractile fibril, and the sarcolemma.

The muscle fibrils are not arranged without order in a single bundle occupying the whole muscle fiber, but are divided into small bundles by layers of sarcoplasm; to these small bundles Kölliker gave the name *columnae muscolares* or muscle columns. Due to this disposition of the contractile fibrils, cross-sections of striated muscle fibers show a peculiar mosaic aspect; they appear, that is, to be divided by thin lines into many little fields of different but generally polygonal shape. This arrangement was described by Cohnheim (20), (Cohnheim's fields), shortly afterwards confirmed by Kölliker (64), and subsequently by all other authors. In longitudinal sections also, the muscle columns appear separated by lines representing the sections of the interstitial sarcoplasmic septa. Recently the question has been put at issue whether we can really speak about primitive contractile fibrils as histological elements, and whether it is right to speak about muscle columns as parts having, in any case, an identical morphological value. The most serious objections were reviewed by Heidenhain in

[1] It is known that one current of thought holds that the sarcolemma derives from connective tissue (Margo, 75; Martinotti, 79; Mondino, 85) but the most reliable embryological researches seem to contradict this opinion.

several of his works (52–54); he emphasized the fact that some muscle fibers (classical examples are found in some larvae of Lepidoptera) in cross-section appear divided by thick protoplasmic layers in a small number of extended fields and that these fields are subdivided by thinner layers into smaller and more numerous fields, and so on. Heidenhain wonders where the muscle columns are in such cases and to what the term Cohnheim's fields can be applied, since with the means of investigation available we see a series of successive subdivisions into smaller and smaller fields. As far as the primitive (fundamental) fibrils are concerned, Heidenhain questions the possibility (stated by Kölliker, Engelmann, and Rollett) of observing them in cross-sections of muscle fiber or of isolating these fibrils mechanically or with selective staining techniques, because we never observe filaments, which from the uniformity of their appearance and from the regularity of their size can be considered histological elements of the same order. All the fibrils that stain selectively or are isolable with the various techniques leave us always in doubt as to whether they are real primitive fibrils or bundles of primitive fibrils. On the basis of these observations, Heidenhain has constructed a general doctrine concerning the structure of the fibrillar substance of muscle fiber. He suggests that this structure depends fundamentally on the linear arrangement of the smallest contractile particles (contractile molecules or Engelmann's "inotagmi").[2] The linear series of inotagmi or molecular fibrils would be the real primitive fibrils, which are invisible in the microscope; these, grouped in bundles, would form the fibrils, which are visible in the microscope ("*histologische Fibrillen*"); these, in their turn, grouped into bigger and bigger bundles, would form the muscle column (of different orders, according to Heidenhain), and finally the whole muscle fiber. From my own researches, data have not emerged that would allow me to state my position either for or against Heidenhain's ideas; I mention them only to explain the nomenclature I thought I should adopt so as to avoid confusion, since many authors use the two terms, fibril and muscle column, without a fixed rule, almost interchangeably. I shall use the expression muscle fibrils in a collective sense when I want to indicate the whole of the contractile elements of muscle fibers. In such cases, I think, the expression is right, if we admit that the primitive fibrils are real histological elements, visible and isolable, and if we admit that they in turn are formed of series of molecules which cannot be visualized with present techniques of investigation. In all other cases—namely, when we speak about isolated longitudinal parts of muscle fibers or of filaments selectively stained, or of divisions into fields of the cross-sections of the

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[2] The phrase "contractile molecules" has been vigorously criticized in a very recent paper by Apáthy (*Anat. Anz.*, Bd. XXI, N. 2, S. 80).

fiber—I shall use the expression muscle column, or the corresponding one, "*sarcostili.*" The muscle columns are made up of a succession of parts different in physical characteristics (*i.e.*, different behavior in polarized light) and different probably in chemical characteristics (*i.e.*, different affinity for stains and different behavior under the action of various reagents, alkali, acids, etc.). This regular succession of different parts is the fundamental reason for the striation. I shall adopt the letter nomenclature of Rollett to describe the striae; when I happen to use some of the older terms, I shall put the corresponding letter in parentheses. That part of a fiber included between two successive lines of Amici (Z) shall be indicated sometimes by the terms muscle unit or muscle segment—but this only for ease of description and without attributing to this expression any meaning to the effect that the muscle segments constitute real distinct parts of the muscle fiber.

FUSARI WAS THE first to think of applying the black reaction of Golgi to the study of striated muscle fiber. He published three successive notes on the subject in 1893 and 1894. In the first (33), he reports having observed in mammalian tongue muscle fibers treated with the black reaction a series of transverse lines formed of brown granules connected to one another by very fine filaments of the same color, while the rest of the fiber is colorless. The striation is sometimes simple, sometimes double; that is, in some instances there are single stripes following one another at equal intervals, and in other instances pairs of stripes each separated by a distance smaller than that which separates two successive pairs. The granules are in simple series in the double or paired lines and in the simple lines of the most finely striated fiber; in all other cases they are in double series. Transverse sections show a most elegant reticulum formed of the finest fibers with granules at their nodal points. When the line is double, observation in polarized light reveals that each series of granules is localized at the boundary between mono- and birefringent material; when the line is simple, Fusari cannot say with certainty whether the fusion is made at the expense of one or the other stratum, in other words, he cannot say whether the two lines unite and fuse together in the monorefringent or in the birefringent stratum.

In the second paper (34), Fusari gives an account of results obtained in continuing the study in a broader field. He confirms the preceding findings and, in addition, announces the discovery that in mammals the transverse reticula (which appear in longitudinal section as lines or stripes) are united to each other by longitudinal filaments running in the spaces between the contractile fibrils. However, many of these fibrils—the larger ones, according to Fusari—while appearing to be fibrils are in reality optical sections of impregnated sarcoplasm lamellae. The nuclei appear as colorless spaces, and the protoplasm surrounding them is usually colored and appears formed of a mass of granules in continuity

with the large longitudinal (ostensibly) fibrils. In transverse sections, Fusari confirms the existence of elegant reticula of selectively stained granular fibrils limiting the fields of Cohnheim, which sometimes are subdivided into smaller fields of finer and more homogeneous fibrils. In developing fibers, the granules and longitudinal filaments are more evident, the transverse reticula less evident. From the whole of his observations, Fusari reaches the conclusion that in general one must recognize three transverse reticula in each segment or muscle unit: one coincident with the line of Amici (Z), the other two with the boundary between the mono- and birefringent substance. Only in rare cases, however, can one expose all three reticula at the same time. Usually when the reticulum of the line of Amici (Z) is stained, the other two remain uncolored, or *vice versa.* In insects the number of stripes, and therefore of transverse reticula, is even greater; besides the three reticula cited above, there exist probably two additional reticula coincident with the accessory discs of Engelmann (N). In the wing muscles (dissociable muscles) of insects, there exist transverse reticula surrounding the fibrils with their meshes, and the lontitudinal filaments are lacking. In the same publication, Fusari also mentions another staining effect that obtains in some cases with the black reaction, namely, the selective impregnation of the birefringent tracts of the muscle columns; these tracts appear as prismatic bodies more intensely stained at the extremities than in the middle part, and some appear divided transversely into two equal halves.

In the last note (35), besides defining better several details, Fusari faces the general questions of the significance of the stainable reticula he discovered and their behavior during contraction. On the first question he expresses the opinion that the transverse reticula and the longitudinal filaments that join them should be considered formed from the primitive unmodified protoplasm of the striated muscle fiber (sarcoplasm of Rollett). He offers the following reasons:

1. Because the longitudinal filaments are larger and more conspicuous in the developing fibers;

2. Because in red muscles the longitudinal filaments are more developed and richer in granules than in white muscles;

3. Because in many fibers, there is below the sarcolemma a thin and continuous layer with stainable granular substance belonging to the sarcoplasm.

Concerning structural modification during contraction, Fusari calls attention again to the fact that the reticula situated at the level of the line of Amici (Z) appear closer to each other and (in transverse sections) the mesh of the reticulum appears broader in the contracted fiber than in the relaxed fiber. Furthermore, in the contracted fiber the double reticulum is lacking. Fusari explains its disappearance with the hypothesis that the substance of which it is composed (sarcoplasm), although stationary between the segments of primitive fibers con-

stituting the birefringent disc in the resting muscle, shifts at the moment of contraction towards the line of Amici.

Fusari's findings concerning the muscles of insects (both in limb muscles and in dissociable muscles of the wings) coincide, as far as it is possible to judge from the descriptions unaccompanied by illustrations, with those published several years earlier (1890) by Ramon y Cajal in two preliminary notes (95, 96), and immediately thereafter described and represented in an extensive work (97). However, there is a profound difference between the two authors' methods of interpreting the probably identical facts they observed: according to Fusari, the transverse reticula, joined with longitudinal filaments that are stainable with the black reaction in the muscles of insects, represent a structural detail of sarcoplasm and show precisely how much primitive sarcoplasm remains in the interstices between the muscle columns; according to Ramon y Cajal, the reticular apparatus is formed not by filaments but by canaliculi continuous with the tracheal canals and representing, therefore, a most complicated system of intracellular tracheal capillaries ramifying and anastomosing among themselves. Ramon y Cajal agrees with Fusari on the position of the reticula relative to the striae of the fiber in limb muscles, except that Ramon has seen only the two reticula situated at the boundary between the mono- and birefringent strata (at the limit of the birefringent layer, as he puts it), and has not observed the third reticulum coinciding with the line of Amici which, in fact, stains only with difficulty in the insects when the other two are stained (Fusari). Their results agree also with regard to the dissociable muscles of wings. Ramon, in fact, describes some transverse reticula at the level of the birefringent segments joined with short longitudinal filaments; as we have seen, Fusari also describes the transverse reticula, but he does not determine with precision their position with respect to the striation, and leaves in doubt the existence of the longitudinal fibrils. In the same work (98), Ramon hints that in the muscles of mammals, the Golgi method often stains "*deux séries de granules placés près de la ligne de Krause* (*Z*) *dont l'aspect et la situation coincident avec les disques accessoires de Van Gehuchten,*" and warns that the reticula formed of tracheal capillaries are not to be confused with the reticula of protoplasmic nature found at the membrane of Krause which, according to Ramon, are never stained by silver chromate. It is apparent also that the two series of granules seen by Ramon in mammals correspond to the double stripes Fusari saw in the same animals. We shall see in what follows how the two formations—namely, the series of granules in the muscles of mammals and the transverse reticula (considered tracheal capillaries) in insects—can, with a more profound study of the question, be brought together into a single series of invariant formations (even if of rather diverse character) in all striated muscle fiber.

EXPERIMENTAL TECHNIQUES

Of the methods of Apáthy, I have applied widely on the muscles of both vertebrates and invertebrates the one he called *Nachvergoldung*. Of the liquid fixatives I preferred the solution of sublimate, and sublimate with osmic acid. I followed scrupulously the method advised by Apáthy and obtained satisfactory results in most cases.

I should say the same concerning the method of Heidenhain with ferric hematoxylin: here I preferred to use pieces of tissue fixed in a mixture of sublimate and osmic acid; the results are of noteworthy fineness, when one succeeds in stopping the process of decoloration at the right moment.

At all events, a few words of explanation seem to me to be necessary on the black reaction of Golgi, the method I used by preference and to which I owe the larger part of the results which form the subject of the present paper. I used consistently the process called double impregnation which, as is well known, consists of four steps: immersion of the very fresh tissue in a mixture of osmium-bichromate; change after a variable time to a solution of silver nitrate; second immersion in a mixture of osmium-bichromate; second change to a solution of silver nitrate—repetition, in other words, of the classical process based on a successive action of a mixture of osmium-bichromate and silver nitrate.

The reaction on muscles is obtained with great ease, one might say invariably, when one takes the precaution of prolonging the initial immersion of specimens in the osmium-bichromate for 5 to 8 days (at an average temperature of about 15°) and of leaving them in the second osmic mixture for a very short period, 24 to 48 hours. The duration of the first or second baths in silver nitrate has no influence on the success of the reaction; only after a very prolonged immersion (months) in silver nitrate, especially under the action of light, do the specimens become black and useless. The succeeding treatment of pieces of muscle is different from that ordinarily used on pieces of central nervous organs because, for the observation of fine structural detail in striated muscle fiber, very thin and uniform sections are indispensable and can be obtained only by inclusion in paraffin.

When it is confirmed by a gross examination that the reaction has taken place, the specimens must be dehydrated quickly by passing them through a series of alcohols. Although rapid, the dehydration must be gradual and complete. Even if the pieces remain in alcohol for a total of 36 hours, the stain loses nothing of its fineness. After dehydration, the specimens are cleared in thick cedar wood oil, in which they can remain without damage even for several days. Usually a few hours is enough for the purpose. Then they are changed to paraffin (I used paraffin prepared by Grübler, according to Graf Spee, melting point 52°); the

duration of the paraffin bath should not exceed 5 or 6 hours. During this time, it is advisable to change the paraffin at least twice to remove the cedar oil completely. I consider it not entirely superfluous to describe the embedding process I used because, although it does not present anything special, the idea is very widely held that specimens treated by the Golgi method cannot be embedded in paraffin, and I consider this opinion entirely erroneous. Sections of the thickness of 5 to 10 μ should be affixed to slides and arranged in series; for removal of the paraffin, one may use xylene, taking account of the fact that sections must not remain [in the solvent] for a longer time than is exactly necessary.

Preparations thus obtained do not keep very well; if they are covered on slides with coverslips and kept in heavy cedar oil, after a few days they begin to become yellow. They keep somewhat longer stored in cedar oil uncovered; in this case it is necessary to apply the section to the lower face of the coverslip and to use a special windowed slide of wood, as recommended by Golgi. To remedy in some way the poor preservability of the preparation, I have formed the habit of saving from every series several sections attached to slides with the paraffin not removed; under these conditions, the colors will be preserved perfectly and one always has at hand some preparations suitable for demonstration. Naturally, at the moment of using them it is necessary to remove the paraffin with a rapid bath in xylene and to mount them in thick cedar oil.

The images obtained with this method (which does not offer the slightest technical difficulty and which succeeds in a rather consistent fashion) are of truly noteworthy fineness and precision. The figures I have drawn with the aid of a camera lucida (Apáthy's model), using two good apochromatic lenses (obj. 2 mm. and 1.5 mm., apochromatic aperture 1.30—Zeiss), give only an incomplete idea of this refinement, since it is not possible to reproduce in drawings the succession of planes observed in the preparation—a succession of planes which in our case is unusually extensive because the contrast between the intense coloration of the impregnated parts and the colorless and transparent background makes it possible to obtain clean images even of details situated in the depths of the preparation.

For study material, I used animals selected from diverse classes of vertebrates, some insects, and some crustaceans. From the mammals, I examined mice (*Mus rattus* and *Mus decumanus*), rats, cats, and several species of bats, at different periods of embryonic development and at different ages of extrauterine life; among the birds, the chicken and the pigeon (embryos and adults); among the reptiles, the lizard (embryos and adults), the green lizard, and several ophidia (*Tropidonotus natrix*); among the amphibia, the frog (tadpoles at various stages of development and adult individuals), and the triton; among the fishes, the *Cyprinus* carp and *Hippocampus brevirostris*. Among the insects, I studied with

greatest attention *Hydrophilus piceus* (larval form and mature insects), *Ditiscus marginalis*, the larvae of *Gastrophilus equi*, the larvae of *Musca vomitoria*; among the crustaceans, *Astacus fluviatilis* and *Carcinus maenas*.

