

# THE STRUCTURE OF INSECT FIBRILLAR FLIGHT MUSCLE

## A Study Made with Special Reference to the Membrane Systems of the Fiber

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### ABSTRACT

The fine structure of fibrillar flight muscle of the mature adult beetle *Tenebrio molitor* is described. Although the very high frequency of contraction of fibrillar muscle has previously been in part accounted for as the result of mechanical specialization of the wing-bearing segment rather than of a correspondingly high rate of motor impulse supply, the problem of the nature of the pathway by which excitation is conducted into these large fibers remained. Therefore, particular attention has been given to the disposition and relationships of the plasma membrane and sarcoplasmic reticulum in this tissue. The invading tracheoles draw with them a sheath of plasma membrane from the surface to all depths in the fiber, and it is suggested that these sheaths, together with the extensive tubular arborisations arising from them, reduce the maximum plasma membrane-to-fibril distance from the radius of the fiber to a value of less than  $2 \mu$ . The evidence presented here confirms Veratti's contention that in fibrillar muscle the "reticulum" is associated with, though entirely distinct from the fibrils. Unlike other muscles so far examined, these flight muscle fibers contain a plasma membrane reticulum only, but it is possible that elsewhere the general "sarcoplasmic reticulum" includes a component derived from the plasma membrane, likewise acting as the pathway for inward conduction of excitation. Profiles of the internalised plasma membrane in *Tenebrio* showing the usual triple-layered 25-25-25 A organization are frequently seen, in sections, in close association with isolated vesicles (defined by "simple" 50 A membranes) which are here considered to represent, in vestigial form, the portion of the sarcoplasmic reticulum which in other types of muscle is complex and highly developed. Such associations, in *Tenebrio*, between these two dissimilar elements are here termed "dyads" and the possible morphological and functional homology between these and the "triads" of other types of fiber is considered.

### INTRODUCTION

It has been known since the work of von Siebold (1848) that the flight muscles of certain insects are of an unusual type. Whereas the typical muscle fiber was considered to be a compact unit, von Siebold, Aubert (1853), Limbeck (1885), and

other early workers supposed from the ease with which this flight muscle fragmented into individual fibrils that true fibers were absent. Subsequently, it was established that this view is erroneous and that "Siebold," "dissociable" or

“fibrillar” muscle, despite its peculiarities, is constructed on essentially the same plan as other striated muscles.

Among the characteristics of this tissue are the large size of the fibrils, their arrangement into fibers which may be several hundred microns in diameter, and the shortness of the sarcomeres, together with the great restriction of the I band. In addition, the sarcosome or mitochondrial content is high, and it has long been realized that the fibers are invaded by a rich tracheolar system; a development which drastically reduces the distances involved in the diffusion of respiratory gases between tracheoles and tissue. The importance of this is stressed by the demonstration that the oxygen consumption of flight muscle (*Apis*) increased 50-fold during active flight (Jongbloed and Wiersma, 1934). The high metabolic rate of this tissue is also apparent from the observations of Pérez-González and Edwards (1954) who found that the oxygen consumption and succinic dehydrogenase activity of the flight muscle of the water beetle *Hydrophilus* was 15–20 times that of leg muscle, and Pringle (1957, p. 45) states that the metabolic rate of fibrillar muscle may be twice that attained by humming-bird flight muscle and ten times that of human cardiac muscle. In view of these facts, it is not surprising that fibrillar muscle is developed in those insects in which the highest wing-beat frequencies occur: in beetles, wasps and bees, and in flies. Among these forms, a rate of 50 to 100 cycles per second is modest, while the fly *Forcipomyia* achieves the impressive figure of ca. 1000 c/s (Sotavalta, 1947).

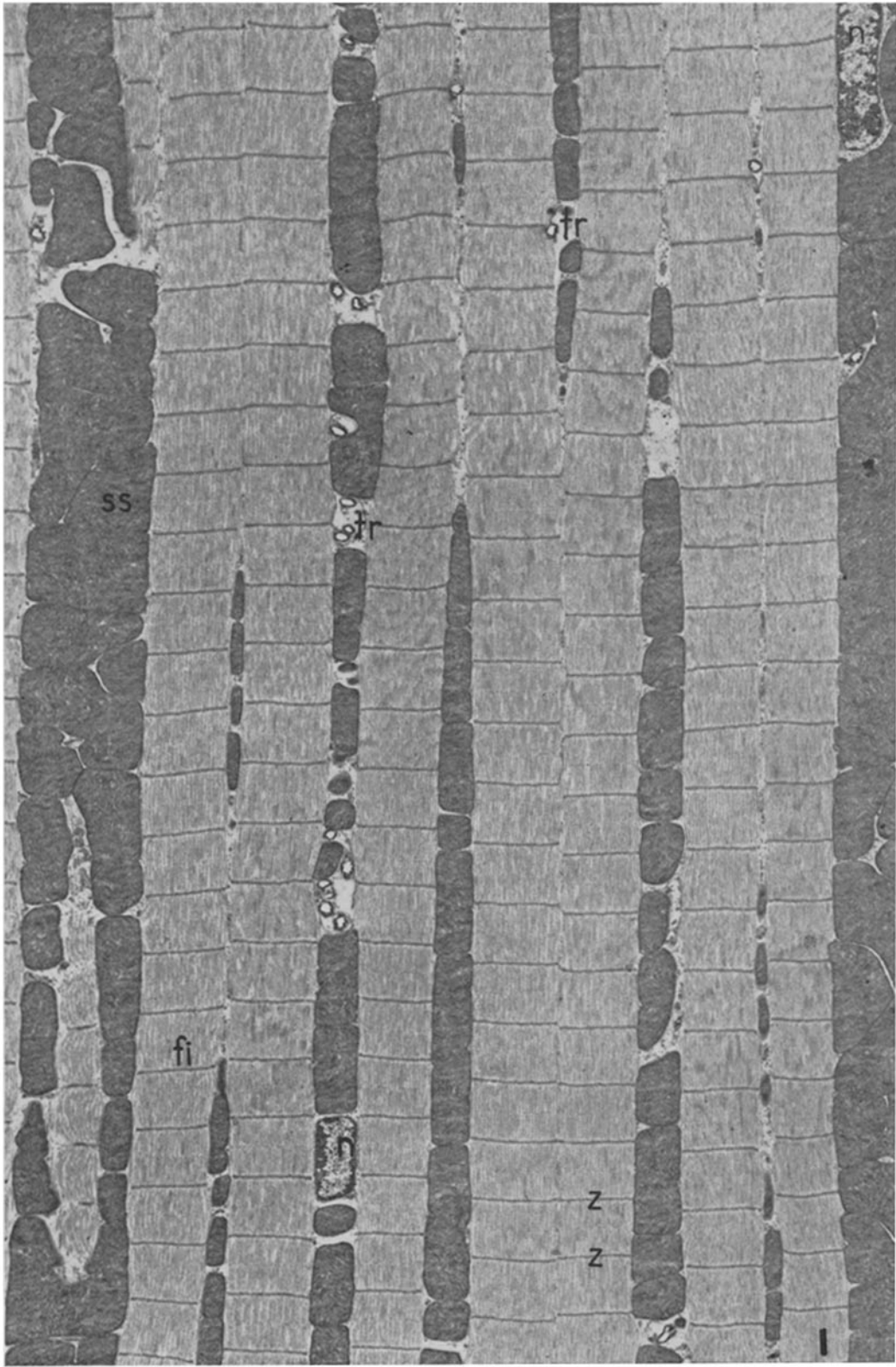
The difficulty of reconciling this degree of activity with general neurophysiological concepts was in part resolved by Pringle (1949) and others, who showed that the frequency of muscular contraction greatly exceeds that of action potentials set up during flight, a phenomenon that will be discussed more fully later. Nevertheless the nature of the internal pathway taken by the membrane-excitation initiated by a nerve impulse at the surface of the fiber was unknown and, in view of the

large fiber diameter involved, constituted an important problem. This will be considered more fully in the discussion, but at this point it may be said that the calculations of Hill (1948, 1949) relating to the diffusion rate of a substance from the depolarised membrane, supposedly activating contraction of the fibrils, stress the need for such a conduction system with reference to vertebrate muscle, while Bennett (1955), Ruska, Edwards, and Caesar (1958), Peachey and Porter (1959), and others tentatively assigned this role to the sarcoplasmic reticulum or to some part of this component of the muscle cell. The aim of the present work is to provide a fuller account of the fine structure of this specialized tissue, and in particular, to investigate the disposition of, and the relationship between, the plasma membrane and the membrane of the sarcoplasmic reticulum of the fiber with a view to establishing, at least on morphological grounds, the probable pathway of impulse conduction.

The fine structure of fibrillar flight muscle, especially from Hymenoptera and Diptera, has been studied by a number of authors: Chapman (1954), Hodge, Huxley, and Spiro (1954), Kisch and Philpott (1955), Philpott and Szent-Györgyi (1955), Edwards, Ruska, Souza Santos, and Vallejo-Freire (1956), Hodge (1956), Huxley and Hanson (1957), and others. While all are agreed on certain general features of this tissue—the large size of the fibrils, the size and complexity of the sarcosomes, and the presence of tracheoles within the body of the fiber—information on the form and distribution of the sarcoplasmic reticulum is almost entirely absent, and cannot be derived from the published micrographs. If, however, such fibers are indeed devoid of, or poorly supplied with this reticular system, then they are unusual by comparison with other insect and vertebrate muscles so far studied. Further, the absence of sarcoplasmic reticulum from these giant fibers would seem to negate suggestions implicating this system in impulse conduction throughout the fiber. Yet examination of fibrillar flight muscle

FIGURE 1

Low power electron micrograph of a longitudinal section of *Tenebrio* flight muscle. Note the short sarcomere length ( $Z-Z$  distance ca.  $1.5 \mu$ ), and the virtual absence of the I band; also the large diameter of the myofibrils ( $f$ ). Two nuclei ( $n$ ) are included in this field while between the fibrils occur rows of large mitochondria or sarcosomes ( $ss$ ) and many profiles of tracheoles ( $tr$ ).  $\times 6,500$ .



fibers of *Tenebrio* has, in the present study, not only revealed that a *continuous* membrane-bound system permeates the sarcoplasm, but has also led to a suggested structural and functional homology between the reticular system of this muscle, and that of vertebrate skeletal fibers.

#### MATERIALS AND METHODS

The tergo-sternal and basalar pleural flight muscles of both newly emerged and older adults of the mealworm beetle (*Tenebrio molitor*) were employed, though the only micrograph of tissue from a newly emerged individual included here is that shown in Fig. 6. For electron microscopy, insects were bisected medially in sucrose-containing 1 per cent OsO<sub>4</sub> buffered with veronal-acetate at pH 7.7. Fixation at 0°C. was continued for 90 minutes, after which the material was transferred directly to 70 per cent ethanol when individual fibers were dissected out, and dehydration was completed. Portions of fibers were embedded in 25:75 Me:Bu. methacrylate containing 1 per cent luperco and 0.01 per cent uranyl nitrate. Sections were cut on a Porter-Blum microtome and examined in and RCA EMU 2 and in a Siemens Elmiskop I. Contrast in the specimens was enhanced by "staining" with 2 per cent uranyl acetate for up to 1 hour, or with lead hydroxide for 2 to 5 minutes according to the method of Watson (1958) and Peachey (1959).

The staining of the reticulum by the "black reaction" of Golgi, was carried out according to the modifications suggested by Veratti (1902). As is apparently always the case, only small portions of the fiber prove to be well stained, presumably because of the impermeability of the deposit of silver

chromate that forms on the fiber surface, but good images of the tracheal and reticular systems within the fiber were obtained with the following schedule:

1. Fix in a mixture of 1 per cent OsO<sub>4</sub> and 3 per cent K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> (1:4), for 8 days.
2. Transfer to 0.75 per cent AgNO<sub>3</sub>; several changes, until the solution contains no precipitate. Leave for 3 days, agitating occasionally.
3. Replace in the fixative for 48 hours.
4. Transfer once more to the silver nitrate solution for 3 days or more. (Note: fixation and silver nitrate treatment was carried out at 15°C.)

The fibers were dehydrated in ethanol, cleared for several days in thick cedar-wood oil, embedded in paraffin wax (56°C.), and sections were cut at 5 to 10  $\mu$ . The wax was removed by brief immersion in xylene and sections were mounted in cedar-wood oil with a cover slip. Veratti found that fading of the preparations was less rapid in the sections without a cover slip, but fading is not appreciable for 3 or 4 days; adequate time to allow examination and photography of the material.

#### *The Sarcolemma, and Its Relationship to the Tracheal System*

In the terminology of the light microscopist, the sarcolemma is the thin surface sheath investing the muscle fiber. Its presence in tubular skeletal and flight muscle was recognized by several early workers, although the situation in fibrillar fibers was more controversial, it being variously supposed that the sarcolemma was here either absent, very thin, or represented by an ensheathing tracheal network. Recently, however, electron microscopy has shown the sarcolemma to be present in all

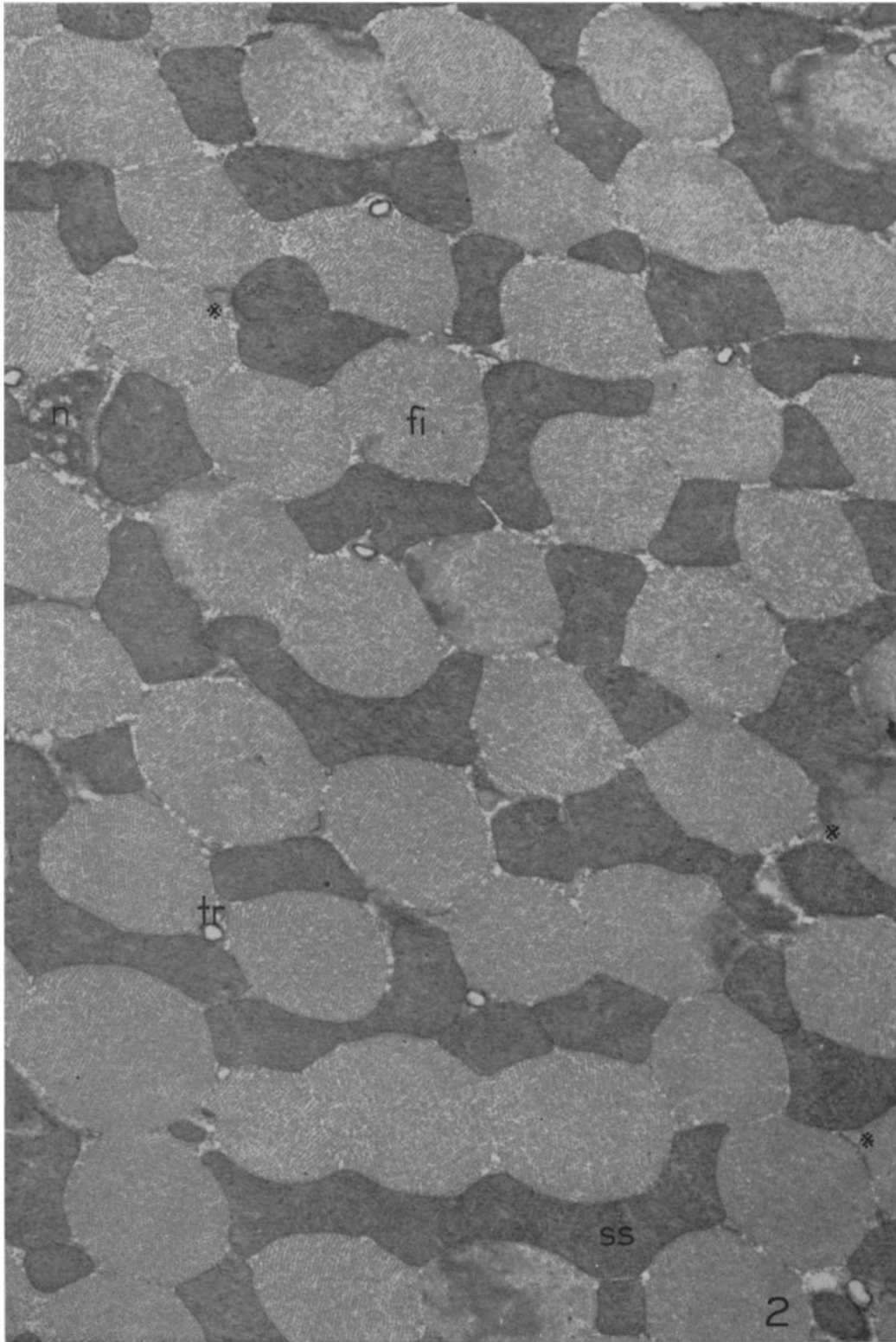
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#### FIGURE 2

A low power survey electron micrograph of transversely sectioned fibrillar flight muscle of *Tenebrio molitor*. Between the fibrils (*fi*) occur irregularly shaped sarcosomes of great size (*ss*); and these two components constitute almost the entire volume of the tissue. Inspection of fields such as this suggests that the sarcosomes account for almost as high a proportion of the total cross-sectional area as do the fibrils; an observation in keeping with the very high metabolic rate of this type of muscle (Pérez-González and Edwards, 1954).

