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On the significance of cross-bridges between microtubules and synaptic vesicles

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[Plates 74 to 81]

Observations on the fine structure of synaptic and non-synaptic axoplasm in the spinal cord of the lamprey ammocoete (*Petromyzon marinus*) are described. Previous studies on this material revealed a close association between spherical vesicles and axoplasmic microtubules near central synapses, and observations were consistent with the suggestion that synaptic vesicles become detached from oriented microtubules in the focal clusters adjoining the presynaptic membrane across which transmitter release takes place. These observations have been extended to include axons containing non-spherical or ellipsoidal vesicles—possibly containing a transmitter chemically and functionally distinct from that in spherical synaptic vesicles. Structural cross-bridges between both microtubules and vesicle populations are now described; these are found not only in the vesicle concentrations bordering synaptic foci, but also in non-synaptic axoplasmic regions where sparsely distributed vesicles are found in association with microtubules. It is suggested that the bridging between vesicles and microtubules may reflect a mechanism for transport of the former—a possibility in accord with cumulative evidence of involvement of microtubules in rapid intracellular translocation in a variety of cells. It is proposed that arrival of excitation at a synaptic site is not only coupled with transmitter release across the axon plasma membrane, but that events at the cell membrane may in turn be coupled with a means of supplying vesicles to the synaptic locus. This hypothesis suggests that the synapse may rely on distant parts of the neuron, perhaps including the cell body, for materials involved in synaptic transmission.

Introduction

Early fine structural studies on nerve cells (de Robertis & Bennett 1955; Palay 1956; Roberts 1956) established that large numbers of small spherical membrane-limited vesicles are concentrated in regions of axoplasm believed to represent the presynaptic component of a synaptic junction, and these authors suggested that the vesicles might be involved in the secretion of chemical transmitters. Subsequently 'synaptic vesicles' have been described in axoplasm adjoining synaptic sites in a variety of central and peripheral neurons in vertebrates and invertebrates. More recently, non-spherical vesicles have been found in axons adjacent to profiles containing spherical synaptic vesicles and it has been proposed (Uchizono 1965; Atwood & Jones 1967; Nakajima 1970; Bodian 1970) that this is a morphological distinction that parallels diversity of sequestered transmitters. An attractive hypothesis of chemically mediated synaptic function has been developed (Katz 1962; Katz & Miledi 1965; Hubbard & Willis 1968; Hubbard, Jones & Landau 1968), according to which the synaptic vesicles are concerned with the storage and/or synthesis of transmitter molecules released into the synaptic cleft at a rate greatly accelerated by arrival of an impulse at the presynaptic terminal—a release including a coupling with excitation via calcium ions or a charged calcium complex within the presynaptic membrane. The general morphological features of a synapse are illustrated here by micrographs of neuromuscular junctions on skeletal fibres of a scorpion, prepared in collaboration with Dr F. E. Russell.

Present concepts of transmitter supply to the postsynaptic surface are primarily concerned with the penultimate step in the synaptic process—the passage of transmitter molecules across

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the presynaptic axon plasma membrane. However, the form of the neuron and the cellular topography of the nervous system often requires that a synapse be located at a great distance from the nerve cell body—a region containing the nucleus and a structurally more complex zone of cytoplasm than occurs in axonal or dentritic extensions of the cell.