Whenever it was possible, I extended my researches to diverse muscles of the organism; in particular, I took care to compare in mammals, birds, and fishes, the muscles with little sarcoplasm (white muscles) and the muscles with abundant sarcoplasm (red muscles) (Knoll, 61, 62); and in insects, the muscles of the limbs and those of the wings. In every case I paid great attention to the functional state, seeking to determine with maximum precision the variation in images in the state of rest and in the various periods or phases of contraction.

MAMMALS

My researches began when, having observed images of singular fineness in newborn mouse neck muscles treated with the black reaction (for other purposes), I was induced to extend my work to other muscles and other animals. In longitudinally sectioned muscle fibers (Plate 1, Fig. 1), I observed a stained reticulum formed by very fine mutually anastomosing filaments with small enlargements or granules at the nodal points. The mesh of the reticulum was predominantly elongated in the direction of the major axis of the fiber. Moving the fine adjustment on the microscope, one could see clearly that the reticulum was not limited to the surface of the muscle fiber, but occupied its entire thickness. The filaments of the reticulum were so thin and the meshes so small that they could not be distinguished at low magnification, and the fibers, where the reaction had taken place, appeared uniformly stained in a light coffee color. Transverse sections of the same muscle fiber (Plate 1, Fig. 2) permitted one to see a reticulum with rounded meshes occupying the entire section of a fiber and formed of threads with nodal enlargements identical in character to those revealed by longitudinal sections.

Two questions arise from these findings:

1. On which constituent part of the muscle fiber is the silver salt selectively deposited in this case?

2. What form does that part of the fiber really possess which is selectively stained and which appears in section as a reticulum of very thin filaments?

Observing the unstained part of the fiber with good apochromatic immersion lenses, one can see very clearly the longitudinal striation and, in certain points where the fiber is well extended, the transverse striation made by the succession of clear bands and less clear bands. With polarized light one sees that the darker bands are birefringent; the lighter ones monorefringent. To have precise information on the form and disposition of the muscle columns in the particular

category of fiber under examination, I made use of Apáthy's method (*Nachvergoldung*, on specimens fixed in sublimate and osmic acid) with which, as has been noted, the columns are selectively stained. With this method I examined several specimens belonging to the same animal (newborn mice) in whose muscles the reaction of the reticular apparatus had been achieved. In these preparations, the muscle fibers appear formed of bundles of straight thin columns arranged parallel to one another; at several points the columns are rather divergent, like the hairs of a brush, and towards the extremity of the fibers one can see single isolated columns. I shall not dwell on the particularities of form of the columns seen in these preparations; these have already been noted. It will suffice for me to affirm how these control studies demonstrate that in the special case, as has already been admitted in general by the majority of observers, fibers have a fibrillar structure and, what is more important, that the form and disposition of the muscle columns remove any doubt that the reticulum shown by the black reaction is due to a total or partial coloration of the muscle columns themselves. In consequence, it is necessary to admit that the appearance of the reticula depends on a staining of something in the interstices between the muscle columns, the sarcoplasm in other words, or a part of the sarcoplasm.

To the first question posed above, namely, into what constituent part of the muscle fiber does the silver salt selectively deposit, we may therefore reply that the silver salt is deposited into the sarcoplasm. It remains to be decided whether the structure stained is the entire sarcoplasm or a part of it (*i.e.*, a particular apparatus existing in the sarcoplasm). We shall see how other researches permit us to conclude that the second hypothesis is the true one.

To reply to the question, what form does that part of the fiber which stains selectively with the black reaction really have, it will suffice to reflect that only an apparatus consisting of anastomosed filaments can give in longitudinal and in transverse sections the images of reticula observed in our muscle fibers. That the filaments constituting the reticulum are true filaments and not artefacts brought about by colored lamellae is demonstrated by the succession of images that obtain through delicate movements of the fine adjustment. In this way, fixing one's attention on a single filament, it is possible to confirm that one is dealing truly with a single isolated thread surrounded on all sides by colorless transparent substance. I also obtained quite easily results identical with those first obtained in the neck muscles of the newborn mouse, in muscles of other parts of the mouse (limbs, dorsal muscles); the reticulum always showed up with the same characteristics and with the same completely irregular disposition. I observed no difference between the appearances of the reticulum in contracted fibers and in those fibers which, from the character of the transverse striation, could be considered fixed in a state of relaxation or in the initial periods of con-

traction. In mouse fetuses a few days before birth, the results were entirely comparable. Unfortunately for me (as it had been for Fusari), I did not succeed in tracing back to earlier periods of development because one arrives quickly at a stage in which the muscle fibers treated with the black reaction stain uniformly brown or black so that one can no longer distinguish any details of internal structure. After birth, however, it is easy to follow the changes in appearance that the stained muscle undergoes during development. In mice a few days old, muscle fibers already show interesting differences from those seen in newborn mice and in fetuses at term. In the muscle fibers in which both the longitudinal

NOTE ON THE PLATES

All figures were made with the aid of a camera lucida (Apáthy model), projecting the image on a sheet placed at the height of the preparation. Unless otherwise noted, the method of preparation is the black reaction of Golgi (double impregnation, rapid process).

PLATE 1

1 Muscle fiber of *Mus rattus* (white), newborn, longitudinal section. Obj. 2 mm. apo. Zeiss oc. 8 comp.

2 Same, transverse section.

3 Muscle fiber of *Mus rattus* (white), adult, fixed in relaxation. Longitudinal section. Obj. 2 mm. apo. Zeiss oc. 6 comp.

4 Same, transverse section.

5 Same, contraction wave, longitudinal section.

6 Muscle fiber of tongue of *Mus decumanus*, adult. Longitudinal section. Obj. 2 mm. apo. Zeiss oc. 8 comp.

7 Muscle fiber of a bat (species undetermined). Transverse section. Same magnification.

8 Same, fixed in relaxation. Longitudinal section. Same magnification.

9 Muscle fiber of fetus of *Cavia cobaya*. Longitudinal section. Same magnification.

10 Muscle fiber of *Columba livia*, adult, fixed in relaxation. Longitudinal section. Same magnification.

11 Same, transverse section.

12 Muscle fiber, 30 mm. long, of *Lacerta muralis* embryo. Longitudinal section. Same magnification.

13 Same, transverse section.

14 Muscle fiber of *Lacerta muralis*, adult. Transverse section. Same magnification.

15 Muscle fiber of *Lacerta muralis*, adult, fixed in relaxation. Longitudinal section. Same magnification.

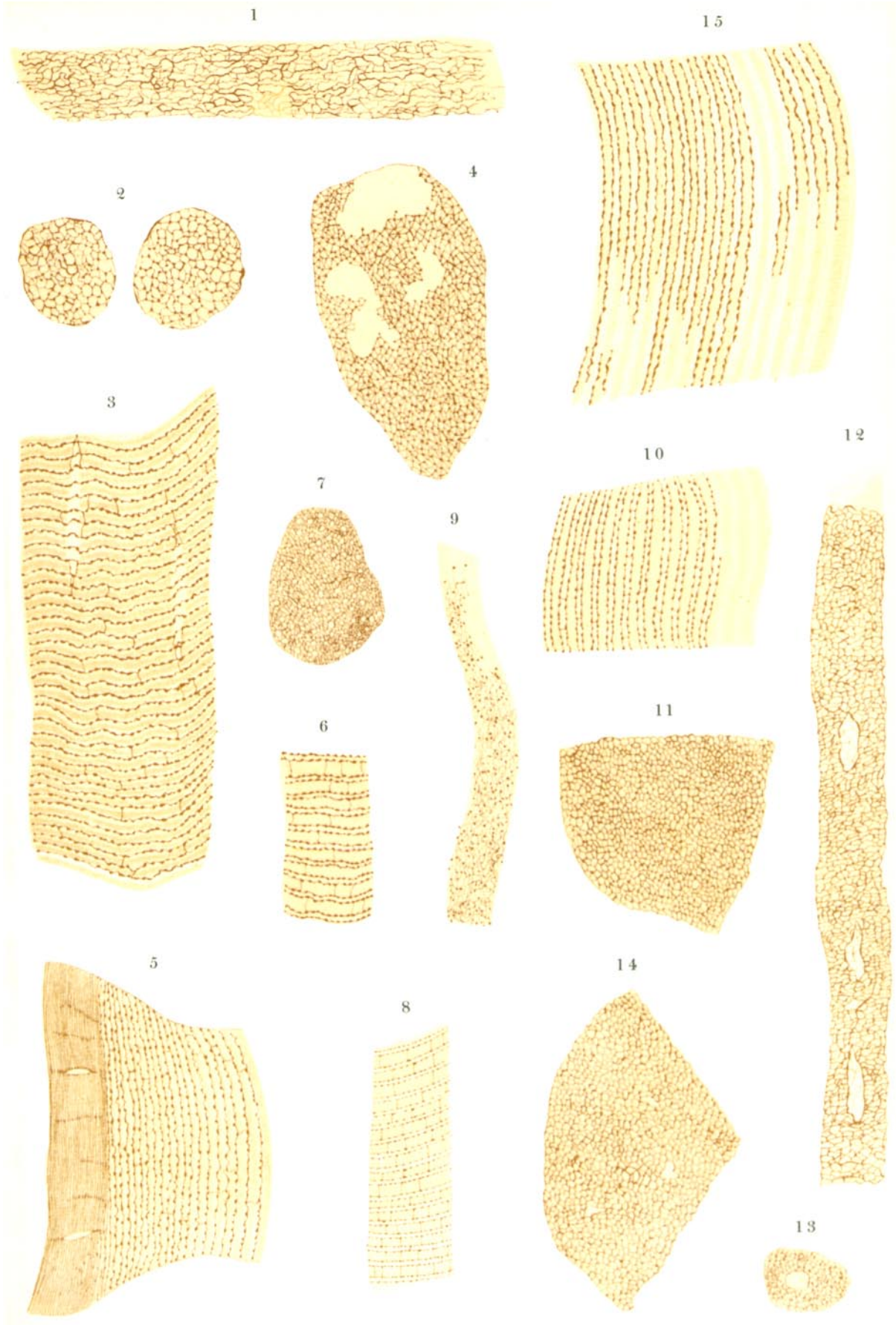

PLATE 1

and transverse striations are evident, a reticular apparatus is stained that occupies the entire thickness of the element, formed of anastomosed filaments entirely similar in form and thickness to those described in the case of the newborn mouse. However, the disposition is not at all irregular; a tendency of the constituent filaments of the reticulum to arrange themselves according to a particular order begins to be manifest. This tendency is more or less accentuated in the different fibers even of the same muscle. The majority of the filaments now have a longitudinal or transverse direction; a few, an intermediate direction. It follows from this that in longitudinal sections the fiber has more or less irregular transverse lines mutually united by longitudinal filaments, whereas in transverse sections one still sees the reticula with rounded meshes as in the newborn mouse muscle. The transverse segments are more abundant; the longitudinal filaments are rare and perhaps thinner; thus at low magnification the fiber in these conditions appears striated transversely by the very fine stained lines following one another at regular intervals.

In the adult mouse the transformation is complete; the reticulum which in the newborn mouse was completely irregular has acquired a geometric regularity: the fiber is crossed at regular intervals by a series of flat transverse reticula made up of the finest fibrils, with granules along their length and at nodal points, running in the interstices between the muscle columns; the transverse reticula are joined together by rather scarce longitudinal filaments. Thus in longitudinal sections the fiber shows equidistant transverse lines (Plate 1, Fig. 3), which are the optical sections of the transverse reticula, united by a few longitudinal lines. In transverse sections (Plate 1, Fig. 4) the reticula are seen from the front; they occupy the entire surface of the sections and define with their meshes the fields of Cohnheim surrounding the sections of the muscle columns. In oblique sections (relative to the long axis of the fiber) there is a combination of the two images. Sections in this direction are very instructive because they afford evidence that the part which stains with the black reaction is a true reticular apparatus made up of true filaments, and because with suitable movements of the fine adjustment it can be established with complete certainty that the transverse lines are nothing else but the edges of single reticula situated on successive planes across the fiber. Moreover, the examination of transverse sections permits the same conclusion and excludes the possibility that the transverse reticula are merely optical sections of a system of stained lamellae occupying the interstices between muscle columns. A secure judgment based on the succession of images obtained in moving the fine adjustment is made easier in the present case by the contrast between the intense staining of the filaments constituting the reticulum and the colorlessness and transparency of the remainder of the muscle fiber. My emphasis on this point will not seem unjustified when one considers the possible

confusion between my results and those obtained many times on transverse sections of muscle fibers, especially with gold chloride. Later on I shall have occasion to consider this argument more broadly and I hope to be able to demonstrate with facts that the similarities between the two series of results are only apparent.

If the images provided by the fibers at complete development are compared with those of the newborn and of the fetus, the difference is quite noticeable at first glance: In the latter we have a reticular apparatus with irregular meshes; in the former in longitudinal sections we only see transverse striations formed by intensely stained filaments, and only by an accurate examination and by comparison with transverse sections are we able to reveal that the striation is only a manifestation of a series of transverse reticula and that these are united to each other by longitudinal filaments. But if we follow step by step the modifications that occur during development and if we compare transverse sections with longitudinal ones, we shall easily become convinced that we are always dealing with the same reticular apparatus which, little by little as the muscle approaches complete development, is becoming more regular until, in muscle that has reached its ultimate form, it is arranged according to an architectonic plan governed by precise and invariant laws.

In adult muscles and particularly in fibers fixed in extension, we see the usual transverse striation made up of a succession of light and dark bands; often evident also is the line of Amici (Z). It is important to establish what position the transverse reticula occupy relative to the striation. On examination in ordinary light of the fiber in complete extension, or better still of fibers which have undergone gentle pulling beyond the limits of maximum physiological extension, one sees already that the stained lines occupy the median part of the light band (I + Z + I) (Plate 1, Fig. 3), that is, the exact place occupied by the line of Amici (Z). The plane of each transverse reticulum, therefore, coincides in each muscle segment with the plane of the line of Amici (Z). Observation with polarized light confirms this conclusion. One can easily see with crossed Nicol [prisms] that the lighter band is situated in the interval between two successive stained lines and is separated from these by a small dark field, while the stained line, consequently the transverse reticulum, occupies exactly the median zone of the monorefringent disc.

Whether the line of Amici that presents itself in fresh muscles or in those stained by the usual methods is due to the presence of the transverse reticulum or whether instead it is independent of the latter and due to some particular structure of the muscle columns (transverse or fundamental membrane), or whether in the long run it represents the optical result of the presence of both the reticulum and a particular structure of the fibril, is a difficult question which I

shall consider by speaking in general of the new interpretations of the images provided by unstained muscle fibers—interpretations that are reasonable on the basis of knowledge of the reticular apparatus present in these fibers. Here it behooves us only to clarify the relationship of the reticula with the striations from a topographical point of view.

In contracted fibers the images are different from those of relaxed muscle only in the fact that the transverse reticula (in longitudinal sections, the transverse stained lines) are much nearer to each other. As already noted, one of the universally recognized facts that characterize contraction is the convergence of the Z lines. Furthermore, the filaments constituting the reticula appear thinner, less granular, and of more regular disposition. To appreciate the full meaning of this last difference one must compare a contracted fiber with one stretched beyond the physiological limit: in the first, the transverse striations will be seen in the form of almost straight threads; in the second, in the form of filaments with a strongly undulated course. One must therefore admit that while in the contracted fiber each transverse reticulum is arranged in a plane, in a relaxed fiber the reticula, considered by themselves, represent undulating surfaces—and in the hyperextended fiber these undulations are exaggerated and more irregular.