Transverse profiles of tracheoles (*tr*) occur throughout the area; eight are present in this figure. A nucleus is seen at *n*.

At this low magnification, it is difficult to examine the reticular plasma membrane elements associated with the tracheoles, and the sarcoplasmic reticulum vesicles, but dyads may be discerned, for example, in the positions marked with asterisks, and elsewhere.  $\times 14,000$ .



fibrillar muscles so far examined, and has also clarified the derivation of this structure. According to the view adopted here the "sarcolemma" includes not only the basement membrane sheath surrounding the fiber, but also the underlying plasma membrane.<sup>1</sup> The complex nature of the basement membrane component of the sarcolemma is well known; Porter and Palade (1957), for example, showed that in rat sartorius muscle it

<sup>1</sup>An alternative view has been expressed and discussed by Bennett (1955 and 1958). Bennett proposed that, in accord with the original definitions given by Schwann and Bowman in the last century, the "sarcolemma" should not include any superficial fibrous layers of collagenous connective tissue; a view with which the present author concurs. Bennett, however, speaks of the structures which in this paper are termed "plasma membrane" and "basement membrane" as "inner and outer sarcolemmal laminae." It is suggested that the observations described here provide good evidence for the existence of a plasma membrane (a triple layered 25-25-25 A structure) and of a basement membrane (of variable depth) in *Tenebrio* flight muscle which correspond, in all essential respects, with those described in other cells. A critique of this subject appears elsewhere in this volume (Mauro and Adams, 1961).

consists of an inner unorganized layer 2 to 300 A in thickness outside which lies a second layer containing thin fibrils.

The basement membrane component of the sarcolemma of *Tenebrio* flight muscle is a well defined structure, and that it consists of at least two distinct regions is most clearly seen in preparations stained with lead hydroxide. In places a third outermost component appears, consisting of a diffuse or unorganized coarsely granular layer (Fig. 8). In such material, the adjacent region appears as an irregular dense sheath about 250 to 300 A in thickness in which, unlike the instance described by Porter and Palade, fibrillar organization is poorly defined. The inner layer consists of a sheath 500 to 1000 A thick, in which no fibrils have been observed. Immediately beneath the basement membrane, or separated from it by a very narrow region of low density, lies the sharply defined plasma membrane, the structure and distribution of which will be discussed later.

The question of the relationship between the sarcolemma and the tracheal cells supplying the fiber remains. It is well known that insects virtually lack connective tissue in the sense of the term used in vertebrates or in other invertebrate groups

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#### FIGURES 3 and 4

Light micrographs of 5 $\mu$  transverse sections of a flight muscle fiber of *Tenebrio molitor*, stained with silver according to Veratti's modification of the classical "black reaction" of Golgi.

Oblique sections of invading tracheoles are seen at *tr*, and associated with these is an extensive reticular system (*rt*), situated in the sarcoplasm between the fibrils (*fi*). At higher magnification, in Fig. 4, it may be seen in the region indicated by an arrow that the reticulum arises not from the tracheole itself, but from a sheath surrounding the tracheole. At frequent intervals, the elements of the reticulum (Fig. 3) bear what appear here to be localized swellings, the "nodes" of Veratti.

These figures should be compared with corresponding electron micrographs (Figs. 6, 19, etc.) where the identity of the reticulum with the plasma membrane tubular system stemming from the sheath surrounding the tracheoblasts is established, and where it is indicated that the "nodes" correspond in reality to the vesicular elements of the sarcoplasmic reticulum.

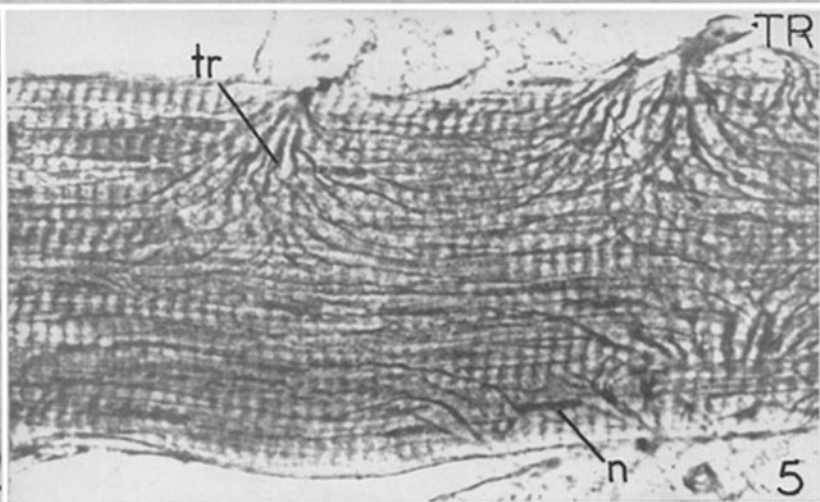
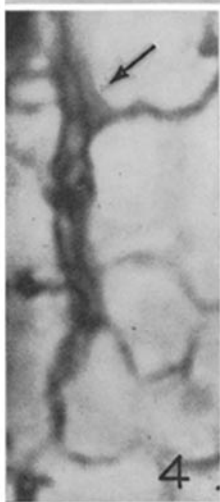
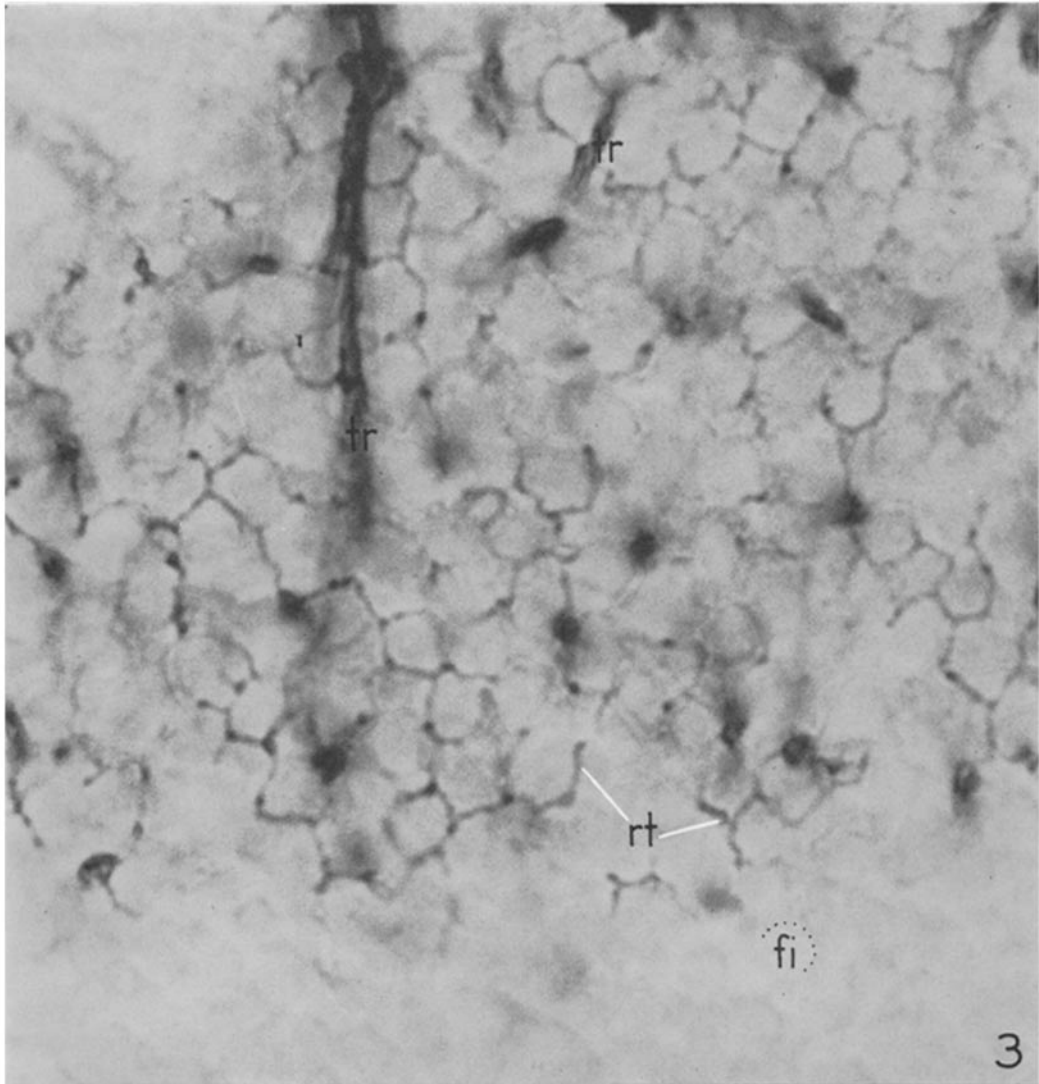
Fig. 4,  $\times$  5,600 (original magnification  $\times$  540).

Fig. 3,  $\times$  3,800 (original magnification  $\times$  540).

#### FIGURE 5

A light micrograph of a 5 $\mu$  section of a flight muscle fiber from the beetle *Dorcus parallelipedus*, stained according to the Romanes silver impregnation procedure.

The groups of tracheoles (*tr*) stemming from the main tracheal trunks, as at *TR*, penetrate into the fiber and spread out to take up longitudinally oriented pathways. The Z bands of the fiber are visible as darkly staining lines, and elongated nuclei (*n*) are scattered throughout the fiber, a pattern of distribution characteristic of this type of muscle.  $\times$  600.



as a definite cellular component containing elastic or collagenous fibers, the latter having been so far reported, in insects, only in ganglion sheaths of *Rhodnius* (Smith and Wigglesworth, 1959) and of *Locusta* (Gray, 1959). An analogous function has often been ascribed to the tracheal network within the insect body, and Edwards *et al.* (1958) summarize this opinion in the statement: "... the basement membranes of tracheoblast extensions, with or without contained tracheoles, merge with other tissues, thus connecting cell groups of either equal or unequal composition" and in particular, they showed that the basement membrane of tracheoblast cells is continuous with the sarcolemmic basement membrane in muscle fibers of the wasp and the cicada. This observation has been amply confirmed here in the case of *Tenebrio* flight muscle. In Fig. 7 is seen an instance in which a tracheoblast cell, with enclosed tracheal intima, has been sectioned obliquely at its point of apposition to the surface of the fiber. The fiber basement membrane is clearly continuous with and similar in appearance to the basement membrane bounding the tracheoblast.

The tracheoles arise within the cytoplasmic outgrowths of the tracheoblast cell, and the entire system of tubes, both wide tracheae and narrow tracheoles, is at all times bounded by cytoplasm and tracheoblast cell membrane, an important fact stressed by Edwards *et al.* (1958). Previously, any tracheole found beneath the surface of a cell and notably in fibrillar flight muscle was termed "intracellular," though the precise relationship between the two cells involved was unknown. Edwards and his coworkers showed that the invading tracheolar cell always preserves its in-

tegrity as a separate system "like a finger poked into the surface of a balloon."

The extent and abundance of the tracheolar system in *Tenebrio* flight muscle are impressive. At the periphery of the fiber the tracheoles, stemming from the tracheal end-cells, splay out and commence their course into the sarcoplasm. Initially their path is usually perpendicular to the long axis of the muscle, but soon most of them take on a preferred orientation parallel with this axis. This is seen in Fig. 5 which represents a light micrograph of a longitudinal section of a fiber of the beetle *Dorcus*, in which the tracheoles are impregnated with silver by the Romanes technique. This orientation is particularly evident in electron micrographs where, as in Fig. 2, a transverse section of the fiber contains almost exclusively transverse sections of tracheoles, which are as abundant in the central region as at the periphery of the fiber. Edwards *et al.* (1958) established the separateness of the tracheolar system within the muscle, but they did not consider the effect exerted by it upon the topography or distribution of the muscle plasma membrane; an effect which will be described later, and which, it is suggested, may be of the greatest functional importance.

Before this, however, a brief description will be given of other components of the fiber—the sarcosomes, the nuclei, and the myofibrils, shown in longitudinal and transverse section in the survey micrographs, Figs. 1 and 2.

#### *The Myofibrils*

With respect to the organization of the contractile material, this work merely serves to confirm the results of previous investigations of similar muscle.

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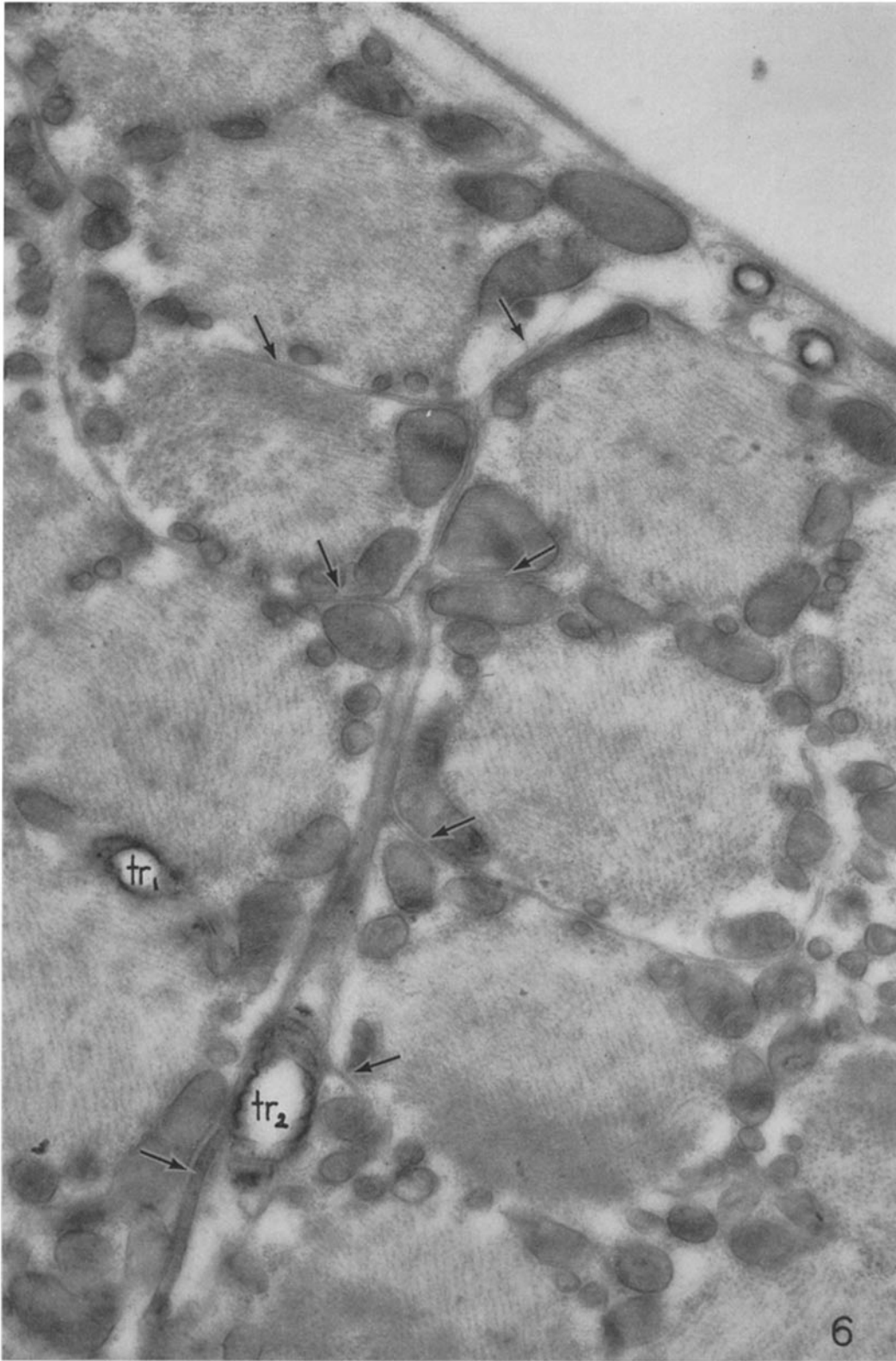
#### FIGURE 6

An electron micrograph of transversely sectioned flight muscle from a recently emerged adult of *Tenebrio molitor*. At this stage of development, the sarcosomes are small, and a rapid and extensive size increase occurs during the first few days of adult life, resulting in the giant sarcosomes depicted in other figures.