The functional relationship between the cell body and distant parts of the cell remains an important and incompletely understood aspect of neurobiology. However, recent work suggests that slow bulk centrifugal axoplasmic flow (Weiss 1969) occurs concurrently with rapid intracellular transport of specific substances and structures (see Discussion). Fine structural studies suggest that in a variety of cells rapid movement of particulates, including chromosomes, pigment granules and various cytoplasmic inclusions, may take place in association with microtubules—a small tubular component of the cytoplasm now recognized as of general occurrence in animal and plant cells (see Porter 1966; Schmitt & Samson 1969 for references). Electron microscopic studies on axons within the lamprey spinal cord have confirmed previous observations on the close apposition of pre- and postsynaptic membranes, together with the association of focal clusters of synaptic vesicles adjacent to the plasma membrane at the synaptic junction. An additional and striking morphological feature of the axoplasm in this spinal cord is the close grouping of synaptic vesicles around axonal microtubules bordering synaptic foci. The possibility that microtubules are involved in physical translocation of synaptic vesicles is strengthened by the occurrence of occasional vesicles, similar in size and form to those in focal clusters, adjacent to axoplasmic microtubules in regions of the cell distant from synaptic sites. It is proposed that arrival of excitation at the zone of membrane in a synapse may be coupled not only with release of transmitter by exocytosis from synaptic vesicles but also ultimately with disjunction of vesicles from adjacent microtubules. The presence of cross-bridges between synaptic vesicles and microtubules strengthens the inference that the close association between these structures is a functional one, and underlines the question of the site of synthesis of axoplasmic vesicles, the mechanism of their translocation and of their interaction with the synaptic foci.

Materials and methods

The material described in this paper was prepared from 12 to 14 cm ammocoete larvae of the sea lamprey (*Petromyzon marinus*) kindly provided by Mr E. L. King, Jr., Acting Investigation Chief, Bureau of Commerical Fisheries, Hammond Bay Biological Station, Millersburgh, Michigan. The ammocoetes were shipped from Millersburgh to Miami and were kept for periods of up to 3 months in shallow tanks of aerated water in a 4 °C room. Preparation of the spinal cord for electron microscopy has been described previously (Smith, Järlfors & Beránek 1970); in summary this involves fixation of the freshly exposed spinal cord with 2.5 % glutaraldehyde with 1 % sucrose in 0.05 mol/l cacodylate buffer at pH 7.4, subsequent washing in similarly buffered 2 % sucrose, treatment with buffered 1 % osmium tetroxide, dehydration and embedding in Araldite. The preparations of scorpion (*Hadrurus hirsutus*) skeletal muscle were fixed according to a similar schedule, except for the inclusion of 0.15 mol/l sucrose in the glutaraldehyde fixative and 0.3 mol/l sucrose in the subsequent wash. Sections were cut with an Ultrotome III (LKB) using glass knives, stained with saturated uranyl acetate in 50 % ethanol and lead citrate and examined in a Philips EM 200. For routine structural studies, sections were treated with the uranyl acetate and lead citrate solutions respectively for *ca.* 10 to 20 min and 2 to 5 min.

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FIGURE 1. Electron micrograph of a field at the periphery of a skeletal muscle fibre (f) of the scorpion *Hadrurus hirsutus*. A motor axon (a) terminating in a bulbous expansion (a') runs across the fibre surface and is flanked externally by a glial cell (g). At four points (*) nerve and muscle cell membranes are locally closely apposed: synaptic vesicles are scattered generally throughout the axoplasm of the terminal, and are focally clustered opposite at least two such regions of membrane junction (arrows). (Magn. $\times 25\,000$.)