One often finds some fibers presenting a fixed wave of contraction. In these cases, if the reaction has taken place, one can observe all the stages from a state of relaxation to a state of maximum contraction and can verify in the best possible way the modification the transverse reticula undergo in aspect and in disposition (Plate 1, Fig. 5). The undulating configuration of the transverse reticula in relaxed muscles can be a source of error in inducing the observer to conclude that there is a duplication in the stained striations. In point of fact, it should be recognized that when the threads making up the reticulum within the muscle fiber are not found all exactly in the same transverse plane, they are projected at different points along the longitudinal axis of the fiber and therefore, if one does not take into account the changes in images obtained in moving the fine adjustment, they can give the illusion of a double transverse striation.

Since the early researches of Fusari had as experimental material muscle fibers from the tongue of the mouse, I wanted to repeat the observations on that same organ. My observations agree exactly with those of Fusari. However, these observations present some differences from observations on muscles of the trunk and limbs. As study material I chose some adult specimens of *Mus decumanus.* I succeeded easily in demonstrating the reticular apparatus in a great number of tongue muscle fibers. In transverse sections there is an elegant reticulum of rounded or polygonal meshes entirely comparable to that already described in adult mouse limb muscles; in longitudinal sections, however, one notes a dif-

ference in the disposition of the striae that represent the transverse reticula: instead of a single transverse reticulum in the plane of the Z line, there are for each muscle segment two reticula situated individually at the boundary between the birefringent (Q) and monorefringent (I) layers (Plate 1, Fig. 6). This disposition is found in the majority of stained fibers. In contracted fibers also, even if the transverse reticula approach each other considerably so that examination becomes quite difficult, it seemed possible to me that one could still recognize the existence of the double reticulum limiting the Q layer. It may happen in these fibers that the difference between the distance separating the two reticula of a pair and that separating one pair from the next couple becomes so small that the individual reticula seem disposed at equal intervals. From this fact one could be led to believe that in certain fibers there is a simple striation (a single reticulum for each muscle segment), whereas in reality there is always the double type of striation and hence two reticula for each muscle segment. No matter how many studies I carried out, I never succeeded in observing any sign that would allow me to suspect that during contraction a fusion or even simply a contact occurred between the two transverse reticula of a single muscle segment. No matter how much they approach one another, these reticula always remain distinct. In the muscles of mouse tongue, the longitudinal filaments are rather scarce; those which join together the two reticula of each pair are rather more abundant than those which join the successive pairs. The filaments making up the reticular apparatus are provided with granules, particularly at nodal points. These granulations are not very abundant and should be clearly distinguished from the granulations (probably of a different nature) which exist in great number in the fibers under examination, but which are related to the reticular apparatus only by contiguity [mitochondria or sarcosomes, *Editor's note*].

In the limb muscles of the adult guinea pig, the results were identical to those described in the corresponding muscles of the mouse; it should only be mentioned that in the newborn guinea pig, the reticular apparatus already displays the regular disposition characteristic of the elements at complete development. In order to find in the guinea pig the irregular form of the reticulum corresponding to that described in the newborn mouse, it is necessary to go back to fetuses about 15 days before birth. It would seem that in the guinea pig, the muscle elements have already at birth attained a degree of development more advanced than in the mouse. This fact should not cause astonishment when one considers the generally greater development found at birth in the guinea pig in contrast to the mouse. Fig. 9 (Plate 1) reproduces an example of muscle fiber in the guinea pig fetus with the reticular apparatus of irregular conformation; only in certain points do filaments with a longitudinal or transverse direction appear slightly more prevalent than those with intermediate direction.

In the rabbit, the disposition, form, and modification of development of the reticular apparatus correspond to those I have described for the mouse.

To complete the exposition of data concerning mammals, a few words about the muscles of bats remain to be said. In my choice of this animal as an object of study, I was guided by a work of Rollett (108) in which certain very interesting observations were recorded. Treating the muscles of several common species of bat with gold chloride and by staining specimens fixed in alcohol with hematoxylin, Rollett was able to establish that in the fiber of almost all the muscles, but particularly in the pectoralis major, the serratus anticus, the intraspinatus, etc., the sarcoplasm (very abundant) was disposed between the muscle columns (very thin) in the form of cords united to each other by thin lamellae. Thus in transverse sections one can see circular areas stained with gold chloride, representing sections of the sarcoplasmic cords, joined to each other by lines which are sections of the lamellae. The precision of Rollett's description having been verified with control preparations, I thought that, were it possible to obtain the reaction of the reticular apparatus in these muscles, the results should be of consequence in deciding whether the structure stained with the reaction is the entire interstitial sarcoplasm or a special formation differentiated within the sarcoplasm. If the entire sarcoplasm stains, one should find in transverse sections of muscles in the bat (in those fibers which present the structure described by Rollett) some reticula formed of thin filaments joining thick circular masses (sections of sarcoplasmic cords); if instead the black reaction selectively stains a particular apparatus existing in the sarcoplasm, the form of the transverse reticula should be independent of the disposition of the interstitial sarcoplasm (in this special case). I shall say at once that it is the second case that proves true! In transverse sections reticula of extreme fineness are observed which show at nodal points only negligible enlargements; in longitudinal sections there is a double striation similar to that described in the tongue of the mouse. There are in each muscle unit, therefore, two stained striations (manifestation of the two transverse reticula) at the boundaries between the Q and I layers. Also noteworthy in the muscles of the bat, besides the already alluded to thinness and regularity of filaments, is the abundance of longitudinal filaments either joining the reticula within a pair or joining successive pairs. Figs. 7 and 8 are examples of transverse and of longitudinal sections of bat muscles reproduced from those fibers in which the structure described by Rollett (which can also be recognized without staining) was very accentuated. Thus we already have a factual reason for assuming that the reticular apparatus that stains with the black reaction does not represent the entire sarcoplasm residuum, but is a special formation differentiated within the sarcoplasm.

In summary, the results obtained by me with mammals are nothing more

than a simple confirmation of those of Fusari, except in the two following points:

1. I did not succeed in persuading myself (despite the powerful influence of Fusari's high authority on me) that the two striae of each pair in a fiber with double striation fuse during contraction into a single stria.

2. The researches with bats (confirmed largely in other animals, as I hope to show in reporting on fishes) compel me to deny that the reticular apparatus stained with the black reaction represents, as Fusari is inclined to conclude, all that remains of the interstitial sarcoplasm.

BIRDS

In birds, too, I started my investigations with embryonic forms. In the muscle fibers of the posterior limbs of chicken fetuses of 15 days' incubation, I obtained impregnation of a reticular apparatus consisting of very slender and quite irregularly oriented filaments similar to that observed in newborn mice and in guinea pig fetuses. I will not dwell on the description because I would have to repeat what I have already said. In the adult chicken and in the pigeon, however, the reticular apparatus presents the characteristic disposition peculiar to the fully developed elements. Figs. 10 and 11 (Plate 1) illustrate muscles in an adult pigeon; Fig. 10 is a longitudinal section, Fig. 11 a transverse section. These figures show that the reticular apparatus consists of a series of flat transverse reticula placed in pairs in each muscle unit and located at the boundaries of the Q layer and interconnected by a few longitudinal filaments. In extended muscles the distance between two reticula of each pair is smaller than the distance between two consecutive pairs; in contracted muscles both the striae of each pair and the pairs themselves tend to converge, but the reticula always remain separate. In spite of repeated examinations, I found no evidence to suggest fusion of two striae into one single stria. In other words, I found no evidence of a transition from the type characterized by the presence of two reticula to the type characterized by a single reticulum in each muscle segment. In pigeons the red muscles (with abundant sarcoplasm) are very numerous. Comparisons of the reticular apparatus in these muscles and the reticular apparatus in white muscles (with scanty sarcoplasm) did not reveal differences worthy of mention. The thickness of the filaments of the reticula in both types of muscles is identical. This fact also might be cited in support of the idea that the reaction does not stain the whole interstitial sarcoplasm; in fact, if that were the case, the reticular filaments in muscles richer in interstitial sarcoplasm would have to be thicker.

REPTILES

Figs. 12, 13, 14, and 15 of Plate 1 illustrate parts of specimens taken from muscles of the lizard. I selected this animal because it was possible for me to obtain the reaction in embryos as well as in adults, so that I was able to make a comparison between appearances of the reticular apparatus at various stages of development. The findings in muscles of the adult green lizard[3] and of the *Tropidonotus natrix* are very similar to those of the lizard. In the muscles of the dorsal mass of lizard embryos 30 mm. in length, the reticular apparatus consists of filaments of extreme fineness distinguishable only at very high power [magnifications] (obj. 2 mm., oc. 8 comp.). The arrangement of the filaments is highly irregular even in fibers that were cut exactly along their major axis (Plate 1, Fig. 12). In older embryos (near birth) one begins to discern the tendency of the threads to arrange themselves according to the laws already mentioned in describing the [ontogenic] modifications of reticula in mammalian muscle.

In adult lizards there is a reticular apparatus formed by paired transverse reticula interconnected by a few longitudinal filaments. The images of these muscles resemble those of bird muscles, as can be easily seen from a comparison of the corresponding illustrations (*cf.* Figs. 14 and 15 with, respectively, Figs. 11 and 10). Needless to say, it was necessary in each category of muscle to repeat the polarized light observations with maximum care in order to establish the position of the transverse reticula with respect to the striations within the fibers, since it did not seem to me that the generic similarity in the arrangement of the reticular apparatus in different animals was sufficient reason to extend specific conclusions from one animal to another. I will add nothing here concerning changes in appearance associated with the different stages of contraction, since I would simply repeat what I said with regard to birds.

AMPHIBIANS

I selected the frog and the newt as examples of this class of animals. To avoid repetitions, however, the description will concern only the frog—and the figures as well are derived exclusively from the frog—inasmuch as from our point of view no differences worthy of mention exist between these two species. Here too I attempted to extend my investigation to different stages of development, but I did not succeed in obtaining results as satisfactory as in the other classes of animals already considered. In very young tadpoles, in which limb formation had not yet begun, I did not succeed in obtaining the reaction on the reticular

[3] *Lacerto viridis.* Trans.

apparatus; most fibers remain unstained, and some stain in a mass of black. The reticular apparatus stains easily in both tail and limb muscles from tadpoles at a more advanced stage of development, but already shows the characteristics that we have seen to belong to fully developed elements. Therefore, it was not possible for me to find in the amphibians the confirmation of that succession of modifications in the structure of the reticular apparatus that, for the constancy with which they appear in mammals, birds, and reptiles, would seem to have the status of a law. The reticular apparatus in tadpoles always shows a regular arrangement; it consists of a series of transverse reticula formed by very thin filaments with a few very minute granules, and by sparse longitudinal filaments uniting the reticula to each other. In each muscle unit one finds a single transverse reticulum coincident with the Z line; there is no trace of the double reticulum at the limit of the Q band. The single transverse reticula are usually very close to one another. The reason for this peculiarity is to be found, I believe, in the fact that the muscles of tadpoles are nearly always fixed in a state of contraction due to the impossibility of holding their ends fast to keep the muscle extended at the time of fixation. Only when one comes upon a fiber that shows a fixed contraction wave can one see in the regions at the ends of the wave some segments that are in extension. One must make use of these cases to study the topographical relationships between the transverse reticula and the striation—in other words, to localize the transverse reticula in the muscle unit—since in contracted areas the reticula (and the stained transverse lines which represent them in longitudinal sections) are so close to each other as to render such a study almost impossible. It was precisely by studying the distal segments of the fixed contraction waves that I was able to establish that in tadpoles there is one transverse reticulum for each segment and that it is located in the plane of Amici's (Z) band. Beyond these general remarks . . . I have observed in the fibers of limbs a peculiarity that is of some theoretical interest: these fibers in young tadpoles are cylindrical in form; the muscle columns do not occupy the whole thickness of the element but are arranged in single or double series at the periphery below the sarcolemma; the axial portion of the fiber is free from the contractile fibrils and is occupied by a mass of undifferentiated sarcoplasm, in which are many nuclei arranged in series. Between two successive nuclei there is a narrow layer—a "partition" of sarcoplasm. Fig. 16 (Plate 2) shows a longitudinal section along the axis of a fiber with the above mentioned structure and in which the reticular apparatus has stained. The protoplasmic septa between the nuclei are crossed by bundles of interwoven filaments continuous with the filaments forming the transverse reticulum. The colored filaments in the sarcoplasmic layers interposed between the nuclei are more granular and have an aspect somewhat different from the filaments in the transverse reticula. This fact indicates

that the reticular apparatus does not exist only in the interstices between fibrils, but extends also into the undifferentiated sarcoplasm, whenever the latter is present in the fibers in noticeable quantity. A corresponding and more indicative finding is present in the muscles of the tadpole tail. In the fibers of these muscles differentiation of the fibrils in the primitive sarcoplasm occurs in accordance with laws different from those [operative] in limb muscles. The muscle columns are collected into a compact bundle occupying only a part of the full diameter of the cell and usually at an eccentric position. A mantle of sarcoplasm, whose thickness varies in the different peripheral areas of the fiber, surrounds the bundle of fibrils; the nuclei are located in this peripheral mass of sarcoplasm. With the black reaction one can see, in both longitudinal and transverse sections, that stained filaments continuous with the filaments that form the transverse reticula often push into the mass of undifferentiated sarcoplasm and reach the sarcolemma. Usually these filaments are thin, homogeneous, without granules, and with few ramifications. This observation has the same significance as the preceding one on limb muscles, *i.e.*, it confirms the conclusion that the reticular apparatus with its branches extends into the undifferentiated sarcoplasm. Then too, detection of selectively stained single filaments within large masses of sarcoplasm furnishes convincing evidence that the black reaction does not stain the whole of the residual sarcoplasm but only a special reticular formation differentiated within it.

In the adult frog the black reaction succeeds very easily. The reticular apparatus corresponds as a whole to that which we have described in the limb muscles of mammals: we have a single transverse reticulum in every muscle unit in the plane of the Z band; the longitudinal filaments between the transverse reticula are rather numerous. As much as I tried in these studies on the frog, varying the reaction conditions in many ways, I did not succeed in staining the two transverse reticula situated at the boundary between the mono- and birefringent substances (between Q and I).

Fig. 17 (Plate 2) shows a longitudinal section of a fiber fixed at the moment when a contraction wave was running through it. The corresponding preparation showed very clearly the structural peculiarities of the reticular apparatus—so clearly that I was easily able to reproduce them photographically. The differences in aspect between the filaments forming the transverse reticula in the segments corresponding to the ends of the wave and in those corresponding to the peak are accentuated. As I have already said, in describing analogous preparations of mouse muscle, the filaments in the extended parts are coarse and undulating in form, with large granules at the nodal points; in the contracted parts they are very thin, laid out rectilinearly, with a few small granules. I think it proper to call attention to the great advantages of the black reaction, in com-

parison with all the methods used so far, in the study of the contraction waves. Even though the stained striae, which correspond to the transverse reticula even in the central area of the wave, may be very close to each other, they are always quite distinct and stand out most clearly against the uncolored background. In the present preparation, for example, the clarity of the image shows how a small group of muscle columns in the area corresponding to the peak of a wave may present a region in a stage of relaxation—one could even speak of "a wave within the wave"—which confirms once again the well known power of one muscle column to contract independently of other columns. Both in tadpoles and in adult frogs, I have turned repeatedly to examination with polarized light to establish the position of the transverse reticula with respect to the striation. Fig. 18 represents a transverse section of a muscle fiber intended to show the arrangement of the meshes forming the transverse reticula when these are seen in one plane.