In this micrograph, profiles of tracheoles within the fiber, are seen at  $tr_1$  and  $tr_2$ . Lying in the plane of section is an arborising membrane-tubule system stemming from the sheath surrounding the lower tracheole. Arrows indicate seven branches visible in this section; some of considerable length, others passing more quickly out of the plane of section. This system represents internalised plasma membrane which, when associated with vesicular elements of the sarcoplasmic reticulum, constitutes the dyad organization seen, for example, in Figs. 16, 18, 19, and 31.

This micrograph should be compared with Figs. 3 and 4 in which the same inter-fibrillar system is visualized with silver, according to Veratti's procedure.  $\times 22,000$ .





In uncompressed sections the fibrils are subcircular in transverse section, and have a mean diameter of  $2.1\mu$ ; an average figure for this type of muscle. Within the fibril, each secondary filament is situated midway between two primary filaments as described in *Calliphora* by Huxley and Hanson (1957). As in other fibrillar muscles the sarcomeres are short (rest length *ca.*  $1.6\mu$ ) and the I band is extremely narrow.

As is especially clear in transverse sections, adjacent fibrils are frequently in contact with each other forming rows which, however, do not have a preferred orientation with respect to the fiber. The bulk of the interfibrillar sarcoplasm contains irregular sarcosomes of great size, the shape of which is determined by the tightness of their insertion between the irregularly spaced myofibrils.

#### *The Sarcosomes*

The equivalence between the sarcosomes of muscle and the mitochondria of other cells is well established on both morphological and biochemical grounds, as is the correlation between their abundance and the metabolic rate of the muscle concerned. The sarcosomes of *Tenebrio* vary greatly in size, the largest attaining a length of  $4\mu$ . Apart from their size and complexity they appear to exhibit no unusual features.

The "double membrane" association bounding the sarcosome comprises an inner and outer membrane, each about 75 A in thickness and separated from each other by a space of *ca.* 100 A. The continuity between the inner of these membranes and the cristae is frequently observed, and while these and the outer membrane very occasionally show a

suggestion of the triple-25 A-layered structure described by Robertson (1959), this is far less evident than in the case of the plasma membrane, and might possibly be a product of focusing. The paired membranes defining the cristae are separated by a gap of about 100 A, and the density of the material within the cristae is slightly less than that of the surrounding matrix, in which small granules are frequently seen (Figs. 29 and 31). Cristae are present in large numbers within each sarcosome and their topography is complex. Typical configurations (Figs. 7, 12, 15) include irregular, subparallel, and concentrically arranged arrays.

The close packing of the sarcosomes between the myofibrils has the result that the surface of the latter may be almost entirely in direct contact with mitochondrial surface. A similarly intimate relationship exists between the sarcosomes and the tracheoles, through the walls of which interchange of respiratory gases takes place. In short, the arrangement of the components of this highly active muscle ensures the maximal apposition of oxygenating surfaces, the enzyme systems of the sarcosomes and the contractile proteins of the myofibrils.

#### *The Nuclei*

To the light microscopist, one of the distinguishing features of fibrillar muscle is the scattered distribution of the nuclei throughout the fiber, a situation contrasting with the central row of nuclei in the fiber of skeletal or "tubular" muscle. This distinction is not an absolute one, however, as the flight muscle fibers of carabid beetles contain a

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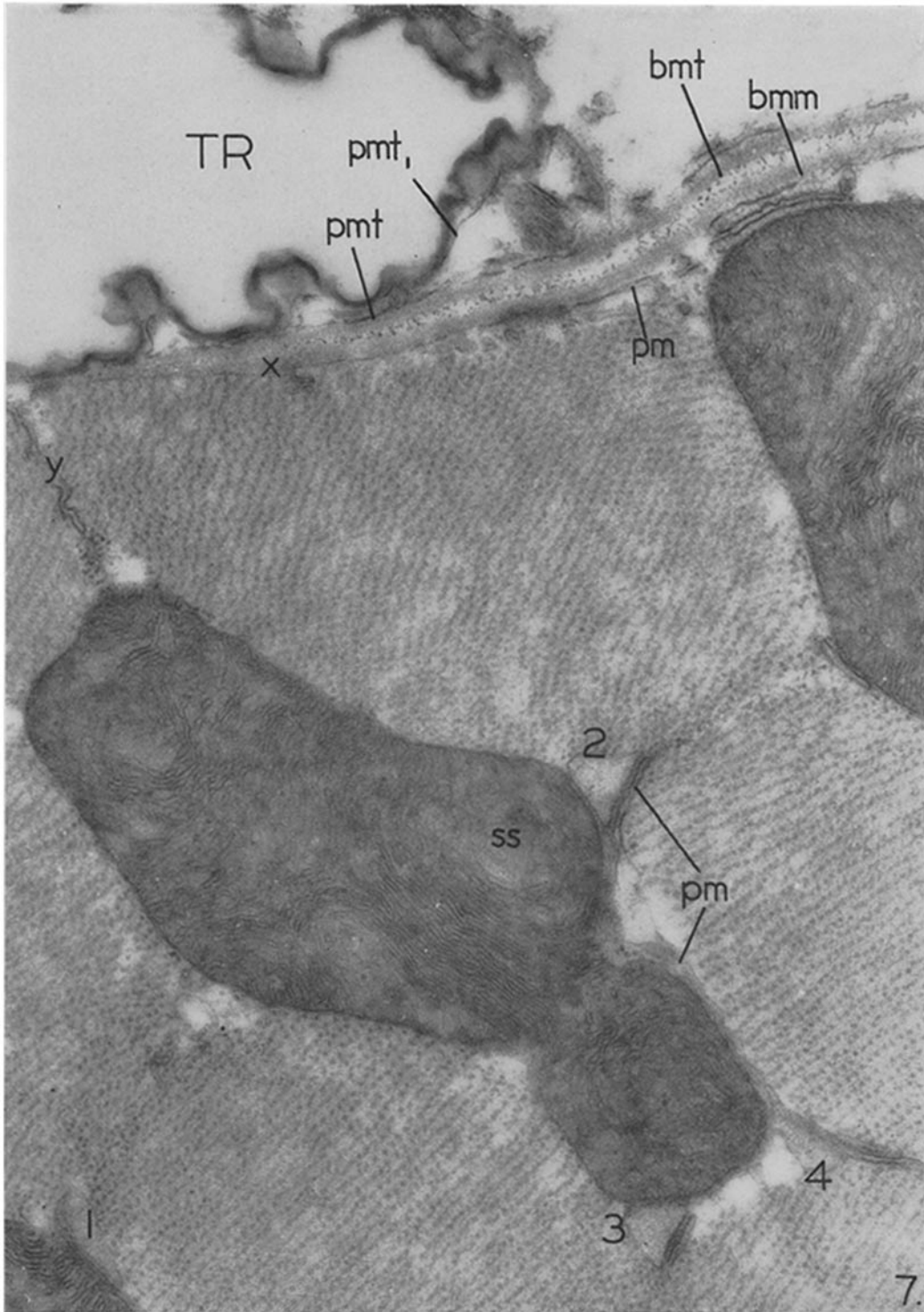
FIGURE 7

This electron micrograph illustrates the continuity between the basement membrane (*bmt*) of a tracheal trunk (*TR*), and that of the fiber (*bmm*). Only the lower portion of the trachea, adjacent to the fiber, is included in this micrograph, and the sheaths of the trachea and of the fiber converge and meet at *x*.

The plasma membrane of the muscle (*pm*) lies beneath the basement membrane, and at *y* makes a short incursion into the fiber, shallower than those seen in Fig. 8. The plasma membrane of the trachea is indicated (*pmt*), while the internalised tracheoblast membrane, inside which the walls of the trachea are laid down, may be seen at *pmt*.

Four dyads are present in this field (at 1, 2, 3, and 4), and plane of section passes through the plasma membrane tubule (*pm*) linking dyads 2 and 4. In the other cases, only the plasma membrane element in the immediate vicinity of the sarcoplasmic reticulum vesicle is seen.

Note that the large sarcosomes (*ss*) contain cristae showing both subparallel and whorled configurations.  $\times 46,000$ .



“core” of nuclei, and it is possible that the distribution of nuclei is related to the fiber diameter which in the *Carabidae* is unusually small (Smith, unpublished data).

In *Tenebrio* the nuclei, which are usually from 3 to  $5\mu$  in length, are rather sparsely scattered through the fiber, occurring both at the periphery and at all depths within it. The usual double nuclear envelope is present, the two components of which are separated by a gap of 150 to 300 A. Pores in the nuclear envelope are apparently of rare occurrence. Particles about 150 A in diameter, presumably of RNP are profusely spread over the outer membrane (Fig. 24) and they are also found in the sarcoplasm close to the nucleus, both free and, rarely, attached to the surface of isolated vesicles (Fig. 23). A careful search was made for evidence of continuity between the nuclear envelope and elements of the sarcoplasmic reticulum such as has been described in other cells. It will be shown in the next section that in this tissue the reticulum is fragmented or vesicular; apparently a vestigial condition, and one which is reflected in the relationship between the system and the nucleus. Smooth-membraned vesicles are frequently observed in close apposition to the surface of the nucleus (Figs. 20 and 23) and occasionally direct association is apparent (Fig. 22) in places where the outer membrane of the nucleus is raised into a “bleb” corresponding to one of the vesicular elements. It is interesting to note that the surface of these localized regions is devoid of particles. Thus it appears that a direct association is present, as in other cells, between the endoplasmic reticulum and the nucleus which, however, is modified in accordance with the specialized condition of the reticulum in these fibers.

### *The Plasma Membrane: Its Structure and Distribution*

(a) *Structure:* A review of the history of electron microscopists' nomenclature for the structures observed at the cell surface and of the development of the concept of the “plasma membrane” is given by Robertson (1959), who earlier introduced the very useful term “unit membrane” to denote the triple-layered 75 A structure which has now been recognized as equivalent to the plasma membrane of physiologists, and which has been observed in a wide variety of cells. Figs. 25, 27, and 28 show the appearance of membrane in osmium-fixed lead hydroxide “stained” fibrillar flight muscle of *Tenebrio*. Its total width agrees very precisely with the most frequently quoted value of 75 A, representing two osmiophilic regions separated by a region of low density, each of these three components having a width of 25 A. However local unevenness in this organization is sometimes met with; in particular the outer osmiophilic layer may be unusually thick (Fig. 27).

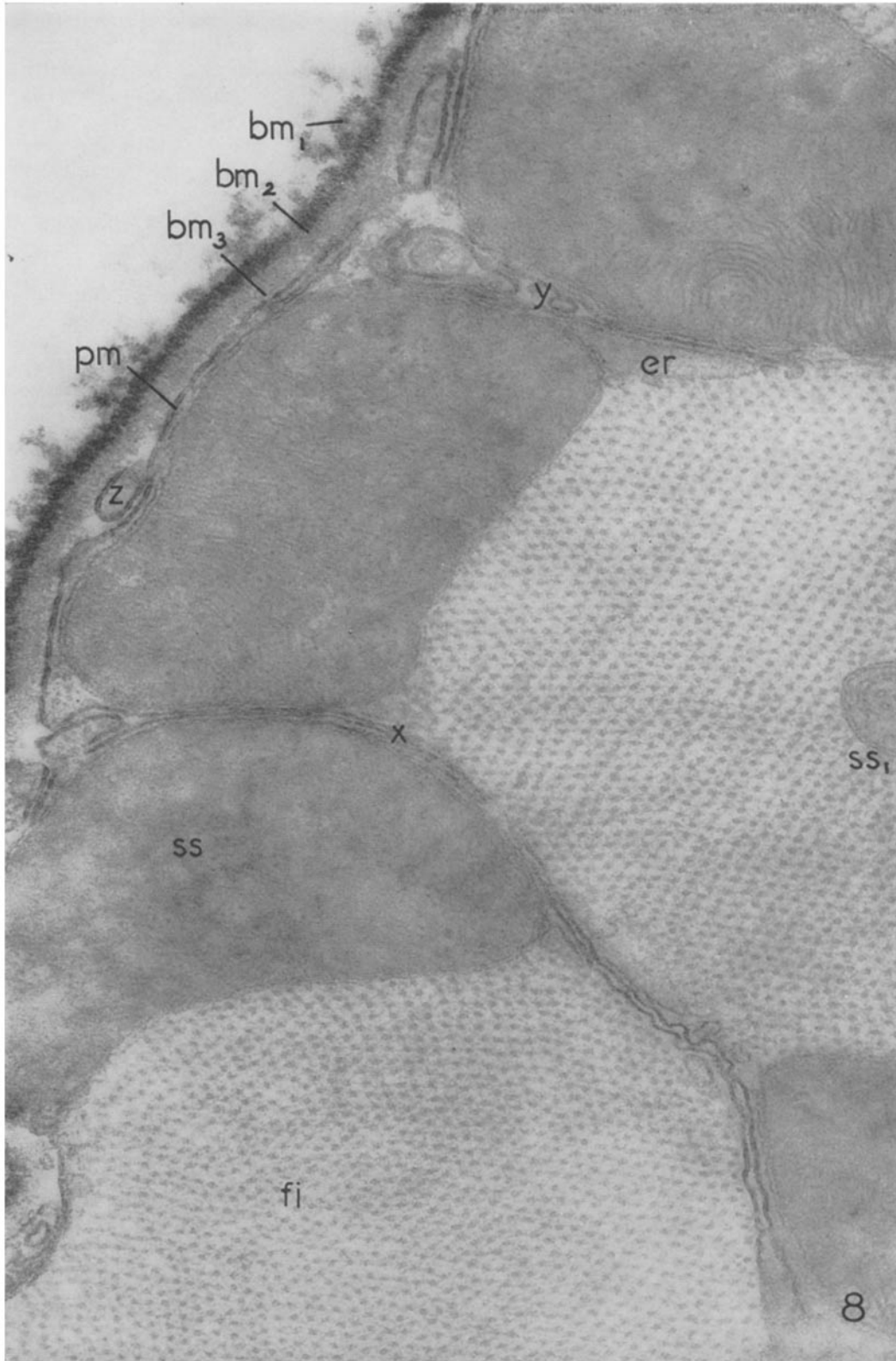
Robertson concluded that not only the plasma membrane but also the membranes of the endoplasmic reticulum and of the “double membranes” of the mitochondria show an identical 75 A triple-layered unit membrane structure. This is clearly not the case in *Tenebrio* flight muscle. The membranes of the sarcosomes and of the sarcoplasmic reticulum vesicles do not show triple layering, even when immediately adjacent to a region of the plasma membrane where this organization is quite evident (Figs. 26, 30, 31). Moreover, while the membranes of the sarcosomes are *ca.* 75 A in width, those of the sarcoplasmic reticulum vesicles are quite distinct, being only 50 A in width.

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#### FIGURE 8

Electron micrograph of a field at the periphery of a fibrillar muscle fiber of *Tenebrio*. Note the diffuse unorganized outermost layer (which is unusually marked in this region) at *bm*<sub>1</sub>, the adjacent very dense layer *bm*<sub>2</sub>, and the innermost region *bm*<sub>3</sub> beneath which lies the plasma membrane *pm*. This last shows the typical triple-layered organization. At *x* and *y*, the plasma membrane is invaginated into the fiber to the depth of the first fibril layer, a characteristic feature of the tissue, before it rejoins its peripheral course. At *er* a vesicle of the sarcoplasmic reticulum is closely applied to the upper of these invaginations. The ovoid profiles, such as the profile situated at *z*, represent sections through “fingers” or “folds” of the plasma membrane, the regions or origin of which lie out of the plane of section.

The matrices of the sarcosomes (*ss*) contain many dense granules, *ca.* 75 A in diameter. In the myofibrils (*f*) only the primary array of filaments is evident in this instance and the small intrafibrillar sarcosome at *ss*, is a rarely occurring anomaly.  $\times 57,000$ .



(b) *Topography of the Plasma Membrane:* Although for most of its course the plasma membrane lies just beneath the basement membrane of the sarcolemma, it is periodically reflexed into the fiber. These irregularities in the circumferential disposition of the plasma membrane are most clearly seen in transverse sections of the fiber (Fig. 8) where they appear as narrow blindly-ending invaginations running parallel with the long axis of the fiber and enclosing a space of varying width, which may be as small as 200 Å. Such membrane-bound flanges or "curtains" seldom penetrate into the fiber beyond the first layer of myofibrils. In addition to these, isolated circular or elongated profiles of plasma membrane are frequently seen (Fig. 8), representing tubular diverticulae, the origin of which is out of the plane of section.