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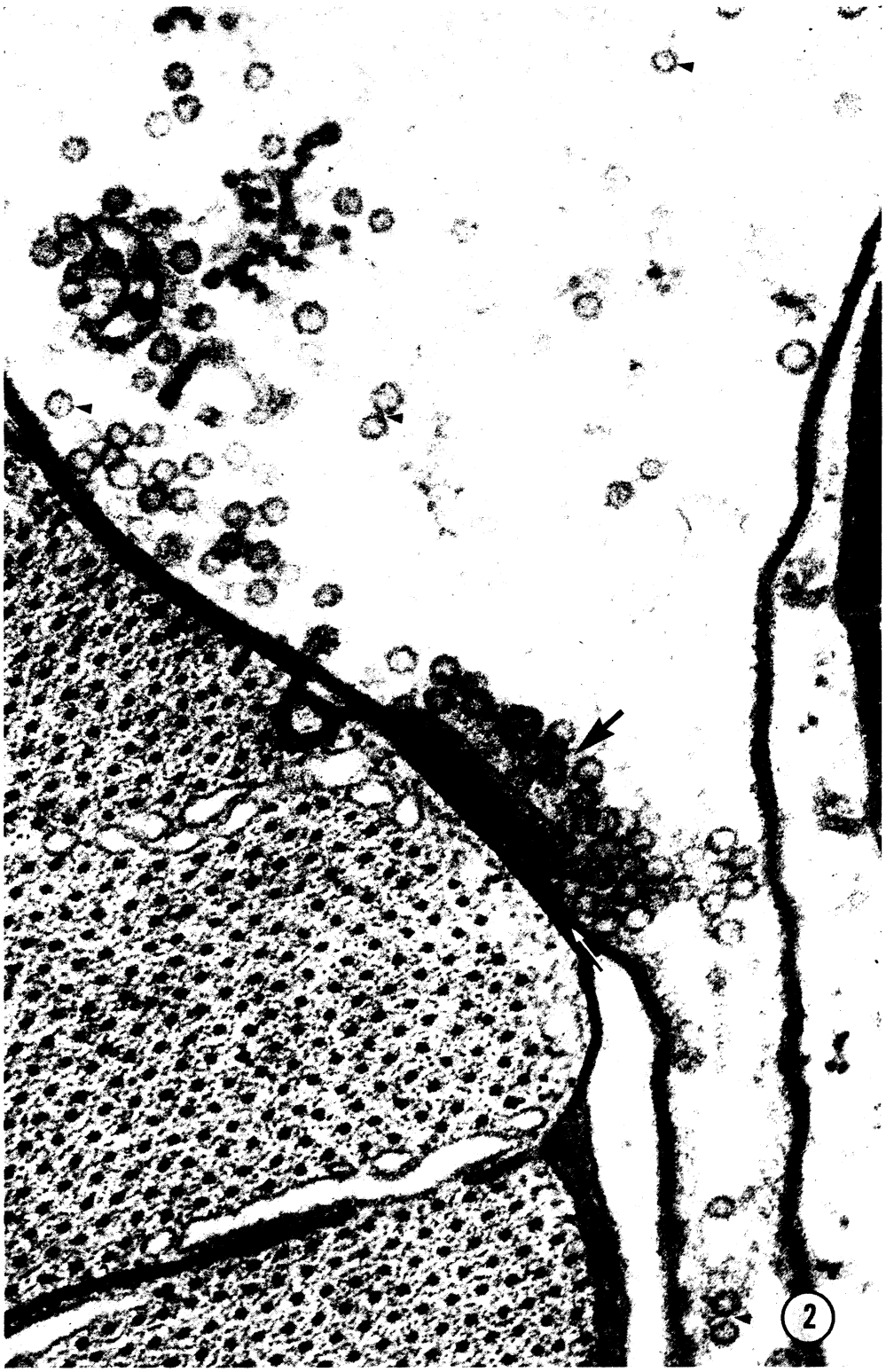

FIGURE 2. *Hadrurus hirsutus*. A portion of the last field, at higher magnification. The terminal axoplasm contains numerous scattered synaptic vesicles 45 to 55 nm in diameter (arrowheads): the tight cluster of vesicles (black arrow) adjoining the axon membrane is believed to represent a focus of transmitter release. Note the narrow intercellular cleft (white arrow) between axon and muscle limiting membranes. (Magn. × 100 000.)