FISHES

In studying this class of animals, the idea that guided me in my choice of species was to find those which were known (through previous studies) to have muscle fibers with abundant sarcoplasm. I began by examining the muscles of the flank of *Cyprinus carpio*. In Kölliker's treatise (Vol. 1, page 358, Fig. 282) a transverse section of one of these muscles is reproduced. From the figure, which corresponds exactly to what one sees in the preparations, one notices that the muscle fibers are formed by muscle columns embedded in a large mass of sarcoplasm surrounded by the sarcolemma. Between the external boundary of the bundle of muscle columns and the sarcolemma, a thick layer of sarcoplasm intervenes, containing nuclei. The muscle column in transverse section has variable forms: in some fibers the rectangular form is prevalent (ribbon-like columns); in other instances, together with columns of the latter form, are columns polyhedral in shape (polygonal in transverse section). Whenever all the columns are ribbon-like, they group longitudinally, the wider surfaces coming into contact in simple series. Thus variably curved lamellae are formed which, intermingling with each other, give a characteristic appearance to the fiber in transverse section. Whenever ribbon-like columns and polyhedral columns are united in one fiber, the former occupy the central part, the latter are arranged in simple series at the periphery (*cf.* Emery, 23). Usually the sarcoplasm shows an abundance of granules stainable with osmic acid. The fibers in which the mass of the sarcoplasm below the sarcolemma is more abundant form small bundles which even with the naked eye can be identified by their brick red color. In these muscles, the black reaction reveals the reticular apparatus to be formed by a series of

transverse reticula, one in each muscle segment at the level of the Z band. There are no differences in the form and thickness of the filaments of the reticulum between the fibers with abundant sarcoplasm and those with less abundant sarcoplasm. The transverse sections of the fibers in which the reaction has occurred show the different forms of the muscle columns. Since the filaments of the transverse reticula run into the interstices between the columns, it is evident that there must be a perfect correspondence between the form of Cohnheim's fields and the form of the reticular meshes. Compare my Fig. 19 with Figs. 38 and 48 of Van Gehuchten's second paper (39). The peripheral layer of undifferentiated sarcoplasm remains unstained. If, when the reaction has gone to completion, we fix our attention on the fibers in which this layer is very abundant, we can see that stained filaments emerge from the reticular apparatus occupying the spaces between the muscle columns, cross the sarcoplasmic mantle in a radial direction, and occasionally reach the sarcolemma on the inner surface of which they seem to attach themselves by triangular processes. These filaments, which are sometimes simple and sometimes branched, are similar in thickness, form, and appearance to those that constitute the remainder of the reticular apparatus. The continuity of these filaments with the filaments of the transverse reticula is absolutely indisputable (Fig. 20). Longitudinal sections of the same fibers provide us with confirmation of the data already described: we see the stained lines, representing optical sections of the transverse reticula, continuing outside the bundle of the muscle columns with fine, occasionally branched fibrils. These fibrils, running tortuously in the sarcoplasmic mantle, go toward the sarcolemma and often reach it (Fig. 21). Most instructive is the comparison one can make in the fibers of red muscles of the carp between the results with the black reaction (described above) and those with the gold chloride method on fresh tissues. Of the various gold chloride methods, I chose that recommended by Ruffini. In fibers so stained one sees in transverse sections that, while the area occupied by the muscle columns shows a reticulum that seems to be analogous to the reticulum stained by the black reaction, the whole peripheral part formed by the undifferentiated sarcoplasm is as intensely stained as the lines which form this seeming reticulum.

The explanation is obvious: gold chloride stains the whole sarcoplasm, that is, both the laminae of sarcoplasm that occupy the interstices between the columns (laminae that in section appear as lines) and the peripheral residuum of undifferentiated sarcoplasm. The black reaction, however, stains only a part of the sarcoplasm whose shape is that of a reticular apparatus, leaving the remainder uncolored. It is natural that the difference between the results of the two techniques become evident only in the peripheral sarcoplasmic mass, because in the transverse sections a thin sarcoplasmic lamina interposed between

two columns and a filament running transversely through this lamina must appear identical whenever the filament is exactly in focus. I stress the condition that the filament be exactly in focus since by moving the fine adjustment we can at once observe in transverse sections differences between the reticulum stained with the black reaction and the "seeming reticulum" which under analogous conditions is demonstrated by gold chloride—in the former case by changing the position of the focal plane we witness the appearance and disappearance of a single filament (on which we have fixed our attention); in the latter case, this does not occur.

Naturally these observations made on the muscles of the carp have a general significance and must, in my opinion, be considered as having established criteria for making comparisons between images formed by various categories of muscle fiber treated with Golgi's method and corresponding categories of fresh fibers treated with gold chloride.

The same reasons that led me to study the red muscles *Cyprinus carpio* guided me also in my choice of *Hippocampus brevirostris*. In this animal I gave particular attention to the motor muscle of the dorsal fin. As has been demonstrated by Ranvier (100, 101), Rollett (107), and Kölliker (66), these muscles consist of fibers very rich in sarcoplasm. The muscle columns are ribbon-like in form (they appear rectangular in transverse section) and unite with each other in simple

PLATE 2

16 Muscle fiber of posterior limb of tadpole of *Rana esculenta*. Longitudinal section. Same magnification.

17 Muscle fiber of *Rana esculenta*, adult. Contraction wave. Longitudinal section. (From a photomicrograph obj. 2 mm. apo. Zeiss oc. proj. 2 [Focal] length of camera, 1 m.)

18 Same. Transverse section. Obj. 2 mm. apo. Zeiss oc. 6 comp.

19 Muscle fiber of *Cyprinus carpio*, adult. Transverse section. Obj. 2 mm. apo. Zeiss oc. 8 comp.

20 Same. Red muscles of the flank. Transverse section. Same magnification.

21 Same, fixed in relaxation. Longitudinal section. Same magnification.

22 Muscle fiber of *Hippocampus brevirostris*. Muscle of dorsal fin. Transverse section. Fixation, sublimate. Stained with carmallume. Obj. 2 mm. Zeiss oc. 4 comp.

23 Muscle fiber of *Hippocampus brevirostris*. Muscle of dorsal fin. Transverse section. Obj. 2 mm. apo. Zeiss oc. 6 comp.

24 Muscle fiber of limbs of *Carcinus maenas*, fixed in relaxation. Longitudinal section. Same magnification.

25 Same.

26 Same, transverse section.

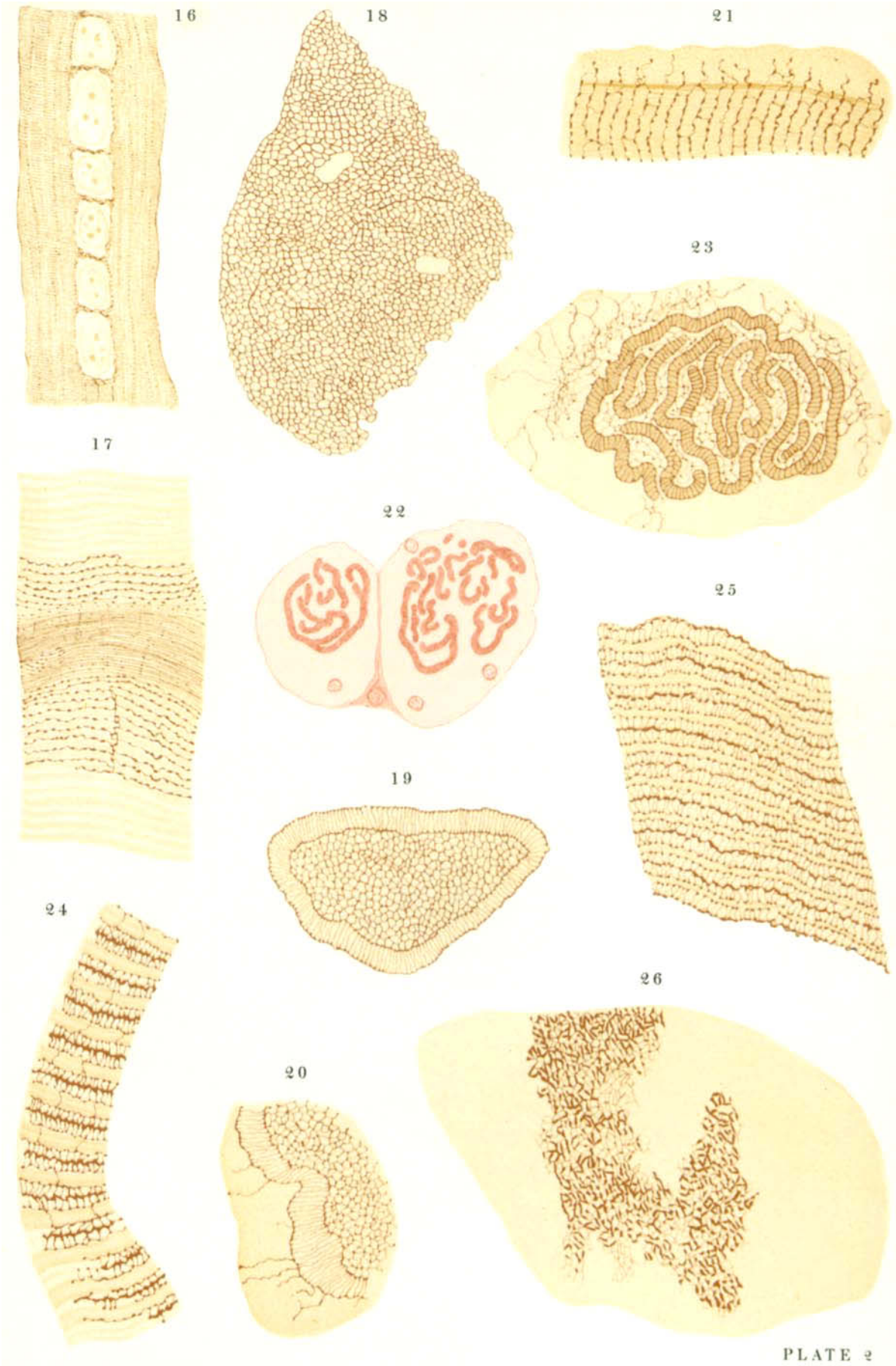

PLATE 2

series along their wider surfaces so as to form small laminae. While in red muscles of the carp the laminae formed by the meeting of a series of muscle columns are (at least in adult animals) tightly grouped in the central region of the fiber, in the sea horse the laminae are separated from each other by a considerable space occupied by sarcoplasm; in addition, in the sea horse a considerable layer of undifferentiated sarcoplasm separates the sarcolemma from the more peripheral muscle columns. In one and the same bundle there are fibers in which the number of laminae is reduced to two or three embedded in a large mass of sarcoplasm containing nuclei toward the periphery, and other fibers in which the number of laminae is greater and accordingly the amount of undifferentiated sarcoplasm is less. Fig. 22 (Plate 2), which is a transverse section of one of the fibers poorest in contractile fibrils and which corresponds exactly to the figures in Rollett's paper, gives a better idea than the text of the structure of these singular elements.

Other interesting peculiarities in muscles of the sea horse are revealed by the black reaction. In longitudinal sections, the fibers appear striated transversely by equally spaced stained lines located in the plane of Amici's (Z) band, in a way similar to that observed in the carp. In transverse sections, one sees that the lines depend also on the existence of transverse reticula but that these lines have a special form related to the great abundance of interstitial sarcoplasm by comparison with the mass of the muscle columns. The filaments constituting the transverse reticula in the contractile laminae form meshes that surround the single columns and adapt themselves to the rectangular form of the latter, and run within the thin sarcoplasmic laminae interposed between one column and another. In addition, they extend into the undifferentiated sarcoplasm, both between the laminae and into the peripheral area, and reach the sarcolemma. The meshes, which are, as I have said, rectangular and regular in the lamellae, become irregular and rounded in the undifferentiated sarcoplasm; the filaments show no differences in the two positions, either in form or in thickness. Fig. 23 shows a preparation in which the reticular apparatus was very clearly stained; the filaments running in the large protoplasmic mass which separates and surrounds the contractile lamellae are very numerous, and many of them can be seen to extend as far as the sarcolemma.

These findings seem to me to demonstrate the existence in the sarcoplasm of a reticular apparatus independent of the contractile fibrils that must be considered as a separate element of striated muscle fiber. In the muscles of the sea horse, that stainable filaments extend, branch, and anastomose in a mass of undifferentiated sarcoplasm far from the muscle column cannot be doubted and, in my opinion, the interpretation ought not to encounter objections. In view of the preceding descriptions, it will not be difficult to demonstrate that the

arrangement of elements seen with singular clarity in the specific muscle under discussion here is found in all muscles. Nor will it be difficult to establish a series of intermediate steps between forms that at first sight appear most dissimilar.

CRUSTACEANS

I obtained the best results from limb muscles of *Carcinus maenas*; treated with the black reaction, the muscle fibers of this animal give images of noteworthy subtlety and elegance. In essential features the structure of muscle in this animal corresponds to that in vertebrates; the black reaction selectively stains a reticular apparatus located in the interstices between the muscle columns and formed of a series of transverse reticula united by longitudinal filaments. While in the animals described so far there are in each muscle segment either one transverse reticulum (in the plane of the Z band), or two (between Q and I), in the present case the transverse reticula are three: one in the plane of the Z band, *and* two at the boundaries between Q and I (*i.e.*, at the two extremities of the I band). We have thus realized the arrangement that Fusari held to be characteristic of all muscles in general (with good reason, in my opinion), although he was only in rare instances able to expose the triple reticulum in one and the same fiber. On this account, Fusari supposed that a kind of incompatibility with the black reaction might exist such that when one reticulum stains, the other two reticula fail to stain, and *vice versa*. The reticulum located in the plane of Z is joined to the two reticula at the extremities of the I band by numerous longitudinal filaments which cross the monorefringent layer, and by a lesser number of filaments that cross through the birefringent layer. The reticulum coincident with Amici's (Z) band usually appears to be formed of filaments thicker than those that form the other two reticula and the connecting longitudinal strands (Plate 2, Figs. 24 and 25). By moving the fine adjustment it is possible to follow these thick transverse reticula through the whole interna of the fiber (when the fiber is sectioned in a plane oblique to its longitudinal axis). By this procedure, it is easy to form a clear and exact concept of the architecture of the reticular apparatus in its entirety and to be persuaded that it really consists of a series of transverse, flat reticula united by longitudinal filaments. The coarse appearance of the reticulum in the Z band is not a constant finding. On the contrary, when the black reaction turns out nicely, this reticulum shows itself to be formed by thin and delicate filaments similar in all respects to those of the other two reticula. In some instances, finally, within one reticulum are seen parts consisting of thick filaments and parts consisting of thin filaments (Fig. 25). I think these findings may be explained in the most obvious way by admitting that in the areas where the reticulum appears thicker, the black reaction has stained not only the reticular apparatus but also a portion of the sarcoplasm which surrounds the

filaments of the apparatus itself; in the areas where, instead, the reticulum appears thin, the black reaction has stained selectively only the filaments. There are other instances of similar events: the black reaction may at times stain a whole element, at times some parts of the same element. One need only recall the staining behavior of nervous tissues—in one preparation we can find some cells uniformly stained in black and other cells in which the reticular apparatus of Golgi stains selectively in the midst of an unstained protoplasm. Sometimes in one cell one part is massively stained in black, and another is unstained and contains a well differentiated reticulum.