Associated with these peripheral plasma membrane invaginations are specialized regions, resembling the adhesion plates described in other cell types. While these structures have hitherto been described primarily as linking cell membranes of adjacent cells, it is clear that in *Tenebrio* flight muscle fibers they serve as zones of attachment between opposite faces of blindly-ending

invaginations of the same fiber-syncytium (Fig. 9), and show a rather close structural similarity to the adhesion plates between human epidermal and cervical epithelial cells described by Odland (1958) and Karrer (1960) respectively. One other instance of adhesion plate linkage between different regions of the plasma membrane of the same cell has been reported by Hama (1959) who observed this situation in the Schwann cell of giant nerve fibers of the earthworm *Eisenia*.

The two plasma membrane profiles in the adhesion plates are separated by a space of ca. 300 Å. These profiles correspond to the "attachment plaques" of Odland, but in this case the adhesion plate zone is not defined by a thickening of the cell membrane; Fig. 25 shows that the characteristic 75 Å triple-layered unit membrane structure persists; but instead this area is defined by the localized deposition of material on each side of the plasma membrane. As in epidermal cells a line of higher density is sometimes seen midway between the apposed membranes, as in Figs. 10 and 11. "Tonofilaments" have not been observed, but in their place is found a layer of dense homogeneous material, similar in appearance to that occurring between the membranes.

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These electron micrographs illustrate the structure and topography of specialized regions, resembling adhesion plates, which occur in the peripheral zone of the fiber in association with the invaginations of the plasma membrane. It is important to note that whereas adhesion plates characteristically link together the plasma membrane of adjacent *cells*, in this case they are performing this function between regions of the *same* cell, as the muscle fiber is syncytial.

**FIGURE 9**

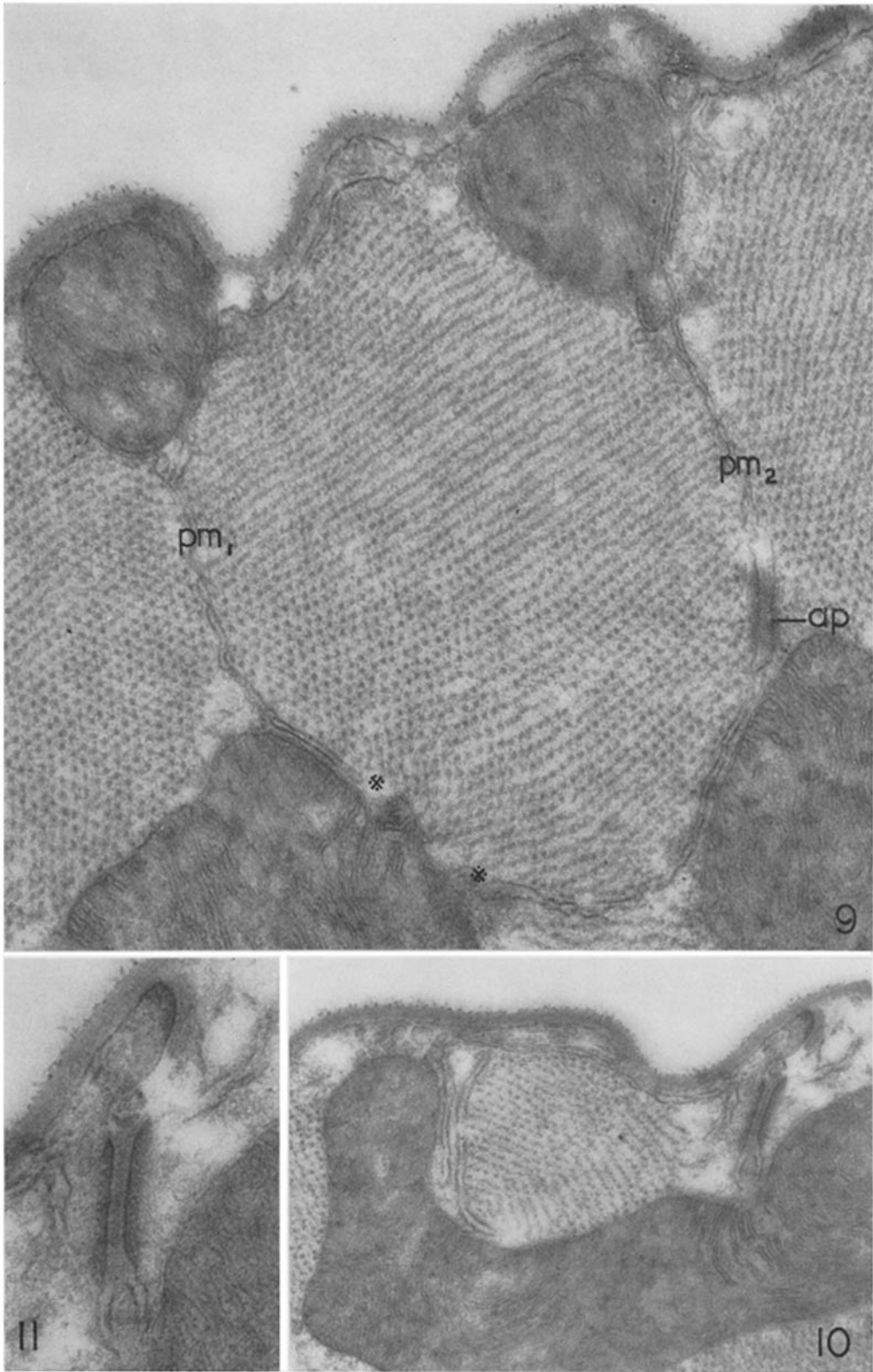
Two plasma membrane invaginations are present in this field,  $pm_1$  and  $pm_2$ , the blindly-ending extremities of which are indicated by asterisks. In the adhesion plate region ( $ap$ ) the two sides of the invagination are flanked by regions containing material of high density, which also occurs between the membranes; a situation resembling that described in a wide variety of tissues. At higher magnification, in Fig. 25, the triple-layered organization of each of the plasma membrane elements in an adhesion plate is resolved.  $\times 49,000$ .

**FIGURE 10**

Another example of an adhesion plate, occurring immediately beneath the sarcolemma. The structure appears to be more complex than that shown in Fig. 9, the terminal regions being dilated, and each dilatation contains a dense ovoid profile. The significance of the structural variation is unknown, as, indeed, is the function of these specializations.  $\times 33,000$ .

**FIGURE 11**

An enlargement of the adhesion plate shown in Fig. 10, indicating more clearly the dense, apparently unorganized material flanking the membranes, and the characteristic region of higher density occurring midway between them; a situation commonly met with in adhesion plates or desmosomes in other types of cell.  $\times 66,000$ .



The length of these structures is not known, but it is probable that the images figured represent profiles of ovoid or elongated areas of attachment as described by Odland, although as seen in Figs. 10 and 11, the peripheral zone of the plate may be more complex than in other adhesion plates. The significance of these structures which are of frequent occurrence in *Tenebrio* muscle is not known, but from their position may be inferred a role in maintaining the form and spatial relations of the plasma membrane invaginations.

Sometimes (as in Fig. 29) these plasma membrane invaginations contain a tracheole at their base, which is of course separated from the muscle by its own plasma membrane and cytoplasm. This situation represents a special case of tracheolar ingression into the fiber. Tracheoles stemming from the tracheal end-cell start their course at the surface of the fiber. Those that penetrate but a short distance sink into the fiber along their entire length, perforce drawing in with them a curtain-like fold of muscle plasma membrane as in the instance illustrated in Fig. 29.

That precisely this situation does not obtain in the case of tracheoles which penetrate deeper into the fiber is clearly shown by the absence of such "curtains" linking them with the periphery. When a tracheole enters the body of the fiber in a more or less radial direction, its point of origin at the tracheoblast cell is, as always, external to the muscle plasma membrane. *The long narrow prolongation of the tracheoblast cytoplasm draws in with it a similar concentric sheath of the fiber plasma membrane.* Thus a tracheolar intima is surrounded

by *three* membranes. Adjacent to the cuticle of the tracheolar tube lies the membrane inside which the intima was laid down.<sup>2</sup> This inner membrane is separated from the peripheral plasma membrane by a very narrow cylinder of cytoplasm which may be as little as 250 Å in thickness. The third membrane, which is sometimes separated from the tracheole by a fairly wide space (Fig. 21) is the indrawn sheath of circumtracheolar muscle plasma membrane. The space between this and the tracheole itself is thus extramuscular and is separated from the hemolymph only by the sheath of sarcolemmic basement membrane.

*Thus the plasma membrane of the muscle syncytium is not restricted to the periphery but as a consequence of the elaborate tracheolar supply infiltrates the entire fiber.* However this ingression of the muscle plasma membrane has so far been treated only in its basic or simplest condition. The complexity of this system will now be considered together with, for reasons that will become evident, the dis-

<sup>2</sup> The precise derivation of this is not entirely certain. The wall of the cavity appears in development within the cytoplasm of the tracheoblast process, either as a truly intracellular product secreted within a membrane-bound vacuole or else at the surface of a tubular infolding of the plasma membrane, linked with the periphery by a "mestraceon," the analogue of the nerve mesaxon. While the latter alternative is perhaps the more attractive, the postulated "mestraceon" has not been seen with certainty in tracheoles, though its presence would be difficult to detect because of the extremely attenuated condition of the tracheolar cytoplasm.

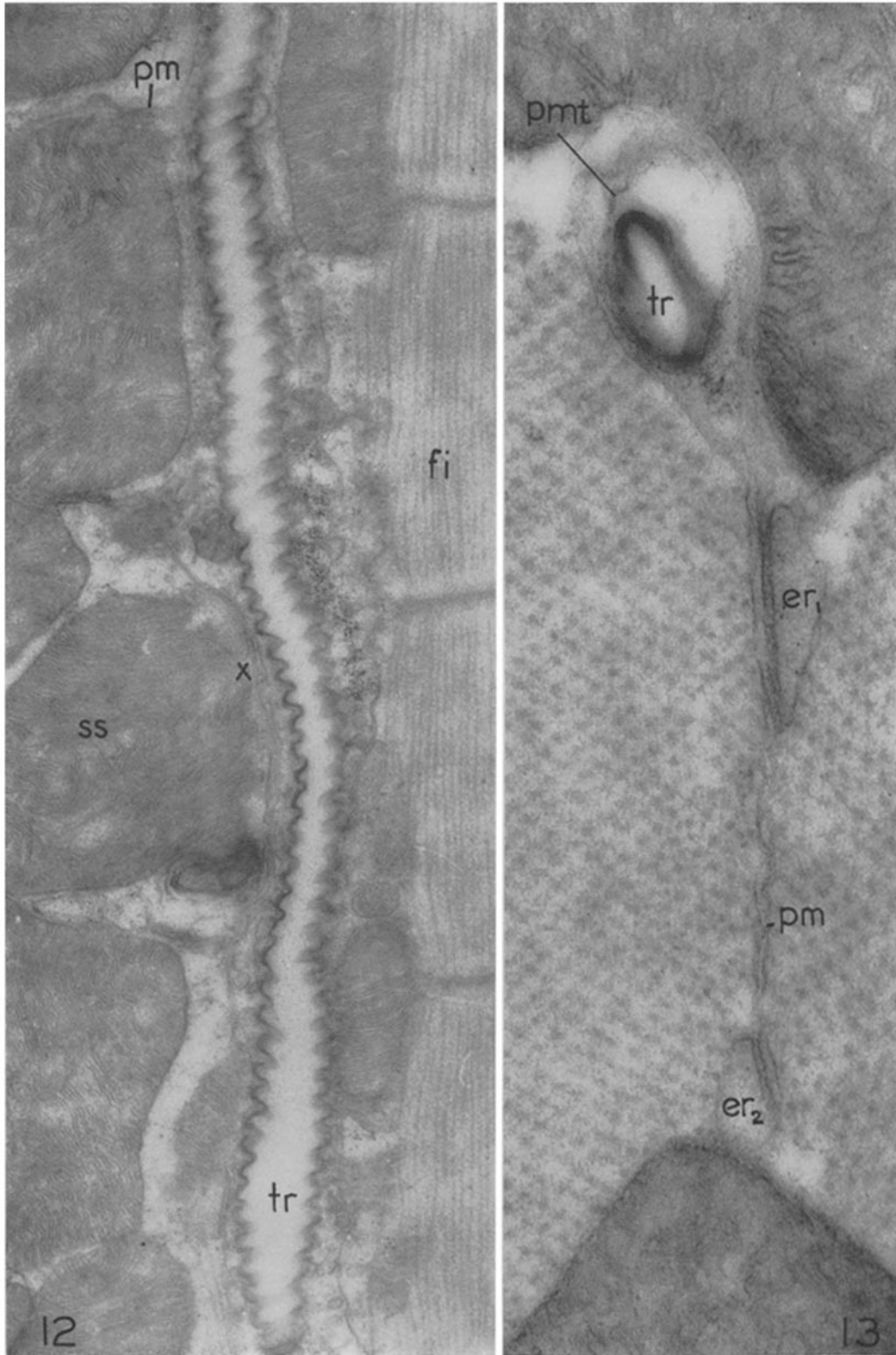
FIGURE 12

An electron micrograph illustrating the intimate spatial relationship existing between the fibrils (*fi*), the sarcosomes (*ss*), and the tracheoles (*tr*). In this instance, the plasma membrane of the muscle is closely applied to that of the tracheoblast extension, though the two may be distinguished, for instance at *x*. At the top of the field (*pm*), a muscle plasma membrane extension is visible, branching from the main sheath, and following a course into the sarcoplasm. As is usual in the case of muscle from a mature insect the tracheoblast cytoplasm is very attenuated.  $\times 42,000$ .

FIGURE 13

In this section a narrow tubular extension of the muscle plasma membrane sheath (*pm*) is seen, passing between two fibrils, while that of the tracheoblast is indicated at *pm*. Two vesicles of the sarcoplasmic reticulum (*er*<sub>1</sub> and *er*<sub>2</sub>) are closely applied to the plasma membrane process, forming typical dyad associations. In any section, most of the dyads appear as isolated profiles, but such fields as this indicate the relationship between these membrane complexes, and the plasma membrane of the fiber associated with the tracheoles (*tr*).  $\times 79,000$ .





tribution and nature of the sarcoplasmic reticulum.

*The Sarcoplasmic Reticulum and Its Relation to the Fiber Plasma Membrane*

The endoplasmic reticulum, originally noted in cultured cells by Porter, Claude, and Fullam (1945) and described by Porter (1953) has subsequently proved to merit the status of a fundamental cell system or organelle. From the universality of this system may be inferred an important role in cell physiology, and the variations in its form and extent are correlated with func-

tional differences between one cell type and another. The highly specialized condition of the endoplasmic (or in this case "sarcoplasmic") reticulum has been demonstrated in vertebrate striated muscle, notably by Porter and Palade (1957) who summarize the characteristics of the system in these muscles as consisting of:

"... Membrane-limited vesicles, tubules, and cisternae associated in a continuous reticular structure which forms lace-like sleeves around the fibrils. It shows a definable organization which repeats with each sarcomere of the fiber so that the entire system is segmented in phase with the striations of the associated myofibrils."

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FIGURE 14

An electron micrograph of a longitudinal section of *Tenebrio* flight muscle. The close packing of the sarcosomes (*ss*) resulting in extreme reduction of the intervening sarcoplasm is striking. Small membrane profiles are found in the sarcoplasm, however, but their distribution is not coordinated with respect to the repeating sarcomere pattern. These profiles comprise dyads (arrows) shown at higher magnification in Figs. 16 and 18, and sections of single tubular elements (\*) of the muscle plasma membrane intrusions, one of which is shown at high magnification in Fig. 17. The appearance of the dyads is similar in both transverse and longitudinal sections of the fiber; an observation in keeping with the suggestion that the isolated sarcoplasmic reticulum components are vesicles which form close association with the three-dimensional reticular system of plasma membrane elements.  $\times 29,000$ .

FIGURE 15

In this electron micrograph, the plane of section grazes a fibril tangentially (*fi*). The plasma membrane of the tracheoblast is visible (*pmt*), and outside this lies the ensheathing plasma membrane of the muscle fiber. The latter gives off a projecting tube (\*) which passes across the surface of the fibril and passes close to a vesicle (*er*). A transverse section of such a tubular projection is seen in Fig. 17, and it is these elements that provide the plasma membrane components of the dyads illustrated in Figs. 16 and 18.  $\times 42,000$ .