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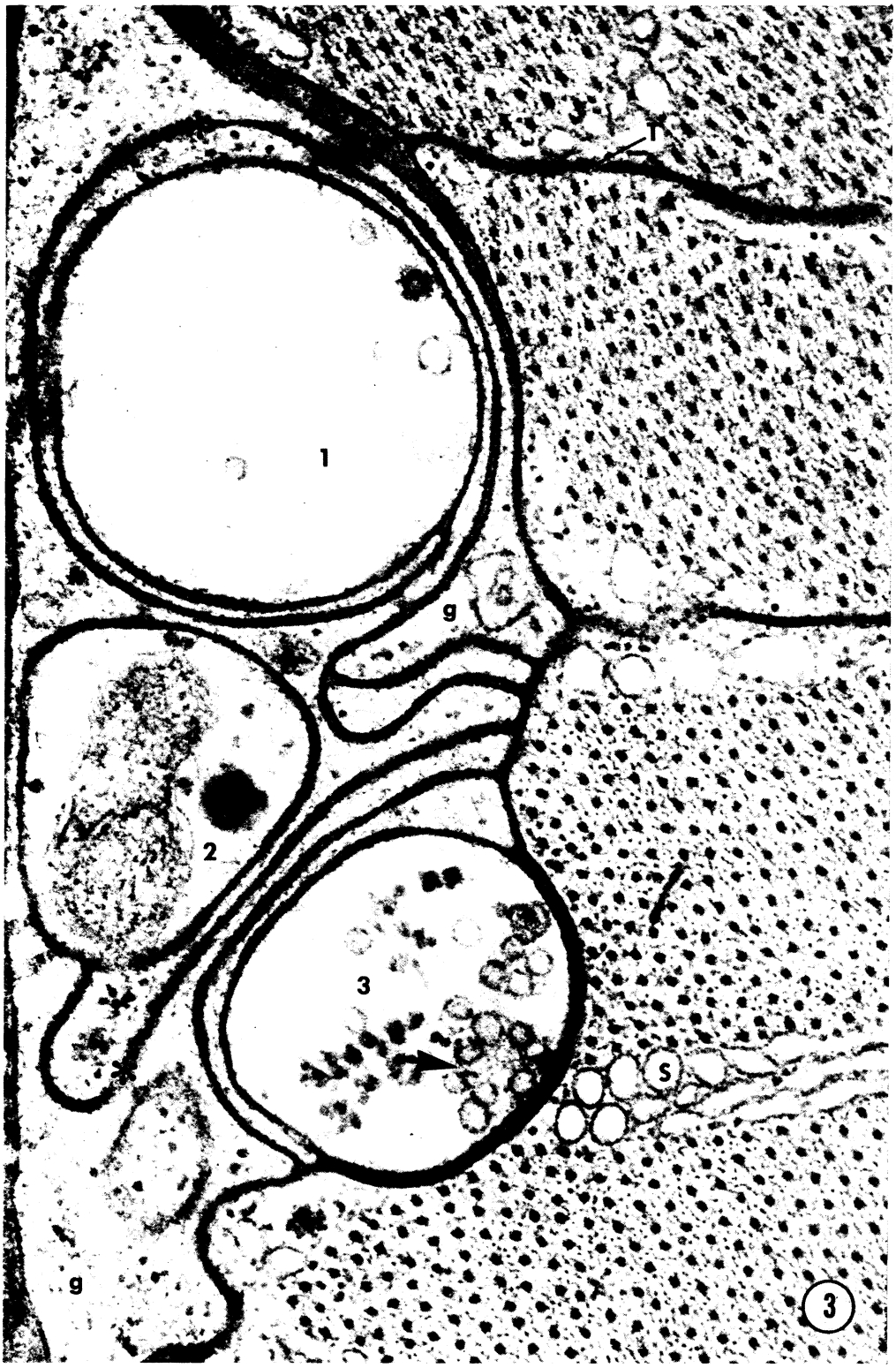

FIGURE 3. *Hadrurus hirsutus*. A field comparable with figures 1 and 2, but in which three axons approaching the muscle surface are sectioned transversely. Axons 1 and 2 are still completely surrounded by the glial cytoplasm (g) and mesaxon membrane invaginations (*). Axon 3 is partially limited by glia, but where this sheath is absent close apposition of nerve and muscle plasma membrane occurs, and in this region a focal cluster of synaptic vesicles (arrows) is present. Note the transverse tubular (T) invaginations of the muscle surface membrane, and the intracellular membranes of the sarcoplasmic reticulum (S). (Magn. × 80 000.)