WE FIND in transverse sections confirmation of the facts set forth as well as new arguments in support of the conclusions we have derived therefrom. Fig. 26 shows a section in the plane of Amici's band; we see the transverse reticulum in one plane. The major portion of the reticulum is formed of gross filaments with considerable thickenings along their course and at their nodal points. In some areas, however, the reticulum is formed of very thin and regular filaments with fine granules at the nodal points. A careful examination of the preparations permits me to conclude with certainty that the thick filaments are continuous with the thin filaments. This confirms the findings in longitudinal sections, *i.e.*, that in some cases the transverse reticulum of the Z band is partly formed by thick filaments and partly by thin filaments. In transverse sections the appearance of the thick portions of the reticulum supports the idea that in these areas a massive coloration has taken place in a part of the sarcoplasm that surrounds the filaments forming the reticulum. A similar event, in my opinion, occurs in those transverse sections in which are seen parts of the thick stained sarcoplasmic trabeculae from which the filaments of the reticulum appear to originate (Plate 3, Fig. 27). Here too we are dealing with massive staining of a part of the sarcoplasm. When the staining turns out well, these trabeculae are not stained; only a reticulum formed by thin and uniform filaments is stained.

In other transverse sections of well extended fibers, in which the transverse reticula are widely separated from each other, two superimposed reticula of different character can be seen. In Fig. 28, for instance, there is at the uppermost surface of the section a reticulum with thick filaments (reticulum of Z band) and at the lower plane, a reticulum with thin filaments (reticulum at the boundary of Q and I). The two tracts of reticulum are not continuous with each other. Their different positions in the vertical can be observed and measured with the fine adjustment on the microscope.

One of the most serious objections to the theory of the reticulum in muscle fibers, expressed most fully in Van Gehuchten's works, is that the (supposed) longitudinal filaments that form the reticulum are never found in transverse sections (Heidenhain, 54). Now, although the reticular apparatus described by

me has nothing in common, either anatomically or functionally, with Van Gehuchten's reticulum, the above objection might be raised to my conclusions also—perhaps with even more reason, given the very intense selective staining the black reaction imparts to the reticular apparatus. It is natural to think that the longitudinal filaments uniting the transverse reticula to each other, especially in cases where the transverse reticula lie at great distance, should appear in transverse section as stained granules. This does in fact occur in muscle fibers of the crab whenever they are fixed in complete extension.

With suitable movements of the fine adjustment, one can see the transverse reticulum disappear, and see appear in its place a series of very minute granules. These granules evidently represent the optical sections of longitudinal filaments that unite the transverse reticulum to the similar reticulum located immediately above or below.

Up to now I have described the appearance of the reticular apparatus in fibers fixed at maximal extension; we shall see now in the crab[4] how the fibers change during contraction. The first event, which occurs at the beginning of contraction, seems to be a drawing together of the three reticula of each fiber: the two reticula at the boundary between the mono- and birefringent layers approach the middle reticulum (opposite Amici's line). Although they draw close to each other, the three reticula always remain distinct from one another (Plate 3 Fig. 29). This agrees with what was seen generally in specimens treated with the

[4] *Carcinus maenas*. Trans.

PLATE 3

27 Muscle fiber of limbs of *Carcinus maenas*, fixed in relaxation. Transverse section. Obj. 2 mm. apo. Zeiss oc. 6 comp.

28 Same.

29 Muscle fiber of *Carcinus maenas*, fixed in initial stage of contraction. Longitudinal section.
Same magnification.

30 Fragment of muscle fiber of *Carcinus maenas*, fixed in contraction. Longitudinal section. Same magnification.

31 Muscle fiber of claw of *Astacus fluviatilis*, fixed in relaxation. Fragment of a longitudinal section. Obj. 2 mm. apo. Zeiss oc. 8 comp.

32 Muscle fiber of limbs of *Hydrophilus piceus*. Transverse section.
Same magnification.

33 Same. Fixed in extension. Longitudinal section.

34 Same.

35 Same, fixed in first stages of contraction.

36 Same. Obj. 2 mm. apo. Zeiss oc. 4 comp.

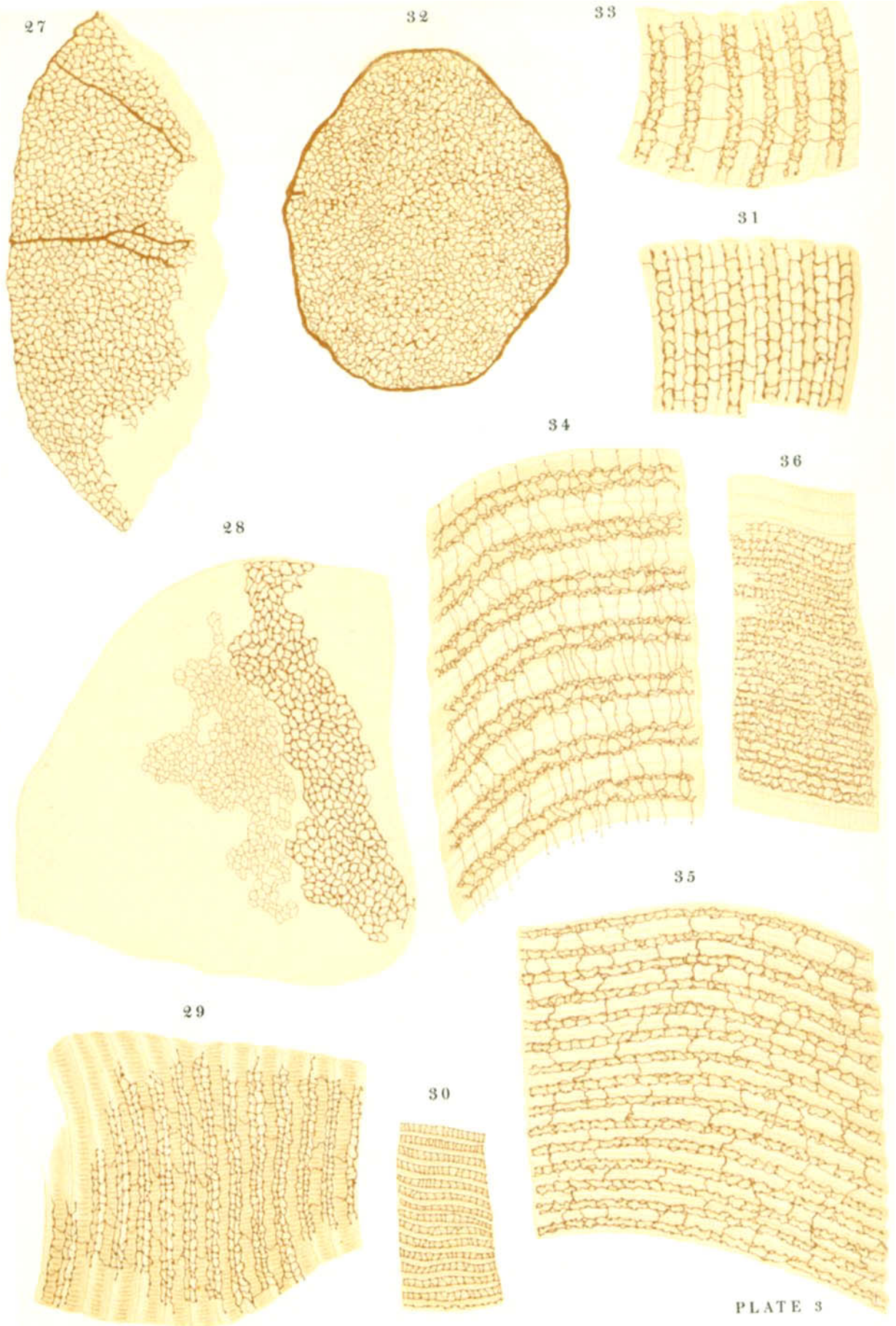

PLATE 3

ordinary methods—decrease in thickness of the monorefringent disc (Merkel, 82; Engelmann, 26–28; Rollett, 106). In a later stage, the groups of reticula draw nearer to one another because of the shortening of the birefringent segment. The initial drawing together may fail to occur when a mechanical obstacle prevents the shortening of a fiber that has gone into contraction (Ranvier's and Renaut's "contracted and stretched muscle"). In fully contracted fibers, the reticulum in Amici's band no longer stains; only two reticula are left in each muscle unit, those at the junction between Q and I, and these are close to each other, but always distinct, and connected by numerous longitudinal filaments. In these muscles too, as was noted in the muscles of vertebrates, the filaments which form the reticular apparatus in contracted fibers appear thinner and more regularly laid out (almost rectilinearly) than in the extended fibers (Fig. 30). This disappearance of (or better, the impossibility of staining) the reticulum in Amici's band in contracted fibers could give rise to suspicions that perhaps, in all these cases in which we have found fibers with only two reticula per muscle unit (mouse tongue, reptiles, birds), we were dealing with fibers in contraction. I believe, however, that this doubt cannot be sustained. Considering the large number of fibers examined, and the fact that in some of these fibers the striation was visible with ordinary light and with polarized light, the dimensions of the muscle segments (distance between two Amici bands) attested that we were dealing with fibers in extension. The doubt remains then that cases where there are only two reticula, as also those where there is only one reticulum, might represent incomplete reactions; and, although the general structure of the apparatus might be three transverse reticula in each segment, we cannot *always* attribute lack of reaction in one of the reticula to the state of contraction. Instead, we must attribute partial absences of staining to those strange selective properties that constitute at one and the same time the most precious quality and the major inconvenience of metal impregnation methods, and of the black reaction in particular.

In the muscles of the crayfish *Astacus fluviatilis*, I found structures similar to those found in *Carcinus maenas*. My investigations were mostly concerned with the muscles of the claws. In the large fibers of these muscles the reticular apparatus stains easily, and is formed both of very fine and regular, and more or less thick and granular filaments. The same explanations for the different aspects of the reticular apparatus in *Carcinus* may be applied to *Astacus fluviatilis*; *i.e.*, it may be reasonably assumed that, whenever the filaments appear thick and granular, the portion of the sarcoplasm surrounding the filaments themselves has also been stained.

Fig. 31 (Plate 3) shows in longitudinal section a fiber of the crayfish claw in complete extension. Shown clearly are three transverse stained striae correspond-

ing to three transverse reticula, one located in the plane of the Z band and the other two at the boundary between the birefringent and monorefringent layers. The longitudinal filaments that unite the three transverse reticula to each other are very numerous. In the preparations, the images are much more complicated than they appear in the figure, due to the fact that parts of the reticular apparatus located in different planes of the fibers are observed at the same time. In order to have demonstrative illustrations that give a clear idea of the arrangement of the reticular apparatus, it is necessary to examine very thin sections or those areas where only a partial reaction has taken place. It does not seem necessary to describe the findings in *Astacus* muscles since they are identical with those in *Carcinus*.

INSECTS

Investigations on this class of animals have a particular interest from several viewpoints. First, it is well known that the muscles of the large Coleoptera represent, so to speak, the material of choice for studying the structure of striated muscle fibers and especially the peculiarities of the striation. Secondly, it is in certain insects that a special kind of muscle is found, *i.e.*, the so called dissociable or fibrillar muscles of the wings, which have been the subject of active investigation and controversy. Lastly, it is on the insects that Ramon y Cajal carried out his investigations using the same methods I employed. In this regard, I feel it is my duty to declare that I have exercised maximum discretion in performing these control studies on the work of Ramon y Cajal. I have proceeded with the utmost circumspection, repeating the observations stubbornly in all those cases where my results did not seem to agree with those of the learned Spaniard. If the final conclusions are very different, I believe that no one can fail to appreciate that they represent a logical deduction from observed facts; nor would one accuse me of immoderate arrogance if I have dared to stand up against the well deserved authority of an eminent investigator, since I have done this solely in the interest of objective truth.

Among the insects I chose, first of all, for reasons of convenience, two species of Coleoptera commonly kept in laboratory aquaria, *i.e.*, *Hydrophilus piceus* and *Dytiscus marginatus*. Later on I studied some larvae of Diptera, to be precise *Gastrophilus equi*, and some species of the family *Muscidae*.

As far as *Hydrophilus* is concerned, I will describe the findings on the limb muscles separately from those on the dissociable muscles of the wings. When the reaction turns out well, images of fibers of limb muscles fixed in complete extension are very clear and elegant. On the unstained background in each muscle segment, two intensely brown stained transverse lines stand out at the boundary

between the monorefringent substance and the birefringent substance and are connected to each other by longitudinal lines (Plate 3, Fig. 33). These bands are none other than the two transverse reticula which are located in the above mentioned planes and extended through the interna of the fiber. In transverse section, these reticula are viewed from the top; the filaments that form these reticula are thin and regular and anastomose with each other to give rise to polygonal meshes with rounded angles (Fig. 32). The longitudinal filaments are more numerous between two reticula in a single muscle segment than between the far reticula of two neighboring segments; in other words, they are more abundant across the birefringent layer than across the monorefringent layer. We have seen that the opposite is true in *Carcinus*, but one need not attach great importance to this difference. Probably the number of real longitudinal filaments is greater than the number visible. As a result of different conditions under which the staining reaction takes place, at times a major portion, at other times a minor portion of the filaments may be stained. At times, the filaments coincident with the birefringent discs are dominant; at other times, those coincident with the monorefringent discs are prevalent. In Figs. 33 and 34 of Plate 3, which show well extended fibers, one sees that two lines representing the optical sections of the transverse reticula are very tortuous and in some places apparently doubled. This apparent doubling is preserved in the illustrations since I considered it important that these reproduce as exactly as possible the configurations visible in the specimens. I believe, however, that this doubling is apparent only, and is due to the fact that the transverse reticula (because they are perhaps pulled beyond the limit of maximum physiological extension) are not arranged in an exactly horizontal plane. From this it derives that when the reticulum is observed in section, the images of some of the meshes—to be more precise, those next to the cut surface—are projected not on one transverse line only, but on different places located above or below the line; therefore the line representing the optical section of the reticulum appears doubled in some places. This partially doubled appearance of the transverse striae is more pronounced in some fibers than in others and it disappears completely at the beginning of the contraction. In contracted fibers, as we will see, the transverse striae are simple and straight along their entire length. In *Hydrophilus* I have not been able to observe a reticulum at the level of Amici's band. Broad analogy in the arrangement of the reticular apparatus in these animals [insects] and in crustacea (in which, as we have seen, the third reticulum is always present) does not allow us to conclude from the absence of the reaction that the third reticulum is absent. The question must be left undecided.

In muscles going into contraction, the same alterations occur as were described in *Carcinus*. Differences are observed in these muscles too, depending on

whether the fibers are free to contract or are stimulated to contract while their ends are held fast. The typical arrangement of the transverse stained lines does not change; I have never observed the two striae in a single muscle unit fuse into a single stria. Although in completely contracted muscles they come very close to each other, they always remain separated. Figs. 35 and 36 show the appearance of fibers in the early stages of contraction.