FIGURE 16

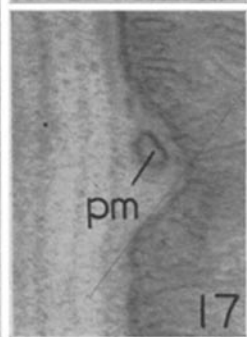
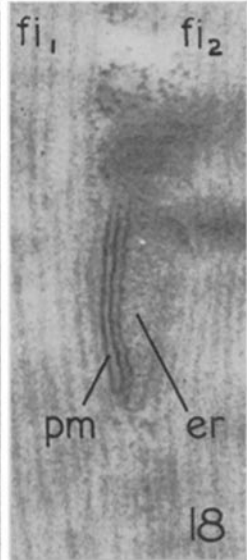
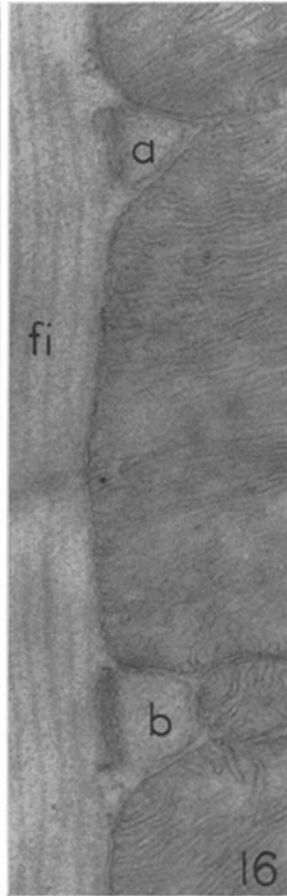
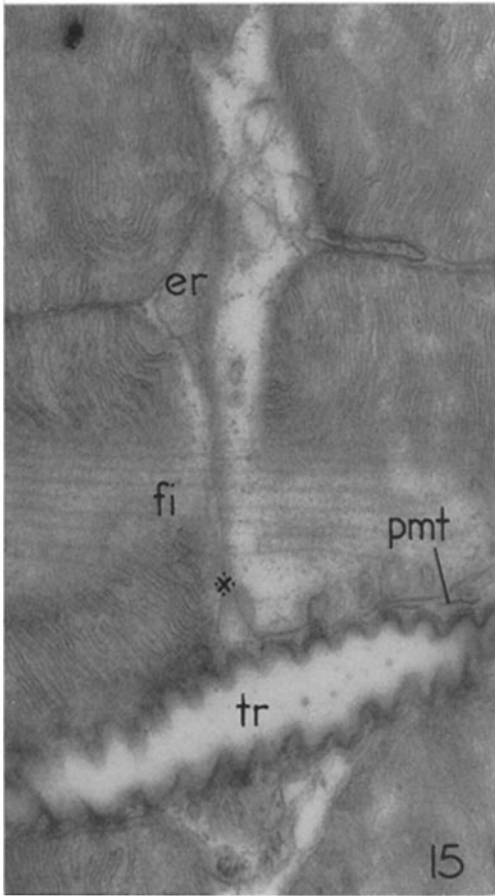
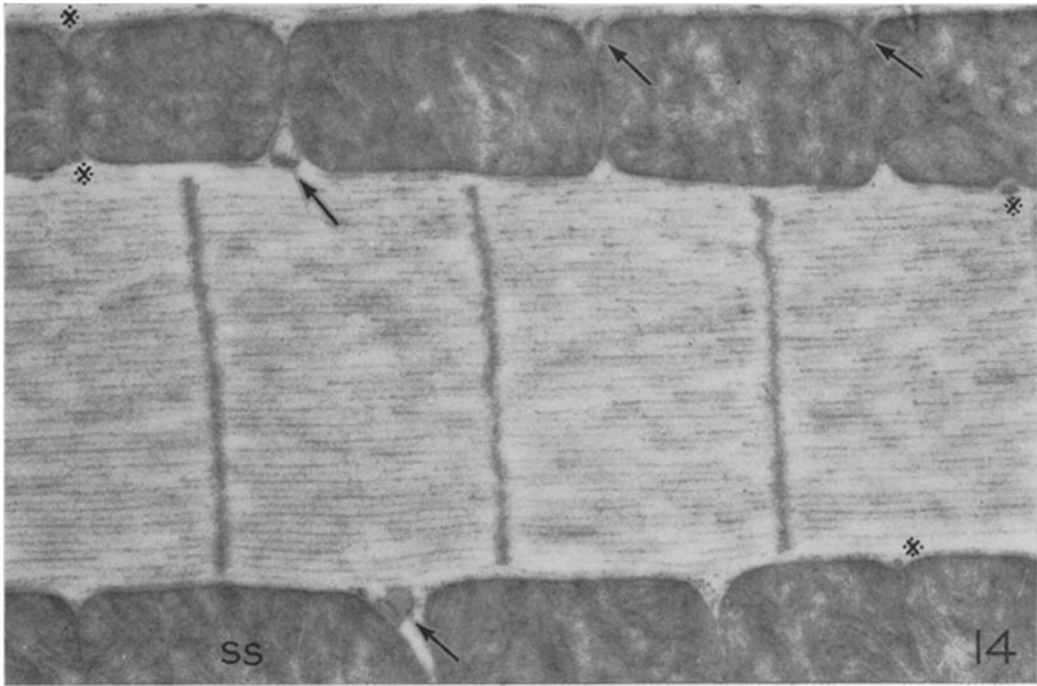
This micrograph illustrates, at higher magnification than in Fig. 14, the relationship between the fibril (*fi*) and the dyads *a* and *b*. In each of these, the plasma membrane element lies between the fibril surface and the dilated vesicle of the sarcoplasmic reticulum.  $\times 63,000$ .

FIGURE 17

High magnification electron micrograph of a transverse profile of a plasma membrane tube, such as that seen at lower magnification in the longitudinal plane, in Fig. 15. Such profiles are abundant in both longitudinal and transverse sections of the fiber, reflecting the extensiveness of the three dimensional reticulum.  $\times 90,000$ .

FIGURE 18

In this instance a dyad is seen, lying between two fibrils (*fi*<sub>1</sub> and *fi*<sub>2</sub>) which are closely applied to each other. Even at this relatively low magnification, the thicker (75A) plasma membrane component (*pm*) is clearly distinguishable from the 50A membrane of the isolated vesicular component of the reticulum (*er*). The appearance of a granular substance within the vesicle is often observed.  $\times 56,000$ .



One of the most striking features of insect fibrillar flight muscle is that the above description, aptly stating the condition in vertebrate muscle fibers, does not apply. From both longitudinal and transverse sections of the fiber, it is evident that the sarcoplasmic reticulum in high frequency flight muscle deviates from the above plan in a striking fashion. In particular, the "triads" of Porter and Palade, comprising intermediary vesicles and lateral terminal cisternae are absent, as is the complex tubular component of the A band region of vertebrate skeletal and cardiac muscle. Instead, the only component referable to this portion of the sarcoplasmic reticulum in the flight muscle of *Tenebrio* is a large number of unconnected smooth-membraned vesicles, distributed throughout the fiber without reference to the repeating sarcomere pattern. Profiles of these vesicles are typically somewhat triangular seen in transverse section, and each is *ca.* 100  $\mu$  in width. The membrane defining them is

*ca.* 50 A wide, and clearly does not show, in these preparations (Figs. 30 and 31), the triple-layered organization, an observation at variance with the findings of Robertson (1959) and Andersson (1957).

While the vesicles are occasionally seen by themselves they are typically closely associated at all depths in the fiber with another component, a flattened smooth-membraned profile. *This double structure is here and subsequently termed a "dyad." Even at low magnification, the membrane defining this second component appears to be appreciably darker or denser than that of the sarcoplasmic reticulum element that it accompanies, and this difference has a fundamental morphological basis, as it has here been shown that the former is continuous with the peripheral plasma membrane of the muscle fiber.* The triple-layered organization of the plasma membrane has already been mentioned and figured (Figs. 25, 27, and 28) and it has furthermore been stated that the thinner membrane of the isolated

FIGURE 19

An electron micrograph of flight muscle so oriented that an unusually extensive view is obtained of the plasma membrane tubules derived from the sheath surrounding the tracheoles (*tr*). The tubule (*pm*) bifurcates, and the branches, in their course between the fibrils, come into association with several sarcoplasmic reticulum vesicles at the points indicated with asterisks, constituting a series of dyads, the plasma membrane component of which is seen to be a continuous system. In other cases (*x, y, z*) only a small portion of the tubular system lies in the plane of section, and the dyad is isolated.

This figure should be compared with the light micrograph of a transverse section of a fiber stained with silver by Veratti's technique (Fig. 3). There, a reticular system is visualized, anastomosing between the fibrils, and evidently arising from a membrane sheath surrounding the tracheoles. In that figure, local thickenings of the reticular elements are visible. It is suggested that the reticular systems shown in Figs. 3 and 21 are identical, and that the "nodes" seen in the light micrograph are, in the electron microscope, resolved into vesicles of the sarcoplasmic reticulum.  $\times 29,000$ .

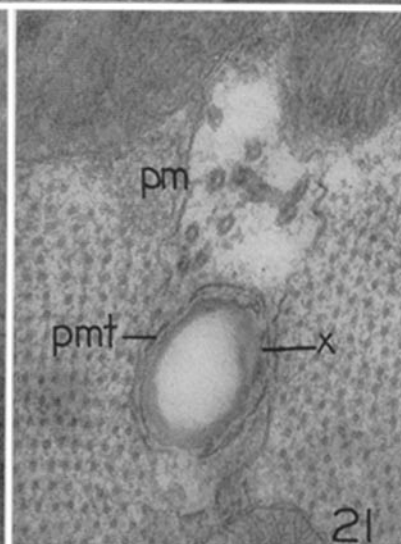
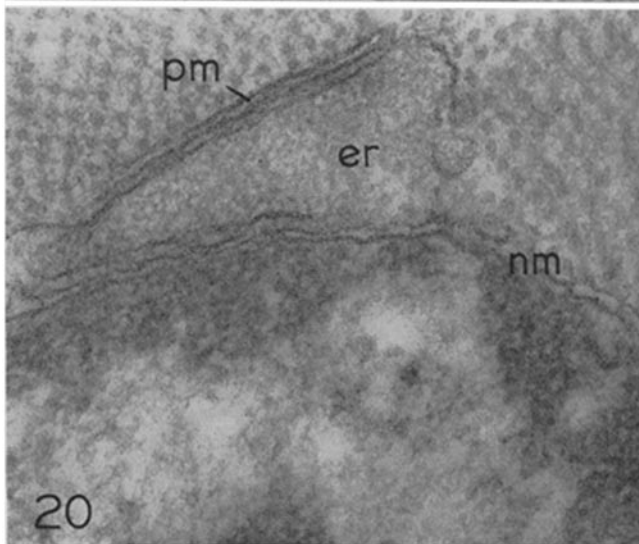
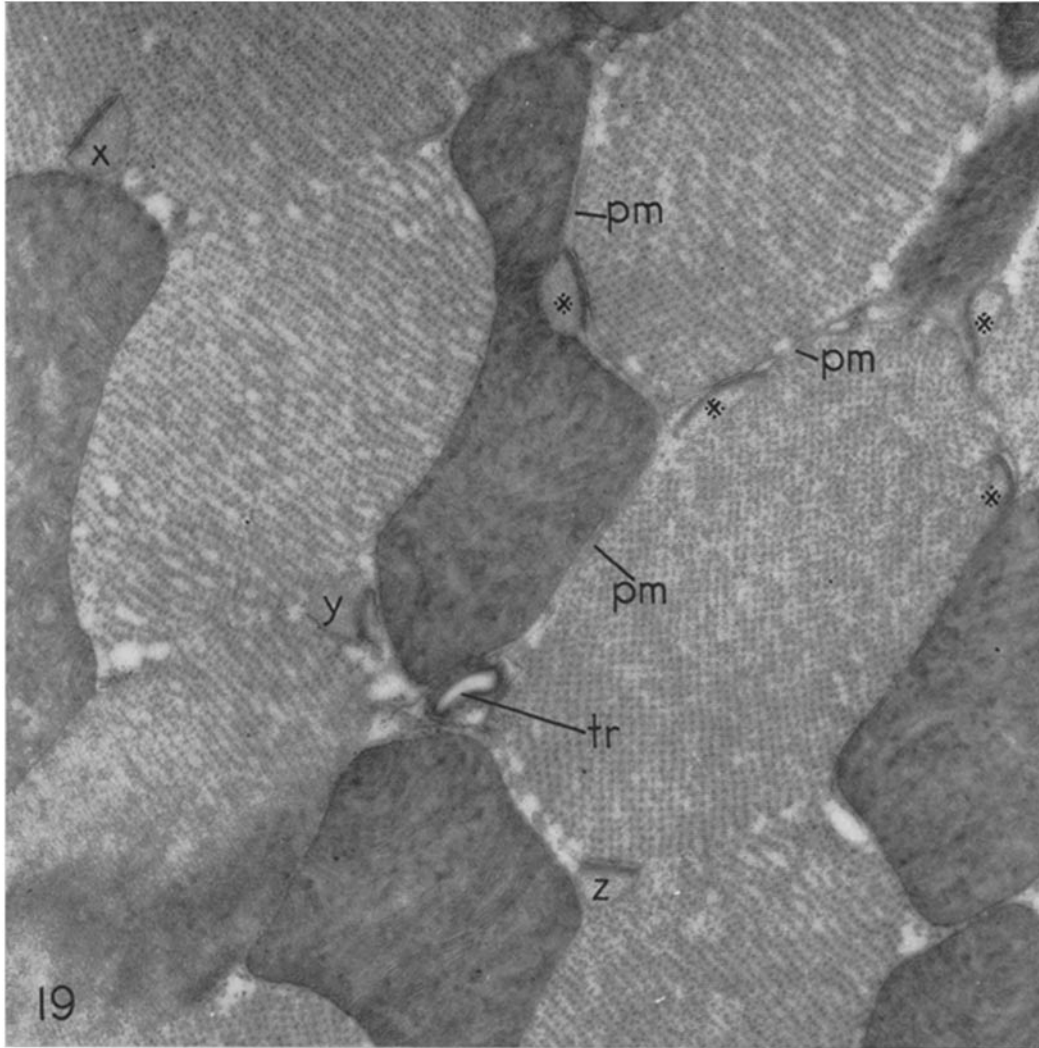
FIGURE 20

Electron micrograph illustrating the intimate relationship which may occur between the nuclear membrane (*nm*) and a dyad, comprising a sarcoplasmic reticulum vesicle (*sr*) and a tubular profile of plasma membrane derivative (*pm*).  $\times 105,000$ .

FIGURE 21

In this electron micrograph, a tracheole is seen in transverse section, lying in the wide cavity bounded by the plasma membrane sheath (*pm*). The cytoplasm of the tracheoblast is extremely attenuated, but its plasma membrane may be seen surrounding the tracheole (*pmt*). As a result of this attenuation it becomes difficult to observe the intra-tracheoblast membrane inside which the wall of the tracheole is laid down, but at *x* a portion of this membrane may be discerned.

The circular or tubular profiles seen in the intima of the plasma membrane-bounded cavity occur occasionally, and while their significance is unknown, they may represent sections through inwardly projecting "fingers" from the plasma membrane sheath.  $\times 50,000$ .



vesicles appears to be structurally simpler. This distinction should therefore be apparent between the two components of a single dyad, and the confirmation of this in favorable sections (Figs. 30 and 31) provides valuable confirmation for the suggested dual derivation of the dyads in this muscle.

The reasons behind the use of the term "dyad" should be explained. Porter and Palade (1957) found that in rat cardiac muscle the "triad" of other fibers was typically represented, through the omission of one terminal cisterna, by a pair of profiles one of which corresponded to the intermediary vesicle component. This association they termed a "dyad." The suggested analogy and possible homology between the dyad or triad of vertebrate muscles and the plasma membrane-vesicle pairing found in *Tenebrio* flight muscle are thus conveniently expressed by the retention of the original term.