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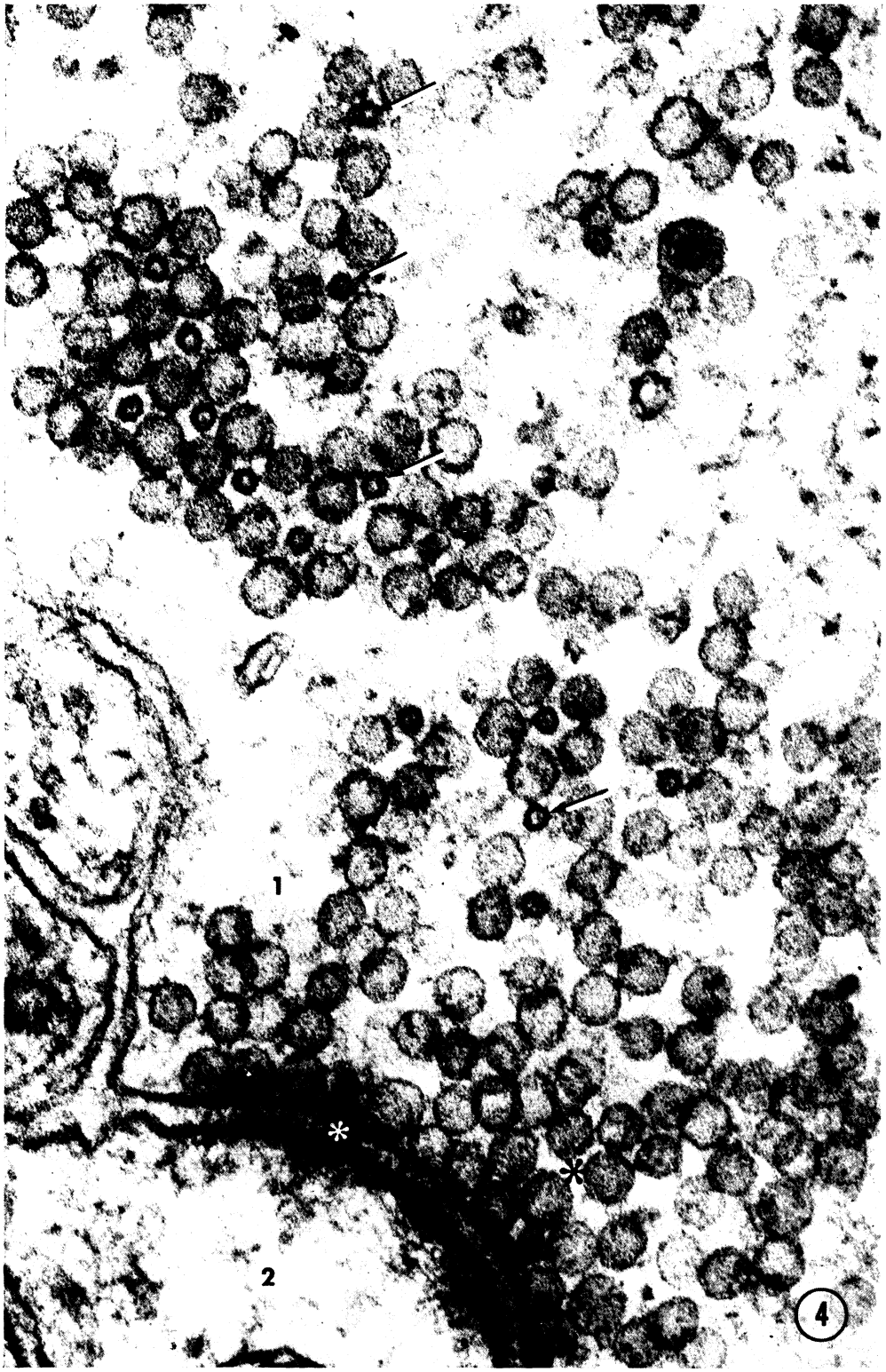

FIGURE 4. Electron micrograph of a field within the spinal cord of the lamprey *Petromyzon marinus*, sectioned transversely to the long axis. A large presynaptic axon profile (1) adjoins a smaller postsynaptic unit (2), across a synaptic cleft (white asterisk) *ca.* 15 nm in width. Large numbers of spherical synaptic vesicles 45 to 55 nm in diameter are present in the presynaptic axoplasm: in the immediate vicinity of the axon membrane, the vesicles are randomly arranged (*) while in a zone of axoplasm commencing about one-third of a micrometre from the cell membrane, many synaptic vesicles are grouped in rosette fashion around microtubules (arrows). A 'complete' rosette involves five spherical vesicles, but incomplete groups and other configurations (cf. figures 6 to 8, 11, 13 to 15) occur in the lamprey axoplasm. (Magn. × 150 000.)