I made some preparations of *Hydrophilus* limb muscles with Apáthy's method, and obtained images of some interest, although they are difficult to interpret. A preparation of this type is represented in Fig. 37 of Plate 4: Amici's (Z) band is intensely stained in violet (nearly black); the birefringent layer (Q) is selectively stained in copper red; the monorefringent layer at both sides of the Z band appears divided into three zones—one almost colorless zone contiguous to Amici's band, a wider middle zone, yellowish in color and of granular aspect, and a third zone similar in both width and color to the first zone between the aforementioned yellowish zone and the birefringent layer. The longitudinal striation is very evident throughout the fiber, and where it coincides with the Q region, one perceives clearly that the fiber is formed of a bundle of filaments—the muscle columns. Do these three lines differentiated in each half of the monorefringent layer (when Apáthy's method is used) correspond to the E, N, and I lines in Rollett's scheme? I cannot say with certainty, but if that were the case, then the fact that the middle layer (N—accessory disc) behaves so differently from the rest of the fiber might have some bearing on the obscure question of the significance of these discs.

We turn now to the muscles of the wings. In *Hydrophilus* these muscles belong, of course, to the category of dissociable muscles (fibrillar or atypical muscles, or Siebold's muscles). Their structure is now fairly well known as a result of the work of Kölliker (65), Aubert (7), Rouget (110), Merkel (82, 83), Biedermann (9), Ranvier (101), von Limbeck (72), Ciaccio (19), Mingazzini (84), Ramon y Cajal (98), Fusari (33–36), Van Gehuchten (38), Schäfer (113), Tourneux (117), Bütschli and Schewiakoff (14), Grummach (47), Luks (74), Petri (93), and others. From Kölliker's classic description, these muscles appear to be essentially formed by two parts—the muscle fibers and an interstitial substance or sarcoplasm. In addition there is an enveloping layer, and nuclei and branches of trachea among the fibrils. It is likely that, at least in some insects, the muscle fibrils of the wings are formed of bundles of thinner fibrils and correspond, therefore, to the columns of limb muscles. The sarcolemma in some species is clearly visible, in others, probably absent, and the primary bundles are covered by a special tissue formed of fat-containing cells. This is true in *Hydrophilus*: the sarcoplasm is very abundant and contains granulations (discovered by Kölliker), the chemical nature of which is unknown, disposed in sets between two fibrils

[sarcosomes, *Editor*]. The sarcoplasm contains also a small quantity of fat droplets. By treating the muscle of the wings in *Hydrophilus* with the black reaction it is possible most often to stain only the tracheal branches which run along the surface of the primary bundles and penetrate between the fibrils. When the reaction is more complete, however, one obtains impregnation of a system of very thin anastomosing filaments running in the interstitial sarcoplasm. The stained filaments form a true reticular apparatus which surrounds the muscle columns with its meshes. All the filaments run either in the longitudinal or transverse direction, so that one could suppose that in these muscles, as in those of the limbs, the reticular apparatus is formed by a series of transverse reticula connected by longitudinal filaments. In the present case, however, it is more difficult to establish the general structure of the reticular apparatus precisely because the reaction as a rule is not complete, but occurs only in small tracts of the fiber. It was also not clear to me what position the transverse tracts of the reticular apparatus occupied relative to the transverse striae of the contractile fibrils, because one cannot be sure in every instance that the reaction is complete, *i.e.*, that all the filaments have been stained. Figs. 38 and 39 (Plate 4) reproduce exactly the appearance of the reticular apparatus in transverse and longitudinal sections, respectively. The filaments which form the reticular apparatus are inserted on the tracheal branches that run in the interior of the fibers. By examining the areas of attachment at very high magnification, one sees that the contact occurs in different ways: at times a fibril of the reticulum enlarges into a club shape and this enlarged end attaches to the wall of the tracheal canal; at other times the filament divides into two divergent branches and these attach themselves to the trachea separately at a very short distance from each other. I was never able to see any image suggesting that the cavity of tracheal canals is continuous, even for a very short way, with the initial part of the reticulum filament that attaches itself to the trachea.

Comparing my pictures of limb muscles and wing muscles of *Hydrophilus* with Cajal's pictures of the limb muscles of *Acridium italicum* and *Ateucus sacer* and of the wing muscles of the latter, it is not possible to doubt that we find ourselves with identical facts. Cajal has concluded that the reticular apparatus shown by the black reaction in both categories of muscles can only be a system of terminal tracheal capillaries which penetrate into the muscle fiber and arrange themselves in it according to specific laws. The main—in fact, the only—argument put forward by Cajal in support of this interpretation is that the extrafascicular tracheae are continuous with the filaments of the reticulum. It is generally assumed that the trachea run on the sarcolemma without penetrating into the muscle fiber. In the dissociable muscles of the wings, on the contrary, most of the investigators assume (especially after Kölliker's and Cajal's work) that tracheal branches,

maintaining their characteristic structure, penetrate the fibers running within the sarcoplasm between the muscle columns. In preliminary observations with the routine methods, I have been able to corroborate the above mentioned facts. It is doubtful that true continuity exists between the tracheal canals located on the surface of the sarcolemma and the filaments of the reticular apparatus, at least in the limb muscles of insects I have examined. I was never able to observe a case in which a filament of the reticular apparatus clearly derived from a trachea. Since the tracheae run at the external surface of the sarcolemma and the filaments of the reticular apparatus come into contact with the internal surface of this membrane, it is natural that very often superpositions may occur such as to simulate continuity. Frequently I too observed images corresponding to Cajal's, that is, muscle fibers that showed fragments of stained tracheal canals adhering to their surfaces, and showed a similarly stained reticular apparatus in their interna. But on close examination I never found a case of definite continuity between the tracheal canal and the filaments of the reticulum. In that case, doubts that what was involved was a mere overlapping could be safely excluded. In the wing muscles, as we have noted, the filaments of the reticulum become attached, in some way, to the walls of the interfascicular tracheal canals. In that case, then, the contact relationships are multiple and clearly demonstrable. My conviction, however, that Ramon y Cajal's interpretation cannot be accepted is based not only on the doubt that may be raised against continuity (in some muscles) between the trachea and the reticular apparatus. I can submit decisive evidence against this interpretation. In muscles of crustaceans (*Astacus fluviatilis* and *Carcinus maenas*) the black reaction stains a reticular apparatus that is altogether analogous to the reticular apparatus described by Cajal in the limb muscles of an insect. Now, one cannot think of trachea or of tracheal capillaries in crustaceans. Comparison of Figs. 24, 25, and 31 with Figs. 33 and 34 will suffice to demonstrate that the reticular apparatus in muscles of crustaceans and the reticular apparatus in muscles of insects merely represent two types of one and the same formation. In both instances the form and the thickness of filaments are identical as are the ways in which the filaments are oriented and anastomose with each other, the general arrangement of the apparatus, and the relationship between the transverse reticula that form the apparatus, and the striation. The only difference in the figures is that in the muscles of *Hydrophilus* the reticulum of the Z band is not stained, whereas it is stained in the crustacean muscles. In this connection, I have already mentioned that, in all likelihood, the absence of the reticulum at the Z band is due to incomplete reaction. Furthermore I have seen this absence of the reticulum very often also in the muscles of the crayfish and of *Carcinus*. In these instances the analogy with the muscles of *Hydrophilus* is complete even in this particular.

Going up higher on the zoological scale, I have observed that the reticular apparatus, sometimes with considerable variation in details, but always maintaining unaltered its general structural characteristics, occurs in the muscles of vertebrates right up to the mammals.

The analogies in structure of the reticulum are very broad [in scope] even between muscles of species widely separated in the animal kingdom. Thus, comparisons between Figs. 2D and 3 in Cajal's paper (97, pages 339–40) with my figures of the muscles of mouse tongue, adult pigeon, and lizard give striking evidence of the almost identical structure of the reticular apparatus in animals of very different organization.

Once it is established that the reticular apparatus is not a special formation of the muscles of insects, but is present with unimportant modifications in the whole animal kingdom, it cannot be admitted that this apparatus in insects is a derivation of the tracheal system. This argument cannot be strictly applied to the dissociable muscles of wings because no muscles other than these in insects have this peculiar structure. In this instance we lack an animal without trachea from which to take a sample that might be used for comparison. On the basis of the analogy, however, I am inclined to assume that even in wing muscles the reticular apparatus is not a system of tracheal capillaries, but a special formation which differentiates within the sarcoplasm, as I have assumed in all other muscles. I recognize, however, that the argument I have brought forward concerning the limb muscles (in my opinion, incontrovertible in itself), is of little value in this special connection. I do not think that much importance should be placed on the contact relationships between the intrafascicular tracheae and the filaments of the reticulum; nothing prevents the assumption that the filaments themselves adhere to the tracheae in the same way they adhere to the internal surface of the sarcolemma. If a comparison between such dissimilar objects were not too daring, I would say that the filaments attach to the tracheae in the same way that neuroglial cell processes become attached to the walls of blood vessels! Against Cajal's interpretation one might also bring the fact that the tracheal capillary system admitted by him to exist in muscles has no counterpart in other organs. As a matter of fact, Holmgren (57), who has studied the tracheae with the black reaction, in referring to Cajal's investigation (without expressing a judgment) is constrained to recognize that, by accepting this author's opinions, the tracheae in the muscles of insects would assume "eine gewissermassen besondere Stellung." The modern authors, Holmgren (56, 57), Wahl (120), Wistinghausen (122), Wielowiejski (121), by now agree in admitting that the tracheae—formed of a cellular layer or matrix (named "peritoneal lining" by some authors) which, like the glands, originates from the ectoderm, and of a chitinous intima produced by elaboration of the

epithelial layer—terminate by penetrating special multipolar cells that anastomose with each other by way of their processes (Schültze's tracheal end cells). In the body of the end cells, the tracheal canal is continuous with canals hollowed out of the cell protoplasm and lined with a chitinous layer. These canals penetrate into the processes and join the canals of the neighboring cells so as to form a system of closed canals, the so called terminal capillary reticulum of the tracheal system. Holmgren, who has been keenly interested in studying the relationships between the tracheal capillaries and certain glandular elements in caterpillars, is of the opinion that the tracheal capillaries (excavated processes of the terminal cells) do not definitely enter into the gland cells, but become embedded in the latter by depressing the cell membrane, so that this membrane always separates the tracheal capillary from the cell protoplasm. Holmgren therefore, in agreement with Kölliker, assumes that the tracheal capillaries actually have a peri- and not an intracellular course, although this is contrary to what one would suppose from gross appearances.

Naturally, I do not intend to conclude either that similar conditions are found in muscles or that the intrafascicular tracheae in wing muscles are to be considered in the same way as the glandular tracheae studied by Holmgren. I will only state that it seems to me difficult to reconcile general knowledge of the structure and embryonic development of the tracheae with the hypothesis that the reticular apparatus demonstrable with the black reaction in muscle fibers in insects is formed of tracheae.

The muscles in larvae of the gadfly were recently investigated by Enderlein (24), who found that some fibers are formed of a thin bundle of fibrils embedded in a large mass of sarcoplasm surrounded by a thick and shining sarcolemma. Coincident with Amici's (Z) band there is a layer of more intensely stained substance which represents a transverse septum that extends even beyond the

PLATE 4

37 Muscle fiber of limbs of *Hydrophilus piceus*, fixed in extension. Longitudinal section. *Nachvergoldung* (Apáthy) method; fixation, osmic sublimate. Obj. 2 mm. apo. Zeiss oc. 8 comp.

38 Muscle fiber of wings of *Hydrophilus piceus*. Transverse section. Obj. 2 mm. apo. Zeiss oc. 4 comp. tr. = tracheae.

39 Same. Longitudinal section. Obj. 2 mm. apo. Zeiss oc. 8 comp.

40 Muscle fiber of larva of *Gastrophilus equi*. Portion of longitudinal section. Fixation, sublimate and osmic acid. Obj. 4 mm. apo. Zeiss oc. 4 comp.

41 and 42 Same. fixed in extension. Fragments of longitudinal section. Obj. 2 mm. apo. Zeiss oc. 4 comp.

43 and 44 Same. Obj. 2 mm. apo. Zeiss oc. 8 comp.

small bundles of the fibrils, across the sarcoplasm and as far as the sarcolemma, with which the septum seems to be intimately connected. Two, and even three, neighboring septa, in the tract coincident with the peripheral sarcoplasmic layer, may reunite into one bundle. Thin stainable fibrils unite the septa by running between the muscle columns. First of all, I carried out control investigations with the methods used by Enderlein (fixation in alcoholic sublimate and staining with hematoxylin) and I have been able to confirm his findings. Continuing the investigations, I found that boiling alcoholic acetic sublimate is a good fixative. Larvae immersed for some minutes in this fluid may be easily stripped of their chitinous covering, while the underlying muscular layer remains intact. Larvae decorticated in this manner are left for several hours in the fixative at low temperatures. After this treatment, embedding the material and preparing serial sections present no further difficulties.

In my preparations all the fibers showed the same characteristic structure: the cavity bounded by the sarcolemma, which is very wide compared to the thickness of the bundle or of the small bundles of muscle columns contained therein, is filled with sarcoplasm. The latter appears as a system of trabeculae or septa separated from each other by empty spaces (wide vacuoles). Actually, it is likely that these vacuoles in the living state are occupied by fluid material or by substances soluble in the reagent used in the preparation technique. This is why it seems more appropriate to say that the whole sarcolemmic sac is filled with sarcoplasm, in which two parts may be identified by means of the reagents: a stainable part, arranged in the form of septa and trabeculae, and an unstainable and homogeneous part. The stainable part contains nuclei around each of which the compact sarcoplasm appears as a mass continuous with the septa and the trabeculae. In sections, the perinuclear sarcoplasmic masses give the appearance of cell bodies; the trabeculae, which originate from the cell bodies, appear to be processes. As shown in Fig. 40 (Plate 4) one would think at first sight that within the sarcolemma of each muscle fiber are multipolar cells which send their processes towards the bundle of muscle columns; the processes with their anastomosing branches would seem to form a system of trabeculae that surround the muscle column at regular intervals. But this interpretation would be completely incorrect. The whole muscle element, *i.e.*, the whole content of the capacious sarcolemmic sac, is morphologically equivalent to a cell or an aggregate of cells within which fibrils are differentiated from protoplasm. The ramifying cell appearance is due to the fact that a part of the protoplasm is not stainable or is extracted by the reagents, so that wide vacuoles are left in its place.

Given this peculiar structure, naturally I wished to investigate the larvae of the gadfly with the black reaction in order to demonstrate the reticular ap-

paratus and, above all, to see how this apparatus behaved in that part of sarcoplasm located outside the muscle columns. In a sense, the larvae of the gadfly present the same favorable conditions as the muscles with abundant sarcoplasm in fishes, insofar as they reveal the reticular apparatus in masses of free sarcoplasm outside the muscle columns.

Technical procedures must be considerably modified to adapt them to the specific conditions of this tissue. As a rule, I use a Pravaz syringe to inject an osmic [acid] mixture into the general cavity of the living larva at sufficient pressure to distend the larva completely. I keep the syringe in place and the fluid under pressure for a few moments, long enough to fix the muscles in extension. Then I divide the larva longitudinally, carefully remove the viscera so as to uncover the muscular layer attached to the rings of the chitinous envelope, and put the tissue fragments into the fixative. The next step is the usual double impregnation. After the reaction has taken place (which I ascertain by making glycerin preparations of lacerated muscle fragments), if the larvae are young, the tissue fragments are embedded in paraffin; if the larvae are large and the chitinous layer cannot be sectioned evenly, the muscular layer is isolated in small shreds which are then embedded separately.