The ensheathing circumtracheolar plasma membrane has already been described, and the next point to be considered is the relationship between these plasma membrane inclusions inside the fiber and the flat profiles associated with the vesicular sarcoplasmic reticulum. Until now, the muscle plasma membrane has been pictured as entering the fiber as a simple cylinder surrounding the tracheoles. Reference to Figs. 6, 13, 15, and 19 will indicate that this is an oversimplification,

for this sheath sends out at intervals narrow tubular or strap-like processes which course between the fibrils and sarcosomes. While sarcoplasmic reticulum vesicles are sometimes seen adjacent to the main circumtracheolar sheath, it is their association with the tubules derived from this sheath that is responsible for the dyad organization. *It is, therefore, in these fibrillar fibers the plasma membrane processes, not the reticulum cisternae, that afford an extensive membrane system within the fiber.*

In fortunate sections, the direct origin of the plasma membrane tubes in the circumtracheolar sheath may be seen, though the dyads appear to be isolated when the plasma membrane element meets its parent sheath at a point out of the plane of section. Figs. 12 and 15 depict tracheoles in longitudinal section. In the first a radial prolongation of the sheath is indicated, and in the second a similar plasma membrane tubule is seen passing tangentially across the surface of a myofibril. The transverse section (Fig. 13) shows a tracheole the plasma membrane sheath of which is produced into a flange or tubule, closely applied to which are seen two vesicles of the sarcoplasmic reticulum. A similar though more extensive view of this organization is shown in Fig. 19, where a plasma membrane tubule branches in its course between the fibrils, and at several points dyad associations are established. The complexity of

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#### FIGURE 22

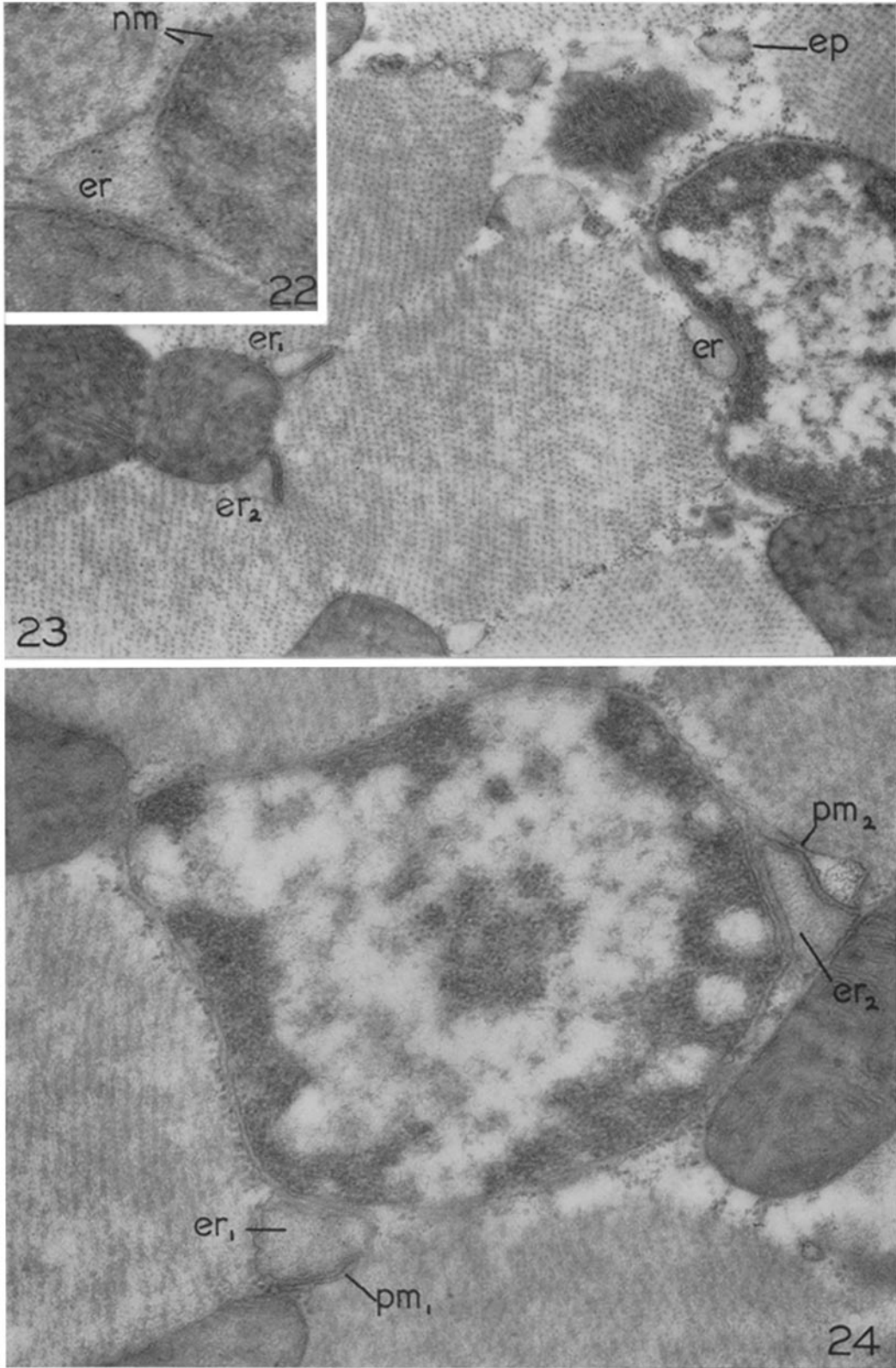
An electron micrograph illustrating the relationship between a vesicular element of the sarcoplasmic reticulum (*er*), and the nuclear membrane (*nm*). The outer of the two components of the nuclear membrane is produced into a "bleb" at *er*, the surface of which is devoid of RNP particles, distributed elsewhere over the surface of the nucleus. Thus the ER-nucleus association in these flight muscle fibers is in accord with that observed in many other tissues.  $\times 90,000$ .

#### FIGURE 23

In this electron micrograph it may be seen that the outer component of the nuclear membrane bears RNP particles, as in other instances, and that similar particles also occur in the immediate vicinity both free and, as at *ep*, attached to the surface of vesicles which are presumed to be elements of the sarcoplasmic reticulum. A smooth element of this system lies against the nuclear surface at *er*, while at *er*<sub>1</sub> and *er*<sub>2</sub> two other such vesicles are present in association with plasma membrane tubular component in the typical dyad configuration.  $\times 34,000$ .

#### FIGURE 24

Another nucleus is seen in this micrograph, in which the particulate outer membrane is more clearly visible. In addition, two dyads are situated beside it, in each of which the sarcoplasmic reticulum vesicles (*er*<sub>1</sub> and *er*<sub>2</sub>) are inserted between the nuclear membrane and the plasma membrane derivatives (*pm*<sub>1</sub> and *pm*<sub>2</sub>).  $\times 56,000$ .



the tubular system is seen to good advantage in Fig. 6 in which the plane of section passes through portions of seven subsidiary tubules all associated with one tracheole. The smallest elements of this arborising system are *ca.* 250 Å in diameter; some ten times smaller than the finest tracheolar branches. In this last example, the muscle employed was from a newly emerged adult of *Tenebrio*, hence the small size of the sarcosomes.

That this plasma membrane system is indeed tubular or strap-like, and does not consist of deep folds is indicated by the rarity of fields such as those just described: fragmentary tubule profiles are typical, and the chance of encountering a plane of section including as extensive a view of the system as that in Figs. 6 and 19 is small. The association between plasma membrane extensions and the scattered vesicles is also found

at the periphery of the fiber, where the latter may be closely applied to the shallow infoldings of the plasma membrane already described (see Fig. 8).

Examination of longitudinal sections confirms the suggestion that the vesicles observed in transverse sections are indeed isolated and do not represent profiles of a system, either extensive or even continuous in the longitudinal direction. Furthermore, from the occurrence of similar profiles of dyads in each plane of section (compare Figs. 7, 13, 16, 18, and 31, for example), it is evident that the plasma membrane system extends in like manner in both the radial and longitudinal directions within the fiber. As seen in longitudinal sections, the dyads are situated alongside the fibrils, lying in the angle between adjacent sarcosomes, usually with the plasma membrane component facing the outermost myofilaments

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FIGURE 25

A high power electron micrograph showing in more detail the structure of an "adhesion plate" association between the two sides of a peripheral plasma membrane invagination. This micrograph should be compared with those in Figs. 9 to 11 which illustrate the appearance of these structures, and also indicate their topographical relationship with the fiber periphery.

The adjacent plasma membrane surfaces each show the 25-25-25Å triple layering, while the space intervening between them and the immediately distal region contains a homogeneous substance of higher density than elsewhere in the adjoining cytoplasm. It should also be noted that the "adhesion plate" shown here appears to be structurally simpler than that illustrated in Figs. 10 and 11.  $\times 360,000$ .

FIGURE 26

An electron micrograph of a portion of a sarcosome (*ss*) situated just beneath the peripheral region shown in Fig. 28. Note that whereas the plasma membrane in Fig. 28 is clearly triple-layered, the membranes defining the cristae of the sarcosome do not show this organization.  $\times 200,000$ . (*fil*: myofilaments).

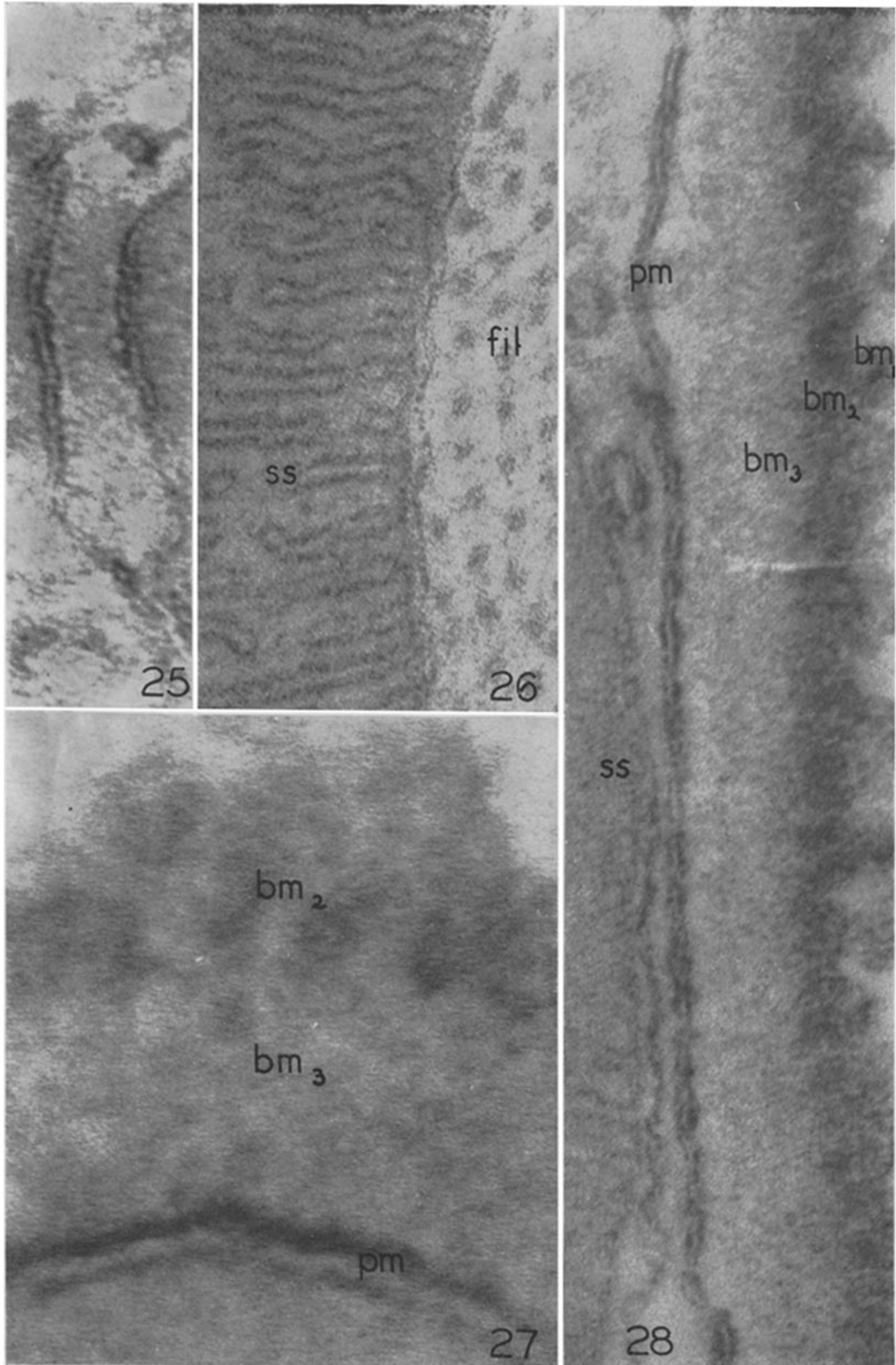
FIGURE 27

High magnification electron micrograph showing the triple layered structure of the plasma membrane (*pm*) beneath the basement membrane (*bm*<sub>2</sub>, *bm*<sub>3</sub>). The localized unequal thickness of the osmiophilic components superimposed upon the basic 25-25-25Å system is frequently observed in the case of this tissue, as has been illustrated in *Amoeba* (Mercer, 1959). Stoeckenius (personal communication) suggests that the variation reflects local irregularities in the disposition of protein which binds either osmium or lead.  $\times 800,000$ .

FIGURE 28

An electron micrograph illustrating the sarcolemmic basement membrane and underlying plasma membrane of a fiber. In this instance the diffuse outermost layer of the basement membrane (*bm*<sub>1</sub>) is less clearly demarcated from the two inner layers *bm*<sub>2</sub> and *bm*<sub>3</sub> than in Fig. 8. Just beneath *bm*<sub>3</sub> and separated from it by a space of *ca.* 150Å lies the plasma membrane *pm* which is resolved into the two *ca.* 25Å osmiophilic components, and the central osmiophobic region. Beneath the plasma membrane is situated the edge of a sarcosome (*ss*).  $\times 250,000$ .





(Figs. 14 and 16) or between two closely apposed fibrils (Fig. 18). In addition, isolated profiles of the tubular or strap-like extensions of the circumtracheolar sheaths are also found (Figs. 14 and 17) and these plasma membrane elements presumably engage in dyad associations with vesicles at points out of the plane of section. The distribution of dyads or unassociated plasma membrane tubules does not correspond to the striation of the myofibrils but, as may be seen in Fig. 14, these structures occur very frequently along each longitudinally sectioned fibril, suggesting that the entire fibril is extensively entwined by the tubule system, with its accompanying vesicles.

The suggested organization of these sarcoplasmic components and their spatial relationship with the myofibrils is illustrated in semidiagrammatic form in Figs. 32 and 33. The former is primarily intended to show the continuity between the basement membrane of the tracheal cells and of the fiber, and the shallow invaginations of the peripheral plasma membrane, associated with tracheoles and otherwise. In this figure both the circumtracheolar plasma membrane sheaths and the sarcoplasmic reticulum vesicles are included, but for simplicity the arborising system of tubules springing from the former are shown only in Fig. 33.

Fig. 33 illustrates the topography of the circum-

tracheolar sheaths and their derivative tubules, and the construction of "dyads" through the association of these tubules with the isolated vesicles of the reticulum.

It is suggested here, and discussed later, that in the distribution of this plasma membrane system lies the resolution of the problem of the great size of these fibers. The wing-beat frequency of beetles is unspectacular compared with that recorded in wasps, bees, and especially certain flies (Sotavalta, 1947), in which the fiber size shows no diminution. In fact it is amongst the Diptera, where the highest frequencies are found, that the largest fibers are said to occur (Tiegs, 1955). The unusual mechanical circumstances responsible for the very high frequency of contraction in these muscles will be mentioned at greater length in the discussion, but the anomaly of the fiber size in fibrillar muscle must still be accounted for.

#### DISCUSSION

It is evident that fibrillar flight muscle contains the same basic components as other types of muscle, although their arrangement shows noteworthy peculiarities. Of these, the reduction of the I band of the myofibrils and the extensiveness of the mitochondrial supply have been established for some time, and their existence has been

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#### FIGURE 29

A transversely sectioned tracheole, lying just beneath the periphery of a fiber, and parallel to its long axis. The plasma membrane (*pm*) invaginates with the tracheole, and in returning to its peripheral course forms a "curtain" containing extracellular space. At *pmi* may be seen the plasma membrane of the tracheoblast extension, within which the intima of the tracheole is laid down.  $\times 63,000$ .