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FIGURE 5. *Petromyzon marinus*. A micrograph corresponding to figure 4, but passing longitudinally through the presynaptic axon (1), close to a point of junction with a small postsynaptic unit (2). Note the opaque material associated with both apposed axon membranes, and the intervening synaptic cleft (*). A glial process containing cytoplasmic filaments is included at g. As in figure 4, synaptic vesicles immediately flanking the presynaptic membrane are unattached and randomly grouped; a short distance away, a passing microtubule (short arrow) is ensheathed with vesicles to the level of the synapse and a second (long arrow) nearby is uniformly surrounded by closely packed vesicles, similar to those in the synaptic focus. (From Smith, Järlfors & Beránek (1970). Reproduced by courtesy of the Rockefeller University Press.) (Magn. × 150000.)

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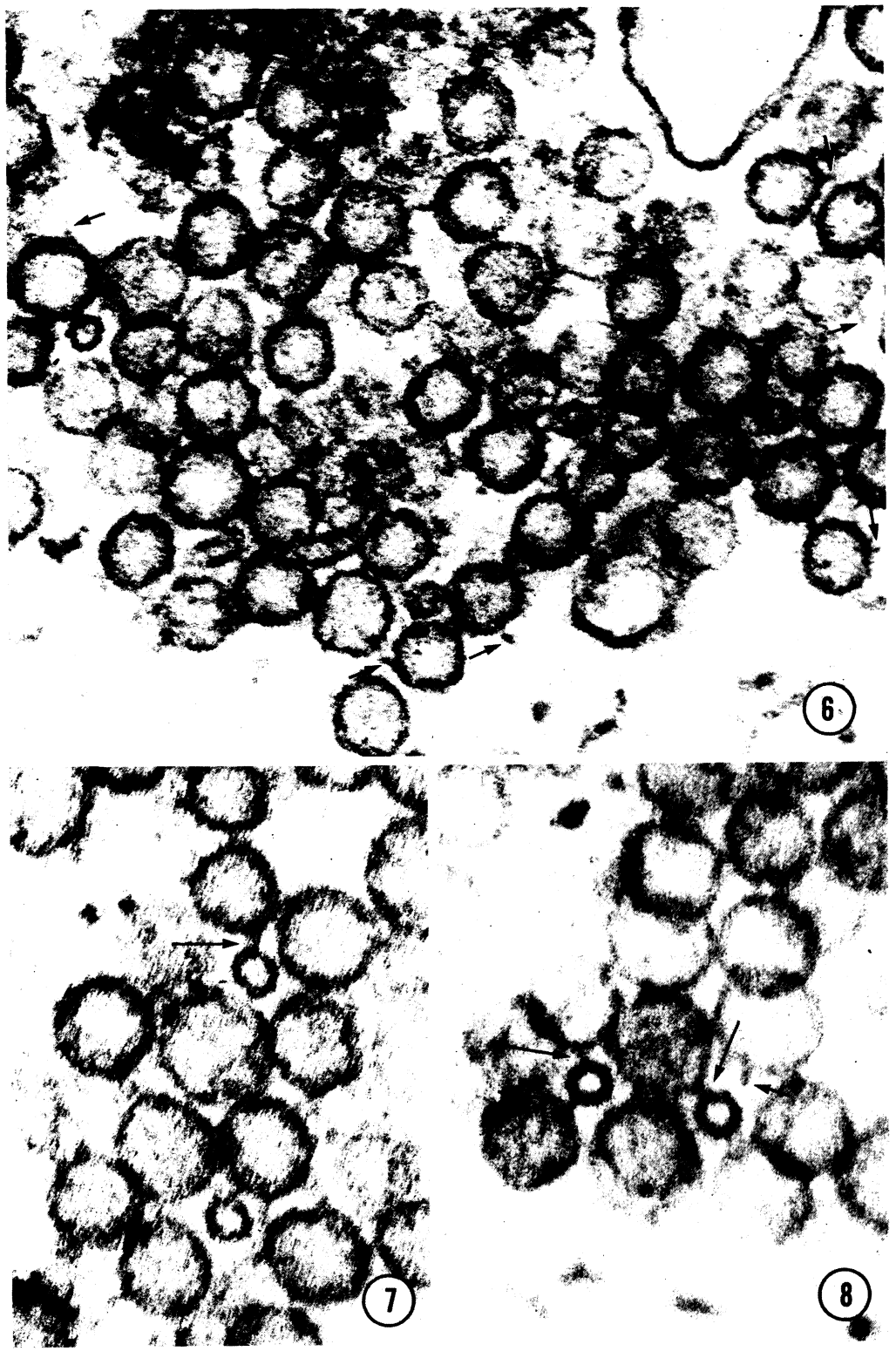