In the muscle fibers of the gadfly larva prepared by this technique, I observed a very complex and delicate reticular apparatus. The fibrils stained with the black reaction that form the reticular apparatus run within the trabeculae of compact and stainable sarcoplasm (mentioned above), and penetrate both into that portion of the trabeculae related to the bundle of muscle columns and into that portion located outside the columns across the wide space between these and the sarcolemma. Where it coincides with the muscle bundles, the reticular apparatus is formed of a series of transverse reticula—three in each muscle segment. One is located in the plane of the Z band, the other two at the boundaries of the birefringent layer (Q). The reticula are connected by longitudinal filaments (very numerous as a rule) running between the contractile fibrils. In the trabeculae, in the area outside the bundle of muscle columns, the reticular apparatus continues in the form of bundles of intertwined anastomosed and twisted fibrils. In the mass of sarcoplasm surrounding the nuclei, there is a very elegant reticulum with rounded meshes that winds around the nucleus and is continuous with the filaments in the trabeculae which go out from the mass of sarcoplasm toward both the central bundle and the sarcoplasm (Plate 4, Fig. 42; *cf.* Fig. 40). In the portion of the reticular apparatus that occupies the interstices between the muscle columns, the analogy both in arrangement and in structure with the findings in the muscles of *Carcinus* is self-evident. We find a close resemblance, especially, in the appearance of the reticulum in Amici's (Z) band. In some preparations, this reticulum appears

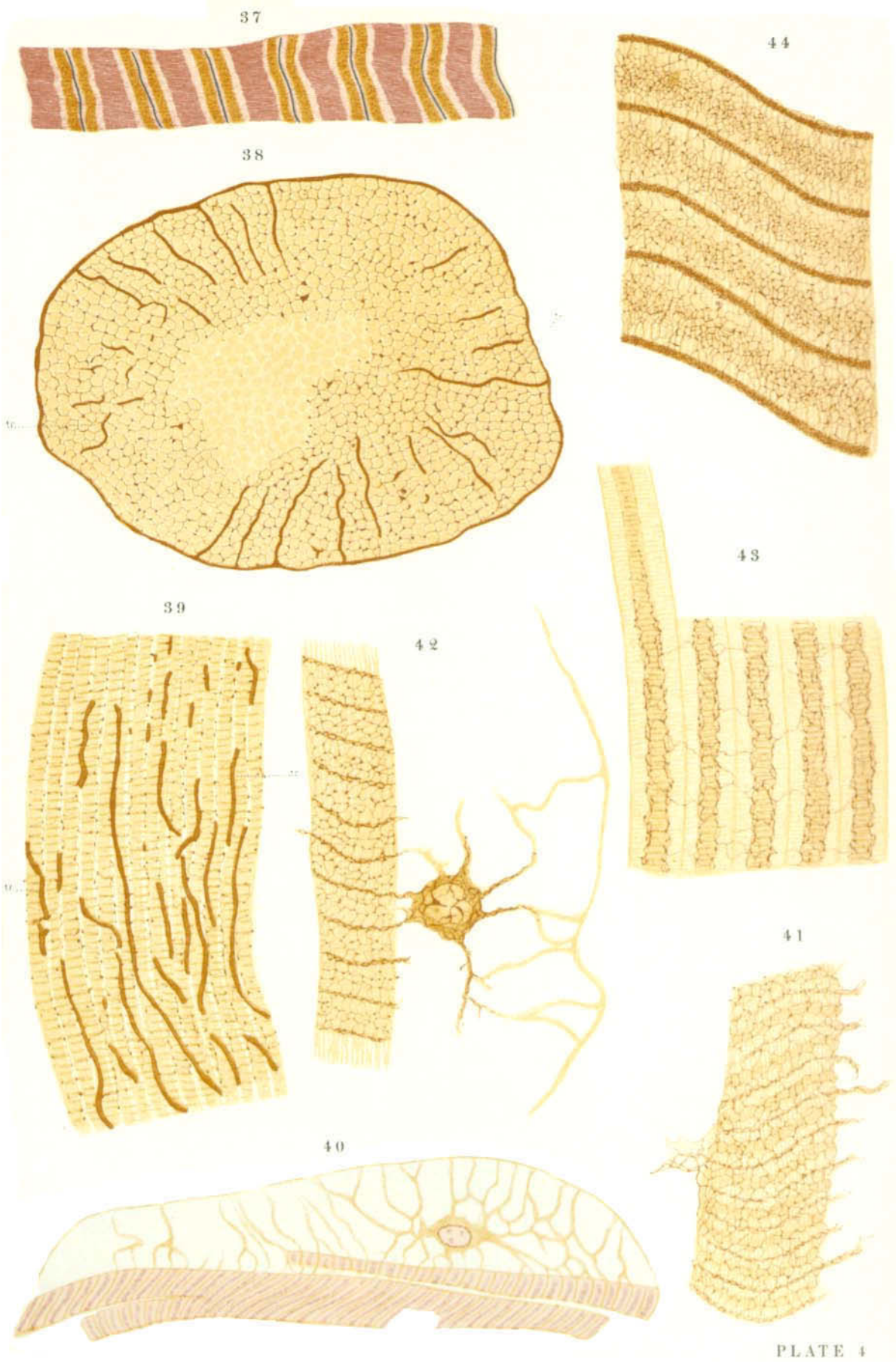

PLATE 4

to be formed of thin and regular filaments quite distinct throughout their course (Fig. 41); in others, the reticulum appears to be formed of thick and granular filaments, sometimes of a mass of granular substance in which the filaments are not distinguishable at all (Fig. 44). It seems to me that one can apply to these last two instances the explanation, proposed and supported with factual data in connection with *Carcinus*, that sarcoplasmic material stained with the black reaction has been deposited on the filaments of the reticulum. The typical arrangement of the reticular apparatus in three transverse reticula per muscle unit can be clearly observed only in certain cases. The two reticula at the limits of the Q band are often more or less irregular; the filaments that unite them are very abundant so that the whole Q band seems to be occupied by an irregular plait of filaments rather than by two distinct reticula at its boundaries (Fig. 44). However, one can see all the transitional stages from these configurations (which probably belong to the overextended fibers) to the perfectly regular forms. Rarely, the reticulum of the Z band is not impregnated, in which case the overall picture of the muscles of the larvae of the gadfly blends with that of the limb muscles of *Hydrophilus* (Fig. 43).

Since we are dealing here with tracheal-breathing animals, and in view of the precedent set by Ramon's studies, it is necessary to give consideration to the hypothesis that the reticular apparatus may perchance be formed of tracheal capillaries, especially because the sarcoplasmic masses winding around the nuclei (ramified pseudocells), (when the reticulum has been stained with the black reaction), roughly resemble the terminal cells of the tracheae. Against this hypothesis, I can only repeat the reasoning founded on the analogous—in fact identical—nature in both arrangement and structure of the reticular apparatus in corresponding systems found in animals without trachea (crustaceans). The reticular apparatus in the muscles of the gadfly and in the muscles of *Carcinus* resemble each other so closely that it would seem absurd to consider them as different structures. The certain and easily detectable continuity between the filaments which form the interstitial portion of the apparatus (the portion associated with the bundle of muscle columns) and those in the free trabeculae and around the nuclei forces us to assume that there is only one formation occupying the whole muscle fiber. With ordinary methods the tracheal canals are seen to run at the surface of the sarcolemma; I could never observe definite penetration of tracheal canals through this membrane. The trabeculae of sarcoplasm stainable by the ordinary methods are very different in aspect from the tracheae and are seen to end by implanting themselves with a triangular enlargement on the inner surface of the sarcolemma. At the points of implantation, the sarcolemma never shows any trace of perforation as would be the case if tracheal branches did penetrate into the trabeculae. Excluding

the hypothesis that the reticular apparatus is formed of tracheal capillaries and granting that it has the same significance here that we have attributed to it in all other animals examined, we see that the idea that the reticular apparatus is a special formation differentiated within the sarcoplasm alongside the contractile fibrils is brilliantly corroborated (especially if close attention is given that part of the sarcoplasm located outside the bundle of fibrils) by the findings in the muscles of gadfly larvae.

UP TO NOW I have reported my findings with the black reaction without mentioning the relationships my studies may have with previous work. I thought it advisable to leave comparisons and critical observations until last because it seemed to me that a report which is too often interrupted by long digressions might be less clear.

Having established that the reticular apparatus stainable with the black reaction is located within the sarcoplasm or, in other words, that on the basis of its response to the black reaction it must be considered as a distinct part of the sarcoplasm (*i.e.*, on account of its particular affinity for the salt formed within the tissues from the combination of potassium bichromate and silver nitrate), and having demonstrated that the reticular apparatus has nothing in common with the muscle columns, but is simply contiguous to them, it is clear that my investigations are not related to the much debated questions of the structure of contractile elements (muscle fibrils), nor to the theories intended to explain the secret mechanism of contraction.

My task is limited to elucidating a fine structural feature of the sarcoplasm. This feature is manifested in a system of reticula up to the present time only partially known, through the work of Fusari. Actually, as Fusari and Ramon y Cajal have pointed out, the black reaction sometimes stains the segments of muscle columns also, preferentially the birefringent segments. But in these cases, which I too have often observed, the images do not appear to me to be clear enough to be taken as a basis for any valuable conclusions on the structure of contractile elements. In the present paper therefore, they have not been taken into consideration. I hope to do this later on, if I am able—modifying the conditions of the black reaction to obtain more precise and definite results.

There are essentially two questions to be considered in establishing the position my findings should have, when confirmed, in the overall field of studies on the fine structure of striated muscle fibers:

1. Has the reticular apparatus already been demonstrated either completely or in part with other technical procedures in its normal form or in a somewhat altered form?

2. Which of the appearances shown by muscle fibers in the fresh state or

treated with routine fixation and staining procedures (striations, polarized light images, etc.) may be considered to be directly or indirectly linked to the existence of the reticular apparatus?

Many investigators have mentioned reticula in striated muscle fibers. Thin (115, 116) in 1874 reported a system of protoplasmic trabeculae among the muscle fibrils gathered into first order bundles which, in their turn, are grouped into second order bundles (muscle fibers). This system of trabeculae is formed of the branching and anastomosing processes of small protoplasmic masses, which Thin believes are "of the nature of cells." The reticulum, called "elastic" by Thin, surrounds with its largest meshes the first order bundles (muscle columns) the transverse section of which corresponds to Cohnheim's fields, and by means of finer trabeculae penetrates into the first order bundles in the midst of the first order muscle fibrils. I do not take into consideration Thin's description of the supposed cellular coverings of the first order bundles, because it would now be only of historical interest.

Bremer (12), in a work on the innervation of muscles, reports briefly on two reticula in the muscle fiber, one thick and the other thin, formed within the protoplasm of the muscle corpuscles (protoplasmic masses containing the nuclei) and more clearly visible in young fibers. The thick reticulum would correspond to Hensen's line (M), the thin reticulum to Amici's band (Z); the two reticula are connected to each other longitudinally by independent fibrils.

Leydig (71) feels justified in concluding that two constituent elements are present in muscles: a solid substance, elastic in nature, that forms a sort of framework differently arranged in striated fibers than in smooth fibers, and a homogeneous, nearly liquid substance which is regarded (strange to say) as the site of contractility, whereas the fibrils that form the framework are regarded as passive elements.

Melland (81), by using gold chloride, acetic acid and osmic acid, claimed to have demonstrated an intracellular reticulum in *Dytiscus* [the diving beetle], in the bee, the frog, the crayfish, and the mouse. This apparatus is regarded as consisting of a number of transverse reticula implanted into the sarcolemma, and of longitudinal fibrils connecting the nodal points of the fibrils that form the transverse reticula. According to Melland, the reticula are formed of isotropic material, whereas all the rest of the muscle substance is anisotropic. The presence of the reticula might explain the transverse striation and other complex appearances by reconciling the many contradictory views held by histologists.

Marshall (77, 78), also using gold chloride in his studies on muscles, found reticula which, by their regular arrangement, might give rise to certain optical effects that would cause the peculiar appearance of the striated fiber. The reticulum is regarded as being formed of longitudinal contractile fibrils

and elastic transverse fibrils, the longitudinal fibrils having the function of shortening the muscle fiber, the elastic fibrils of bringing the muscle fiber back to its original form when contraction ends.

Van Gehuchten (38–40), following Carnoy's idea (18) "*que la cellule musculaire est une cellule ordinaire dont le reticulum s'est régularisé et l'enchylème chargé de myosine,*" has brought together in two celebrated articles a great bulk of observations in order to demonstrate that two essentially distinct elements are present in the striated part of muscle: one of these is a liquid or viscous structureless substance rich in myosin, the other is organized in a very delicate way and forms a framework that occupies the whole interna and the whole length of the fiber—a reticulum of mathematical regularity with meshes elongated in the direction of the axis of the fiber. The planes of the reticulum are united with each other by transverse filaments that go from the surface to the deepest parts of the fiber. The reticulum is considered the essential part of the fiber, and the site of contractility. It is easy to observe images corresponding to Van Gehuchten's figures. With Apáthy's method, especially, it is possible to obtain very delicate staining of Van Gehuchten's supposed reticulum. The theoretical part of Van Gehuchten's work was harshly contested and his ideas were not accepted by the majority, even though the accuracy of his observations has been recognized.

Ramon y Cajal's views (98, 99) approach those of Van Gehuchten, at least insofar as limb muscles are concerned. Ramon also admits the presence of an apparatus formed of longitudinal fibrils and of transverse reticula, all immersed in a homogeneous interfibrillar substance rich in myosin. The latter, when coagulated by the action of fixatives or even by the air, is assumed to give rise to "*fibres moules,*" which are no more than Kölliker's muscle columns, and should therefore be considered artefacts. Only the reticulum is considered to have contractile properties. It should be noted at once that Cajal's theoretical views clash with well verified facts. Regarding insect wing muscles, for example, it is sufficient to recognize that the supposed "*fibres moules*" can be observed isolated in the fresh state, and that in this condition they are able to contract.

The conclusions of Ramon y Cajal and Van Gehuchten, which represent the most complete explication of the theory of the reticulum and the most serious attempt to cast down the theory of the fibrillar structure of the muscle fiber, were opposed by a number of investigators, most important of whom are Kölliker (65, 66) and Rollett (107). (In this connection, see in the Italian literature the work of Mingazzini (84) and Motta Coco (86).)

It is beyond the scope of the present study to refer at length to the debate that turned on this fundamental point in the histology of the striated muscle fiber. I have pointed out from the beginning that I consider it demonstrated that the contractile fibrils are the essential element in muscle fiber. It is neces-

sary, however, to call attention to the fundamental difference between the reticular apparatus described by me and the reticula that form the basis of the theory I have summarized above, especially since the similarity in names might easily give rise to confusion.

All the above mentioned investigators describe as expressions of reticula, images which result from the more or less complete staining of the interstitial sarcoplasm or from concomitant staining of part of the muscle columns altered to a greater or lesser degree, and of part of the sarcoplasm. Rollett (107), in a critique of the work of Melland, Marshall, and Van Gehuchten, based on rigorous observations, says this: "*Die Bilder welche die genanten Autoren auf Fadennetze in Muskel beziehen, kommen nur durch die besondere Anordung des Sarkoplasmas in Muskel zu Stande, welches im Allgemeinen in Form eines Wabenwerkes die gegliederten Muskelsaülchen umgiebt.*" For two reasons, I think this objection cannot be raised against the reticulum I have described: first, because I obtained a selective staining that makes it possible to establish exactly whether we are dealing with filaments or with optical sections of lamellae, and that makes it possible to follow the single filaments which form the reticulum in their course within the muscle fiber; second, because the findings on muscles with abundant sarcoplasm demonstrate that it is not the whole sarcoplasm that is stained with the black reaction, but a system of filaments running in the sarcoplasm (and there is no doubt that in the masses of undifferentiated sarcoplasm we are dealing with filaments). I think that Rollett's views on the [honeycomb] arrangement of the sarcoplasm are in agreement with the proved facts about the structure and the development of muscle fibers. In reporting my findings, in fact, I have always implicitly taken this arrangement to be true.