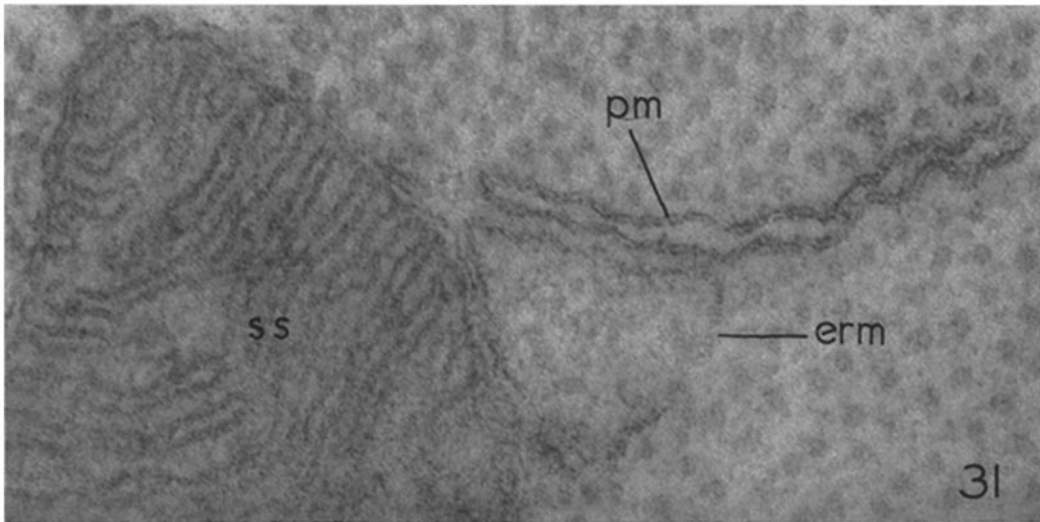
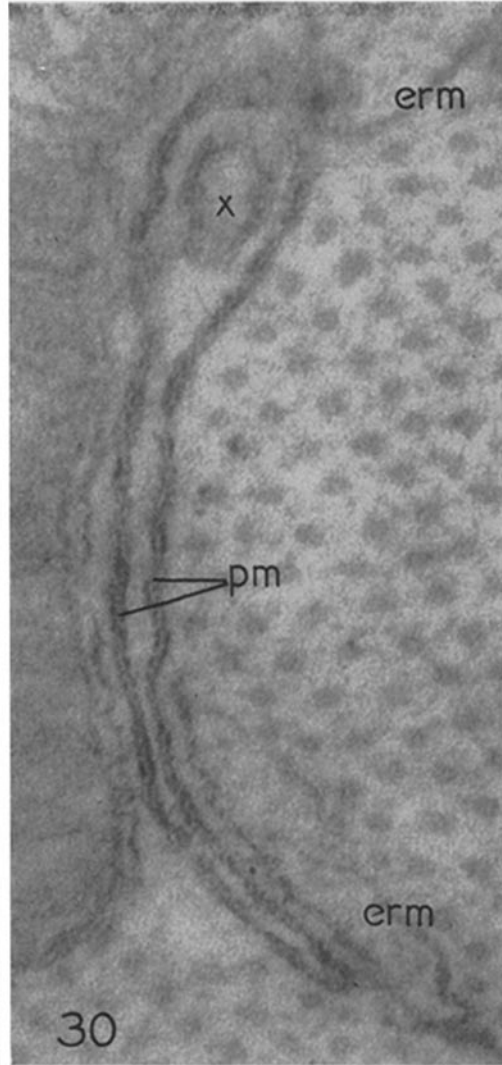
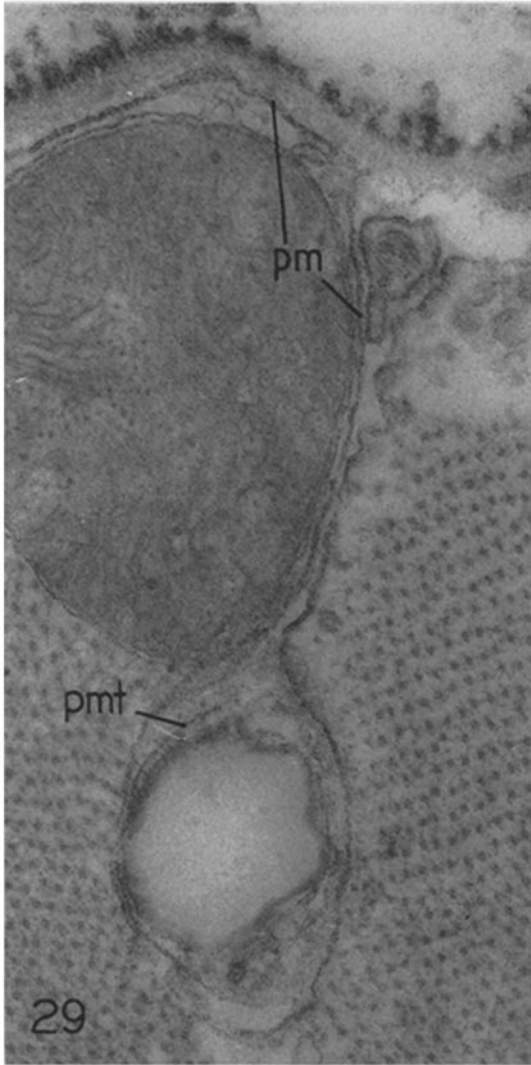
#### FIGURE 30

Electron micrograph of a transverse profile of a fiber plasma membrane tube; an extension of the sheath drawn in with the tracheole, situated deep inside the fiber. The membrane of this tube shows the 25-25-25A triple layering characteristic of the fiber plasma membrane (*pm*). The ovoid profile at *x* may represent a section through an inwardly projecting diverticulum of the tubule or a small extension of the tracheoblast.

In addition, at *erm*, are seen profiles of vesicles of the unconnected sarcoplasmic reticulum component the membranes of which are *ca.*50A in thickness and do not show a triple-layered organization.  $\times 230,000$ .

#### FIGURE 31

An electron micrograph showing an association between a 75 A thick plasma membrane element of the reticulum (*pm*) and an isolated vesicle (*erm*) bounded by a 50 A membrane, in the dyad configuration. No evidence of triple layering is seen either in this last membrane or in the components of the cristae within the sarcosomes (*ss*).  $\times 164,000$ .



reasonably accounted for. Of greater interest, therefore, are the present observations on other sarcoplasmic components, notably the inter-fibrillar membrane systems of the fiber.

Bennett (1955) has drawn attention to early descriptions of a reticular system within the sarcoplasm, visualized by metallic impregnation methods (Thin, 1874; Retzius, 1881; Cajal, 1890; Veratti, 1902; Holmgren, 1908; etc.),<sup>3</sup> and observes that these images correspond to the "sarcoplasmic reticulum" of smooth-membraned cisternae and vesicles seen in electron micrographs. However earlier electron microscopic investigations of insect fibrillar flight muscle failed to show such a reticulum, and hence offered no basis for interpreting the images obtained by application of the "black reaction" of Golgi to these muscles described by Cajal,

<sup>3</sup> It is probable, however, that a number of the early descriptions of reticula in muscle fibers, notably those based on methods of staining with gold chloride, are incorrect, as these result in the impregnation of the entire sarcoplasm rather than of a differentiated structure within it. This subject is discussed more fully by Smith (1961), and a review of modern concepts of the sarcoplasmic reticulum is given by Porter (1961), also in this volume.

Veratti, and Holmgren. As an incidental to the electron microscopic work on *Tenebrio* muscle this method was repeated as carried out by Veratti, and the original results were duplicated. Veratti writes (present translation):

"By treating the muscles 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. . . . 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." However, he continues: "It was not clear to me what position the transverse tracts of the reticular apparatus occupied relative to the transverse striae of the contractile fibrils. . . ." and of especial interest and importance here is the further observation that "The filaments which form the reticular apparatus are inserted on the tracheal branches that run in the interior of the fibers. . . [and] . . . I was never able to see any image suggesting that the cavity of the tracheal canals is continuous, even for a very short distance, with the initial part

FIGURE 32

Illustrating the organization of sarcolemmic, sarcoplasmic, and fibrillar components in the peripheral region of a flight muscle fiber of *Tenebrio*. The following points should be noted:

(i) At the upper left, an obliquely sectioned tracheum makes contact with the fiber through fusion of the basement membranes of tracheoblast and muscle, while at the upper right a tracheoblast extension passes between the basement membrane of the sarcolemma and the plasma membrane.

(ii) The plasma membrane of the fiber makes a number of shallow invaginations, which are thought to run for a short distance only, in the longitudinal direction. Internalised tracheoles running parallel with the long axis of the fiber, however, (upper center) carry with them a "curtain" of plasma membrane, throughout their course.

(iii) Elsewhere, four tracheoles are depicted, passing between the myofibrils and sarcosomes. Each of these is accompanied by a concentric sheath of fiber plasma membrane continuous with that at the surface, at the point of ingress of the tracheole. The subsidiary tubular branches of these circumtracheolar sheaths are here omitted, and are shown in Fig. 33.

(iv) Scattered vesicles of the sarcoplasmic reticulum are shown, lying in the sarcoplasm between the fibrils and sarcosomes. It is these vesicles that engage in dyad associations with the plasma membrane tubules from the circumtracheolar sheaths. Note also the "blebs" formed from the outer membrane of the nuclear envelope; these are thought to represent, in this tissue, the relation between nucleus and endoplasmic reticulum observed in other cell types.

To simplify the diagram, the space between the sarcosomes and the myofibrils has been exaggerated.

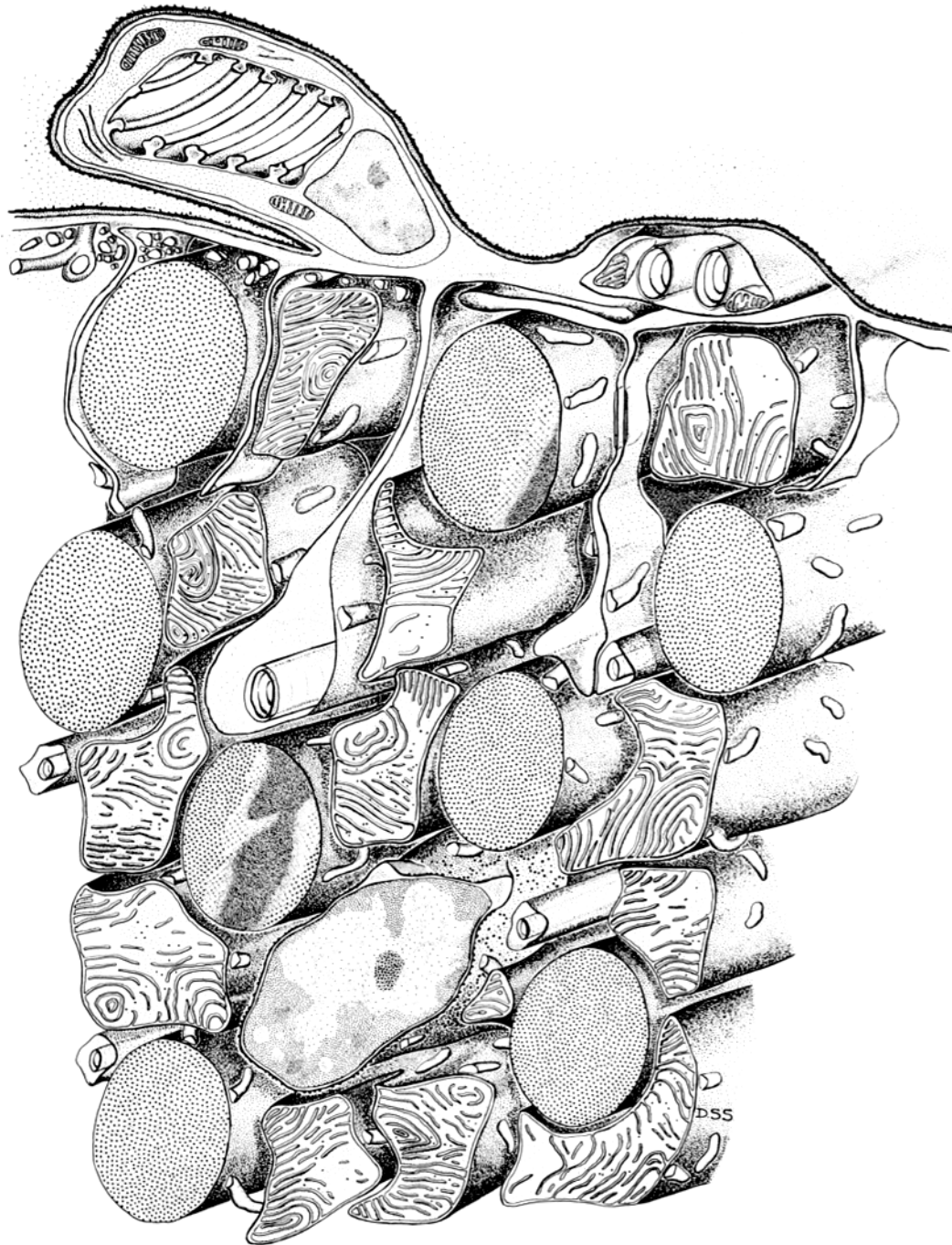


FIGURE 32

of the reticulum filament that attaches itself to the trachea.”

Cajal (1890), on the other hand, had not only supposed that the finer branches of the tracheae, the tracheoles, enter the flight muscle fiber (in the beetle *Ateucus*), but had also employed the black reaction to visualize the reticular system described above by Veratti. To Cajal, however, no distinction was evident between the tracheal and reticular systems (the “*capillaires trachéens*”); the latter were held simply to represent the finest anastomosing extremities of the former. He further claimed that in the leg muscles, the intracellular reticulum was similarly connected with the tracheae, in this case running along the surface of the fiber; a contention that Veratti again refuted, on a basis both of direct observation and also by analogy, pointing out that the reticulum in these muscles closely resembles that found in the crustaceans *Astacus* and *Carcinus* which, of course, lack a tracheal supply.

From the correspondence between the images obtained by Veratti in vertebrate muscle, and the interpretation of the organization of the sarcoplasmic reticulum made by Porter and Palade (1957) and others, it is evident that it is this sarcoplasmic component that is stained with silver in the “black reaction.” It is equally evident that Veratti’s fibrillar flight muscle reticulum corresponds to the internalised plasma membrane system described here, a system that lacks the oriented repeating unit structure of the vertebrate skeletal muscle sarcoplasmic reticulum. Furthermore, the “nodes” present throughout the network described by Veratti (see also Figs. 3 and 4) probably represent the isolated vesicles which are associated with the plasma membrane tubules in the dyad configuration.

From the invariable presence of a reticular

system in the wide variety of muscles studied, Veratti was justifiably unwilling to concede a special position to the fibrillar flight muscles, while from the observations to be described here emerges the significance of his correctly drawn distinction between the intracellular tracheolar and reticular systems. During the past fifty years this distinction has rarely been recognized or even alluded to; a fact principally due to neglect of the original work or to confusion arising from failure to appreciate its comparative aspects. Tiegs (1955), for example, wrote in connection with this muscle:

“It cannot be said of the Golgi preparations that they have solved the question of the intracellular tracheae. That the tracheae can be displayed by the technique is certainly so; but it is disconcerting to find that similar networks have been reported by this method in crustacean and even mammalian heart muscle.”

The confirmation that has stemmed from electron microscopy of the presence of a reticulum or derivative in all striated muscles so far examined, rather than proving disconcerting, has provided valuable pointers to a clearer understanding of muscle function, and the work reported here is an attempt to incorporate one rather specialized example into the wider comparative framework.

In connection with this fibrillar muscle reticulum, two questions must be answered. First, what is its functional role and second, as a corollary of this, what is the role played by the internal membrane systems of other types of muscle?

The nature of the coupling mechanism linking fiber-membrane excitation and contraction is still uncertain. Whereas in vertebrate “twitch” muscle, containing a single motor end-plate on

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### FIGURE 33

This drawing illustrates the extensive system of subsidiary tubules, springing from the plasma membrane sheaths surrounding the tracheoles, two of which are shown here. Note their relationship with the fibrils, and with the vesicular component of the sarcoplasmic reticulum. In many places, the plane of section passes through both a plasma membrane tubule and a closely applied vesicle, the two components of a “dyad.” The striation of the myofibrils is indicated, and it may be seen that the distribution of tubules and vesicles does not correspond to this repeating pattern.

At the lower left, a portion of a nucleus is included, showing the raised “blebs” of the outer membrane of the nuclear envelope. These blebs are often (as represented here) accompanied by plasma membrane tubules, just as are the isolated vesicles of the sarcoplasmic reticulum.