FIGURES 6 TO 8. Micrographs illustrating structural linkage between microtubules and vesicles in the vicinity of synapses in the spinal cord of *Petromyzon*. They suggest that a bridge component is provided by the vesicles.

FIGURE 6. The synaptic vesicle membranes bear short (*ca.* 10 nm) projections (arrows) generally set at an angle with respect to the vesicle radii. (Magn. ×220000.)

FIGURE 7. A structural link (arrow) between a microtubule and a member of a synaptic vesicle rosette (cf. figure 4). (Magn. ×300000.)

FIGURE 8. An axoplasmic field bordering a synapse illustrating (as in figure 7) structural bridges (long arrows) between microtubules and synaptic vesicles. In addition, a projection from the surface of a vesicle is resolved (short arrow). (Magn. ×300000.)

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The cross-bridges between vesicles and microtubules described here show less affinity for these reagents than do other structures, and were resolved most clearly in sections in which the above times were increased two- or threefold.

Results

(*a*) *Innervation of scorpion skeletal muscle*

Skeletal (leg) muscle fibres of the scorpion (*Hadrurus hirsutus*) conform to the general arthropod plan in possessing multiterminal and polyaxonal neuromuscular junctions. Unlike the motor nerve junctions on a vertebrate fast fibre, which is literally an 'ending' characterized by concentrations of synaptic vesicles (Birks, Huxley & Katz 1960; Katz 1962), each axon associated with muscle fibres, such as that illustrated in figure 1, is synaptically linked at several points with the adjoining muscle cell. Synaptic vesicles are scattered along the axon as it courses across the muscle; these are not conspicuously aggregated in the terminal expansion, but are focally clustered adjacent to restricted regions of close nerve–muscle membrane apposition. A portion of this field, including a synaptic focus, is illustrated at higher magnification in figure 2: the spherical 40 to 50 nm vesicles are closely packed along a 0.5 μm length of the presynaptic membrane, which flanks that of the muscle across an intercellular cleft of *ca.* 15 nm. Figure 3 includes a group of axons approaching the surface of a similar muscle cell. Arthropod peripheral nerves lack myelin; instead the ensheathing glial cell envelops the axon via loosely wound mesaxon folds in the 'tunicated' arrangement initially described, in insects, by Edwards, Ruska & de Harven (1958). Presynaptic axon profiles still completely surrounded by glial folds infrequently contain synaptic vesicles. Along the terminal region of the axon, where the glial sheath is absent (cf. figure 1) from the inner surface of the nerve process, the concentration of synaptic vesicles increases abruptly, and as illustrated in figure 3 the distribution of vesicles includes focal clusters believed to flank sites of release of chemical transmitter into the synaptic cleft. The fine structural details within the muscle cell illustrated here include the transverse tubular (T-system) invaginations of the surface membrane, considered to initiate contraction by conducting a signal coupled with surface excitation into the fibre, and the intracellular sarcoplasmic reticulum, involved in the control of contraction and relaxation respectively via Ca^{2+} release triggered by the T-system signal, and subsequent sequestration of calcium ions required to trigger the myosin ATPase operative during contraction (see Smith (1966) for references).