At this stage I cannot resist the temptation to quote Rollett again: "*Ob das in solcher Weise angeordnete Sarkoplasma als solches noch eine feine besondere Struktur besitzt . . . müssen erst noch weitere Untersuchungen lehren.*" To a certain extent, the reticular apparatus I have described answers Rollett's expectations. It does indeed represent a "special fine structure" within the sarcoplasm—a system of fibrils running in the walls of sarcoplasmic alveoli.

Retzius' findings (104) on muscle fibers appear to be to a large extent similar to Fusari's and to my own. Retzius too admits the existence of transverse reticula that have relationships with the nuclei or, to be more precise, with the sarcoplasmic masses which surround the nuclei. These reticula are arranged at regular intervals along the fibers and are united to each other by fine longitudinal fibrils. Retzius describes a first order reticulum in the plane of the Z band, a second order reticulum in the plane of M, and in some cases reports two more reticula of a third order in the space between the other two. Haswell's conclusions (49) are similar to those of Retzius. Given the methods Retzius em-

ployed, it is very likely that the whole interstitial sarcoplasm was stained, so that reticula described by him are subject to the objection advanced by Rollett. In fact Heidenhain also says, in reviewing Retzius' work, that he has not recognized the real form of the substance between the columns, that true longitudinal filaments do not exist, and that, on the contrary, the sarcoplasm is arranged in such a way as to form a system of septa, which is equivalent to Rollett's alveolar formation—" *Wabenwerkes.*"

In a more recent paper, Retzius (105) has reported again on striated muscle and reached conclusions which, from my viewpoint, have considerable interest. In this paper, in fact, the concept is expressed for the first time that in the sarcoplasm "parts are found which are specifically modified and are to be considered developmental products, similar to the contractile fibrils, of the primitive protoplasm of muscle cells." In the sarcoplasm of preparations preferentially fixed in chrome-osmic-acetic mixture and stained with special aniline dye techniques, Retzius reports granules (sarcosomes) and fibrils whose grouping in the monorefringent layer at the sides of Amici's band might be the reason for the appearance of the two lines called accessory discs (N).

I cannot discuss the question of whether Retzius' interpretations of the accessory discs may still be maintained, now that it has been criticized by Rollett (109), and in view of his demonstration with the polarizing spectroscope of the existence of birefringent tracts in the muscle columns in the plane of the accessory discs. I simply wish to call attention to the possibility that the series of sarcosomes found by Retzius may be no more than altered and unselectively stained parts of the same apparatus that is completely visualized by the black reaction.

MacCallum (15–17), using Kolosow's method (involving precipitation of metal in tissues impregnated with osmic acid by treatment with a mixture of tannic and gallic acid), has reported, first in myocardium cells and later in voluntary muscle fibers, a peculiar structure in the sarcoplasm. According to this investigator, each muscle fiber is formed of a bundle of little muscle columns separated by sarcoplasmic layers. Each little column occupies the central area of a column formed by a series of superimposed sarcoplasmic discs. The discs of a series are separated from each other by thin membranes continuous with Krause's membrane (MacCallum is certain the latter really exists). The external surface of the discs is lined with a similar membrane. These membranes are selectively stained with Kolosow's method, and each sarcoplasmic disc is subdivided by a series of radial lamellae.

From this report it may appear that no relationships exist between the structure observed by MacCallum and the reticular apparatus, but on examining his illustrations one is not convinced that the stained parts do actually represent

a system of lamellae and not a system of filaments. If the latter were the case, then there would be a clear analogy between MacCallum's findings and mine.

In order to resolve the doubt that cannot but he generated by the apparent discrepancy between MacCallum's descriptions and his illustrations, I made control preparations. The results I obtained, although incomplete, confirm rather than remove the doubt.

In sum, from the above review of the reports on structures which might have some relationship with the details described by me, one may conclude that no one (with the obvious exceptions of Fusari and Ramon y Cajal) has observed the internal reticular apparatus of muscle fiber; only Retzius, in the second memoir, and perhaps MacCallum observed images that may be related to partial staining of the apparatus itself.

We come now to a second group of questions: Which among the several aspects shown by the muscle fiber treated with methods other than the one that selectively stains the reticular apparatus can be regarded as due to the existence of the apparatus? Along these lines, it is necessary first of all to consider the relationships between the reticular apparatus and the transverse striations. As has been shown, the flat transverse reticula forming the reticular apparatus occupy within the fiber a determinate position with respect to the striation. Not all the fibers possess the same number of transverse reticula in each muscle segment. When they exist, however, the reticula invariably occupy determinate planes, if the positions of Amici's band and of the mono- and birefringent tracts are taken as reference points. If the results of the black reaction were reliable even when they are negative (*i.e.*, if it were permissible to conclude that a particular formation did not exist from the fact that in a number of attempts this formation could not be stained), then, on the basis of the number of reticula in each muscle unit, I would be able to distinguish three types of muscle fibers: (1) fibers with only one reticulum, in the plane of the Z band; (2) fibers with two reticula, at the limits of the Q disc; (3) fibers with three reticula, one in the Z band and two at the limits of the Q disc. But such a classification would be of no value since it is known that with metallic impregnation methods, the selective staining often fails in some areas in spite of all attempts—for reasons unknown! On the other hand, the fact that in the same muscle, occasionally in the same fiber, two types of structure may be found (*e.g.*, in the larvae of the gadfly, in fibers of the same bundle; in *Carcinus*, in different equally extended segments of the same fiber we can find the three-reticulum and the two-reticulum types) suggests that in all likelihood there is only one type of structure; that in all muscles, three transverse reticula are present in each muscle unit, and that only because of uncontrollable conditions in the staining reaction does it occur that sometimes all three reticula are stained, and

sometimes only one or two. This conclusion is in conformity with Fusari's ideas. In those muscles in which the staining of the three reticula is easily obtained, the reticulum of Amici's band usually does not stain in fibers in a state of even incipient contraction (*vide Carcinus maenas*). In muscles which usually show two reticula, these are maintained during contraction, the only modification being that the two reticula in each pair and the pairs themselves are brought nearer. In muscles with only the one reticulum in Amici's band, that reticulum is easily stained even in areas fixed in a state of contraction, the difference consisting in the fact that in these areas the single reticula are very close to each other.

In the study of changes in aspect of the reticula during contraction, serious obstacles are encountered because of the difficulty, especially in vertebrates, of obtaining fibers fixed in a state of complete relaxation and of obtaining a series of well impregnated elements in different stages of contraction. Another difficulty is met because it is impossible to establish, given the differences in aspect, whether these differences are due to the contraction or to the reaction which, as a result of factors we cannot ascertain, may change the selective staining properties of the elements. For these reasons, the few data I have given here on the functional modifications of the reticular apparatus are to be considered an expression of isolated facts, from which we cannot derive any general laws. A study of this subject that might lead to definite conclusions would require a great deal of comparative research.

As soon as a transverse reticulum has been demonstrated in the interstitial sarcoplasm at the level of Amici's band, the question arises whether Amici's band itself—as it appears in fresh muscles or in muscles treated with routine fixation and staining methods—depends on this reticulum. Many investigators nowadays admit that Amici's band is formed of elements within the muscle columns and elements running within the sarcoplasm in the interstices between columns—in other words, that the dark and stainable line depends on the presence of differentiated parts in the muscle columns and differentiated parts in the sarcoplasm—both situated exactly at the same level. Hence, Heidenhain distinguished two parts in the Z band: Zf (the part formed by differentiated elements of the fibrils), and Zs (the part formed by differentiated elements of the sarcoplasm). This distinction is fully corroborated by my observations inasmuch as it is easy, especially with Heidenhain's and Apáthy's methods, to stain selectively a transverse line corresponding to Amici's band in the isolated columns. On the other hand, in certain muscles (especially in the larvae of the gadfly), even by using routine methods, Amici's line is seen to extend through the protoplasmic septa located between the columns. Heidenhain's distinction smooths the path for us to advance a hypothesis on the role that may be played by the reticulum in giving rise to the image that we are accustomed to designat-

ing as Amici's band. Probably, the peculiar aspect of the sarcoplasm at the level of Amici's band is due to the existence of the transverse reticulum (the Zs part of the Z band depends on the reticulum), whereas the differentiation that occurs at the same level (Zf part of the Z band) is completely independent of the reticulum. One could object that there is a possibility that the differentiated elements of the columns which form the Zf part of the Z band are due to a portion of the reticular meshes which remain adherent to the columns when these are mechanically isolated. But this objection is not supported by the fact that the Zf tracts of the columns are selectively stained by Heidenhain's hematoxylin and by Apáthy's method, whereas the filaments which form the reticulum are not stained at all by these techniques. Much more difficult and complex is the problem concerning the two reticula at the limits of the Q layer because here the problem is to establish whether the presence of the two reticula influences the appearance of those two dark lines known in the literature as the lines of Tourneux after the investigator who first described them (117). It is necessary, furthermore, to establish what relationship exists between the reticula and the series of sarcosomes described by Retzius, mentioned by Schäfer, and substantiated by Heidenhain with iron hematoxylin. I will not pretend to summarize the question of the accessory discs, one of the most controversial and one of the most obscure points in muscle fiber histology, especially since different elements in different muscles have been described under the same name by many investigators.

I wish only to point out that among the best ascertained facts, we have on the one hand the existence of two series of sarcosomes (specially differentiated parts in the sarcoplasm) at the two sides of Amici's band in the monorefringent layer (Retzius), and on the other hand, the existence in the same position of a birefringent layer in the muscle columns (Rollett). I believe it is justifiable to assume that the transverse reticula described by me and the series of sarcosomes described by Retzius in the same region of the fiber are two aspects of the same formation, the difference in appearance depending solely on the difference in preparatory techniques used. If the hypothesis is tenable and if the conclusions of Rollett are accepted, it must then be accepted that the dark lines known as "accessory discs" are the resultant optical expression of the presence of both differentiated (birefringent) segments in the muscle columns and the transverse reticulum in the sarcoplasm. If this is the case, the accessory disc, like Amici's band, should be considered to be formed of two parts: one belonging to the fibrils (Nf), the other to the sarcoplasm (Ns).

FROM THE considerations so far brought to light one can see that my own investigations occupy a secondary position in the larger scheme of investigations

on striated muscle fiber. My work does not touch on the weighty questions of the intimate structure of the contractile substance, nor does it give any new data to explain the mechanism by which the muscle fibers contract.

My investigations concern a quite different subject; they tend only to show a peculiarity in the structure of the sarcoplasm. In this sense they may be considered corroboration and continuation of Fusari's studies and as partial correction, as far as interpretation is concerned, of Cajal's studies. I am not qualified to speak on the morphological and functional significance of the reticular apparatus I have described. It is well known that in the last few years Golgi (42–46) demonstrated with the black reaction peculiar intracellular reticular apparatuses in neural elements, and that, later, similar apparatuses were demonstrated in other types of elements, both epithelial (Negri, 89; Pensa, 91) and connective (Pensa, 92) in nature. Between those apparatuses and the apparatus I have described in muscle fiber there are, along with many differences, analogies that must be considered in forming a comparative judgment. As in nerve cells, the protoplasm contains two different and apparently independent formations—the primary nerve fibrils which represent the conductor element, whose existence cannot be doubted since the work of Apáthy, Bethe, and Golgi, and Golgi's reticular apparatus; so in muscle fiber there are two different and apparently independent formations—the muscle fibrils (contractile elements) and the reticular apparatus described by Fusari and myself. Though the analogy may be complete, it does not allow us to conclude that the two reticular formations in the two types of cellular elements are identical, not even from a merely morphological point of view. I have pointed to this analogy only as a simple fact. From a physiological standpoint we are even less in a position to come to any conclusion because Golgi, as we know, has not expressed any hypothesis on the significance of the reticular apparatus that he discovered. In addition, the attempts made by various investigators (Holmgren, Retzius, Studnicka) to interpret the reticular apparatus, however ingenious, have not been documented with sufficiently precise and certain anatomical data to be unconditionally accepted.

CONCLUSIONS

1. There exists in striated muscle fiber a reticular apparatus consisting of anastomosing filaments that stain selectively with the black reaction. This apparatus lies within the sarcoplasm and must be regarded as a product of differentiation of the primitive protoplasm of muscle cells. The apparatus extends both into the sarcoplasmic trabeculae in the interstices between the

muscle columns, and into the sarcoplasmic masses which lie outside the columns (when these sarcoplasmic masses are present).

2. The reticular apparatus has a simple contiguity relationship with the muscle columns and nuclei; it appears to take on a more intimate relationship with the sarcolemma on the internal surface of which single filaments of the apparatus occasionally implant themselves by means of triangular enlargements.

3. The reticular apparatus (at least in mammals, birds, and reptiles) exhibits modifications at different stages of development. In developing fibers, the reticular apparatus is formed of anastomosing filaments running in the interstitial sarcoplasm at random in all directions; in fully developed fibers, on the contrary, the direction and the arrangement of the filaments that form the reticular apparatus are regulated according to a generally precise and constant architectonic plan. The reticular apparatus in the adult animal may be considered to be formed of a series of transverse reticula united to each other by longitudinal filaments. The transverse reticula occupy fixed positions relative to the striation. One finds fibers showing only one reticulum in coincidence with the Z band, other fibers showing two transverse reticula at the limits of the Q band, and other fibers showing at the same time one reticulum in the Z band and also the two Q band reticula. It is likely that in reality all fibers have three reticula and that the failure of one or another reticulum to stain depends on the conditions in which the reaction takes place.

During contraction the appearance of the apparatus is profoundly modified: of these alterations (for which it has not been possible to establish laws), the most constant is the approximation of the transverse reticula. However, the two adjacent reticula never fuse into one.

4. We can conclude that the reticular apparatus that stains with the black reaction in the limb muscles of insects (described by Ramon y Cajal) cannot be interpreted as a system of tracheal capillaries. This decision is based on the identity in form and arrangement of the reticular apparatus in insects and the corresponding apparatuses in animals without tracheae.

5. The above mentioned reasoning (No. 4) is not valid for the dissociable muscles of wings, for which there is no precise comparison in non-tracheate animals. But the analogy tends to preclude the possibility that the reticular apparatus demonstrable in this type of muscle can be interpreted as a system of tracheal capillaries—even though relationships may exist between the reticular apparatus and the tracheal branchings running amongst the muscle columns.

6. There is a morphological analogy between the reticular apparatus of muscles and the apparatuses described in many types of epithelial and connec-

tive tissue cells by Golgi and his students. This analogy is not such, however, as to permit us to affirm the identity of the two types of structures.

7. There are no data on which to base any hypothesis on the functional significance of the reticular apparatus of muscle fiber.

This series of investigations on voluntary muscle fiber having come to an end, many interesting questions arise. Future investigations should be directed especially toward establishing: firstly, whether a reticular apparatus similar to that in voluntary muscles exists also in myocardial muscle elements (striated muscle cells and Purkinje cells); and secondly, what the relation is between the reticular apparatus and motor nerve endings, the transition zones between muscle and tendon in vertebrates, and the attachment points on the chitinous laminae in insects.

I hope to be able to continue my studies of the subject along these lines.

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