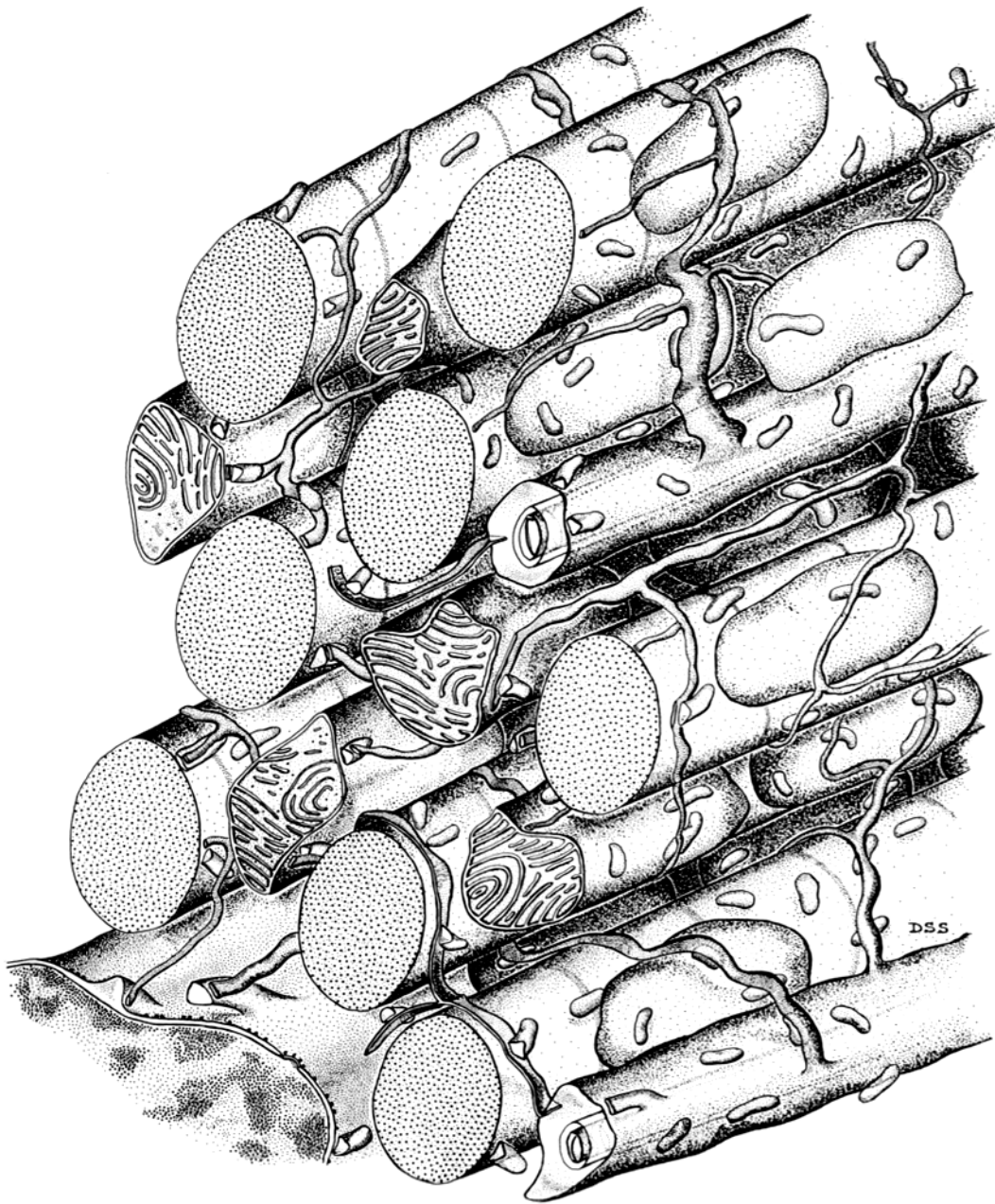


FIGURE 33

each fiber, the arrival of an impulse initiates a propagated wave of membrane depolarisation which spreads out rapidly from the end-plate region, each insect muscle fiber is supplied by a number of fine motor nerve branches and the impulse sets up a local area of depolarisation at each myoneural junction, rather than a propagated response. In each instance membrane activity triggers contraction of the fibrils, and while the pathway by which this coupling occurs is essentially unknown, in terms of the response made by the muscle itself, that it may well be mediated by one or more steps of a chemical nature has often been suggested, for example by Hill (1948 and 1949), while the work of Csapo (1959) and others implicates calcium in this coupling process.

However, if activation of the fibrils is mediated by a substance liberated at the site of membrane depolarisation, that is, at the front of the propagated excitatory wave in vertebrate muscle or at the several sites of depolarisation in insect muscle, then the time interval before the onset of contraction must be compatible with the diffusion rate of the substance from the activated membrane to the most distant fibrils. This problem has been clearly stated by Hill, by Peachey and Porter (1959), and by Huxley (1959), and others, and was investigated on a theoretical basis by Hill (1948, 1949) who concluded that inward diffusion of the activating substance could not be completed within the observed time interval between excitation and the onset of contraction, if it is assumed that in this period a reasonable concentration of the substance must be reached at the axis of the fiber, and that the distance to be traversed is equal to the radius of the fiber.

But if plasma membrane depolarisation is not merely a surface phenomenon; if the membrane is in some way internalised within the fiber then the situation is very different. Such a situation has been observed in the case of skeletal muscle of *Carcinus* by Peachey (manuscript in preparation), where the very wide fibers are deeply penetrated by arborisations of the plasma membrane which reduce the diffusion distance from the radius of the fiber to, at the most, a few microns. Another instance which is in some respects analogous occurs in *Branchiostoma* where, by virtue of the 1  $\mu$  thick plasma membrane-covered lamellar fibers, the maximum diffusion distance is only

0.5  $\mu$  (Peachey and Porter, 1959, and in this volume Peachey, 1961).

In vertebrate skeletal muscle the position is as yet less clear, for no *uninterrupted* internal plasma membrane system has been observed. However, Porter and Palade (1957) reported a close association between the intermediary vesicles of the sarcoplasmic reticulum triads in the subsarcolemmic region of *Amblystoma* muscle, *via* a continuous series of vesicles closely resembling those situated between adjacent terminal cisternae. In cardiac muscle of the rat they found a homologous situation—an elongated membrane-bound element extending between the plasma membrane and each circumferential Z band.

A strong pointer towards the physiological importance of the triad level, and very possibly of the intermediary vesicles themselves, stems from the work of Huxley and Taylor (1955 *a* and *b*; and Huxley (1958 and 1959). They found that depolarisation of the frog sartorius fiber surface by a current too small to initiate a propagated response, applied over a diameter of only 1 to 2  $\mu$ , caused a local contraction only when applied opposite an I band; never when opposite an A band. In this muscle, as in *Amblystoma* myotome fibers, the triads straddle the I bands. In the muscle of *Lacerta*, on the other hand, as Porter and Palade (1957) reported for rat muscle fibers, *two* triads are present beside each sarcomere, situated near the A-I junction (Robertson, 1956; Huxley, 1959) and similarly Huxley found that contraction only occurred when the membrane was depolarised opposite a triad; each half of the I band in *Lacerta* was found to respond independently.

Thus, earlier suggestions based on circumstantial grounds that the sarcoplasmic reticulum is involved in impulse conduction, have received some experimental support, with the qualification that only *part* of the reticulum may be implicated. It is possible that if the triad is indeed a physiologically differentiated complex, then morphological distinction may be sought between the membranes of the intermediary vesicles and the reticulum cisternae, paralleling the difference described in this paper between the vesicles ascribed to the reticulum, and the plasma membrane element of the dyad in fibrillar muscle of *Tenebrio*. The reasons for calling this membrane association in *Tenebrio* a "dyad" have already been mentioned. It is suggested that while the



intermediary vesicles of the dyads or triads of other fibers may not form a continuous system, they may perhaps be homologous with the internalised plasma membrane component of the fibrillar muscle dyad which is, on this basis, to be regarded as an evolutionary specialization developed in connection with the physiological peculiarities of the muscle. The sarcoplasmic reticulum of other types of insect muscle (notably "tubular" or skeletal muscle) has received less detailed attention than has vertebrate muscle, but appears to resemble the latter in many respects. Repeating profile associations similar to the dyads or triads of Porter and Palade have been described in *Locusta* (Vogell *et al.*, 1959) and in dragonfly (*Aeshna*) flight muscle (Smith, in press).

In interpreting the findings described here, certain peculiarities of fibrillar muscle should be stressed. Roeder (1951) showed that while the flight muscles of many insects exhibit a 1:1 relation between motor impulse and contraction frequency, those insects that have developed "fibrillar" muscle show a myogenic rhythm at a frequency which may greatly exceed that of the impulse supply; a rhythm the existence of which was first demonstrated by Pringle (1949) in *Calliphora* flight muscle. Boettiger and Furshpan (1950; 1951; 1952), from examination of the mechanics of wing action in the blow-fly *Sarcophaga*, suggested that the rhythm described by Pringle could be the result of simultaneous tetanic contraction of the wing elevator and depressor muscles acting against a mechanically determined "click mechanism" imposed by the architecture of the wing-bearing segments of the thorax. However more recently, Boettiger (1957 *a*) and Pringle (in press) have obtained evidence that this hypothesis may be an oversimplification, and that in this oscillating system, a precise length-tension relationship is operating in the fibers. The whole problem is fully reviewed by Pringle (1957).

Nevertheless, although the very high rate of contraction of fibrillar flight muscle may be accounted for in these terms, its maintained activity requires repeated motor nerve excitation, albeit at a much lower frequency, and it is evident that the motor axon branches are presented with fibers of unusually large size, while it is known (Smith, 1960) that these axons do not, in *Tenebrio*, penetrate beyond the fiber surface.

The average radius of a flight muscle fiber in

*Tenebrio* is  $100\ \mu$ ; a great distance in terms of diffusion time needed for the equilibration of the center of the fiber with any chemical disturbance occurring at the periphery. But the situation is very different if the plasma membrane surrounding the tracheoles is taken into account. In analyzing this situation, the concept was employed of a hypothetical cylinder of sarcoplasm, associated with each circumtracheolar plasma membrane sheath. It has been shown that such sheaths permeate the fiber, and that they are principally oriented parallel with the long axis of the fiber. It is suggested that depolarisation of the plasma membrane associated with the arrival of a nerve impulse at the periphery will be transmitted throughout the ramifications of this membrane within the fiber. As each fiber of this type of muscle is multiply innervated by many fine branches of the motor nerves, many areas of depolarisation will be initiated (corresponding to the distributed or multiple nature of the myoneural synapses) by the arrival of each impulse. The internalised plasma membrane system is visualized as mediating the inward spread of depolarisation from each peripheral excited center.

Thus the aforementioned hypothetical column of cytoplasm under the influence of each circumtracheolar sheath becomes the unit upon which to base considerations of excitation-contraction coupling in these fibers. To obtain a quantitative estimate of this, the tracheolar distribution was examined in 10 low power fields of transversely sectioned fibers, each representing an area of  $900\ \mu^2$ . From counts of tracheole profiles, it was calculated that the mean radius of influence of each circumtracheolar sheath is  $2.55\ \mu$ . This figure does not, however, take into account the full extent of the internalised plasma membrane system, as allowance has not yet been made for the tubular arborisations springing from the main sheaths. To supply this correction, a similar survey was made of ten higher magnification fields, each corresponding to an area of  $144\ \mu^2$ , in which not only tracheoles but also dyads were counted, as one component of each dyad, as much as the tracheolar sheath, is presumed to represent an excitable prolongation of the plasma membrane. Other profiles of this system are certainly present in each section, but positive identification is only readily available in the case of the dyad, and as only these were included in the counts, the resulting estimate is a conservative

one. Taking into account both the plasma membrane around the tracheoles, together with the tubular ramifications of this system, it is found that the maximum distance, on a statistical basis, separating any point in the fiber from an element of the plasma membrane is only  $1.72 \mu$ . It should be remembered at this juncture that the average fibril diameter in this species is about  $2 \mu$ , so that in terms of excitation transfer or coupling, it appears that the basic cylinder of contractile material upon which excitation must act is not that of the  $200 \mu$  diameter fiber, but rather a large number of cylinders, each certainly no wider than the individual myofibrils of which the fiber is composed.

This tissue was termed "dissociable muscle" by the early light microscopists on account of the ease with which the fiber could be separated into its component fibrils. It is now apparent that in another sense these fibers may be considered to be "dissociable," this time in terms of the physiology of excitation and contraction.

The fiber diameter of fibrillar muscle varies widely. Within the order Coleoptera, this ranges from the unusually small figure of  $25$  to  $35 \mu$  in certain carabids (e.g., *Bembidion elongatum* and *Harpalus tardus*)<sup>4</sup> through an intermediate range such as *Lucanus cervus* ( $80 \mu$ ) and *Melolontha melolontha* ( $140 \mu$ ) (Darwin and Pringle, 1959), to *Tenebrio molitor* ( $150$  to  $250 \mu$ ) and *Cosmopolitus sordidus* ( $250$  to  $300 \mu$ ).<sup>4</sup> Wing-beat frequencies in this order are not spectacular; Darwin and Pringle (1959) give a value of  $40$  c/s for *Lucanus*, and Sotavalta (1947)  $62$  c/s for *Tenebrio* and for others, values of up to  $175$  c/s (*Attagenus schaefferi*). The fiber diameter of the hymenopteran *Apis mellifica* is  $170$  to  $200 \mu$  (Tiegs, 1955) while Sotavalta (1947) recorded a wing-beat frequency of  $208$  to  $277$  c/s in this species. It is significant that both the highest frequency of beat and the greatest diameter of the flight muscle fibers may occur together, in the Diptera. Sotavalta found the frequency to be  $150$  to  $350$  c/s in female culicid mosquitos, and  $450$  to  $600$  c/s in males, while Tiegs states that the fiber diameter in the related species *Muscidus alternans* is  $170 \mu$ , an unusually low figure for a fly, as is shown by the following examples:  $750 \mu$  for *Trichophthalma bancrofti*,  $500 \mu$  for *Muscina stabulans* (*M. assimilis*  $131$  to  $175$  c/s),

<sup>4</sup> Fiber diameters measured by the author on material fixed in alcoholic Bouin's fluid.

$1$  mm. for *Calliphora stygia* (*C. erythrocephala*  $110$  to  $196$  c/s). Sotavalta recorded frequencies of several hundred cycles per second for many species of Nematocera, with the highest value, over  $1000$  c/s, being recorded in *Forcipomyia* sp., while according to Tiegs the tachinid *Rutilia potina* attains a fiber diameter of  $1.8$  mm., although to confirm these diameters the disposition of the plasma membrane should be studied with the electron microscope as it is possible that these very large fibers may perhaps prove to be compound structures, enclosed within a common basement membrane.

While the mechanics of the high frequency of wing beat in these insects is explained by the work of Pringle, Boettiger, and others, the great variation in fiber diameter in fibrillar muscle must be accounted for. It is suggested that, by virtue of the penetration of the fiber by circum-tracheolar sheaths and their arborisations, the fiber diameter here ceases to be a limiting factor in excitation-coupling considerations. It has been shown that in *Tenebrio* the maximum distance separating any point in the fiber from a plasma membrane surface is ca.  $1.7 \mu$ , and preliminary observations suggest that the same is true of dipteran muscle. It thus matters little, in terms of the inward passage of the impulse, whether the fiber diameter is  $25$  to  $35 \mu$  as in certain carabid beetles or several hundred microns.

Other questions concerning insect and other muscles remain unanswered. The association between plasma membrane tubules and the vesicles of the sarcoplasmic reticulum in *Tenebrio* is striking, but its significance is unknown. Similarly, if in vertebrate muscles impulse conduction is the domain of the intermediary vesicles, then what is the function of the rest of the sarcoplasmic reticulum, the extensive network of cisternae surrounding the myofibrils and corresponding to the isolated vesicular component of the system in *Tenebrio*? The variation between different muscles in the extent and disposition of the fibrils and of the reticulum and sarcosomes is considerable, and the combined enzymatic and electron microscopic study of three functionally and structurally different muscles from a single species (*Locusta migratoria*) (Vogell et al., 1959) demonstrates the value of such an approach. It may well prove that the two principal functional roles suggested

for the sarcoplasmic reticulum, namely impulse conduction and elaboration and transfer of metabolites, occur side by side in what has hitherto been regarded as a single system; that morphological differentiation of the system reflects a corresponding physiological duality.

It is from a comparative analysis of problems

such as muscle contraction and metabolism at the cytological and biochemical levels, that a fuller understanding of the diversity of muscles of one animal group and another, and also between different muscles of an individual, may be expected.

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