The purpose of these micrographs in the present context is to illustrate some general morphological points common to central and peripheral chemically mediated synapses: (i) the close apposition of pre- and postsynaptic membrane surfaces characterized by exclusion of glial processes generally accompanying the axon elsewhere and (ii) the 'recognition' paid to these sharply localized regions in the distribution of synaptic vesicles within the presynaptic axoplasm. These points have been well documented previously in studies on a variety of central and peripheral nervous systems, but the micrographs described in the following section include some recently observed morphological features that may clarify the function of synapses and their relationship with the rest of the cell. Evidence of non-random or linear alinement of synaptic vesicles is sometimes found in the arthropod preparations just described, but in this case axoplasmic microtubules are inadequately preserved and their possible role in vesicle alinement—clearly resolved in profiles of lamprey spinal cord—cannot yet be assessed. In the

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lamprey material, however, not only are juxtaposed microtubules and vesicles sharply defined but evidence of possibly delicate and labile structural cross-bridges between these components has also been obtained.

Lamprey spinal cord

Recent fine structural observations on the nerve cord of the ammocoete larva of *Petromyzon marinus* have revealed morphological features of the axoplasm that may provide new information concerning the functional relationship between synaptic foci and distant regions of the cell. A preliminary account (Järlfors & Smith 1969) has documented close association of synaptic vesicles and microtubules in central axons of the lamprey, and the possible functional consequences of this association have been discussed more fully (Smith *et al.* 1970). Figure 4 includes the significant features of recent observations. As has already been illustrated in the peripheral nervous system (figures 1 to 3) the apposition of pre- and postsynaptic membranes across a narrow cleft is a morphological criterion of chemically mediated synapses; likewise the focal clustering of synaptic vesicles and local deposition of dense material in association with the postsynaptic membrane. Synapses identified through these features are abundant in the lamprey spinal cord, and in figure 4, a small postsynaptic unit is engaged synaptically with a larger vesicle-containing presynaptic member. The synaptic vesicles are randomly arranged and concentrated within a narrow zone, *ca.* 0.5 μm in width, flanking the axon surface; beyond this point vesicle profiles are locally numerous, but are predominantly associated with microtubules which are oriented parallel with the long axis of the nerve process. As has been described elsewhere (Wuerker & Palay 1969; Smith *et al.* 1970) the axoplasm characteristically contains varying proportions of microtubules and neurofilaments, respectively *ca.* 25 and 6 nm in diameter, and the association with vesicles in the lamprey cord is strikingly restricted to the former. The vesicles are disposed around the microtubules in a rosette pattern; five spherical vesicles constitute a complete rosette, but incomplete groups or juxtaposition of single vesicles

Description of plate 80

Figures 9 to 15. Micrographs further illustrating the association between vesicles and axonal microtubules in the *Petromyzon* spinal cord. These figures represent fields not closely associated with synapses.

Figures 9 and 10 At all levels in lamprey axons, microtubules occur, generally singly, but frequently in pairs or larger groups, as illustrated here. In figure 9, four microtubules are associated, and in figure 10 a central microtubule is surrounded by a regular grouping of six similar structures. Bridges linking grouped microtubules are frequently seen, as in these figures; in addition (as in figure 10), similar but 'unfilled' arms project from microtubules within the group. (Magn. × 400 000.)

Figure 11. A spherical vesicle (v), *ca.* 45 nm in diameter, adjoining an axonal microtubule. In addition to the bridge between these structures (arrow), two further similar projections stem from the vesicle surface (arrowheads). (Magn. × 300 000.)

Figure 12. A micrograph similar to the last, but illustrating linkage (arrow) between a microtubule and a non-spherical (flattened) vesicle (v) in a *Petromyzon* central axon. Non-spherical synaptic vesicles are characteristic of certain nerve cells in the lamprey cord (cf. figures 16, 17), and may contain transmitter molecules different from these associated with spherical vesicles. (Magn. × 300 000.)

Figure 13. A group of five axonal microtubules, one of which is apparently linked (arrow) or approaching linkage with a spherical vesicle, similar to those clustered around many central synapses in the lamprey nerve cord (cf. figure 4). Two unattached projections (arrowheads) stem from the vesicle surface. (Magn. × 400 000.)

Figure 14. A group of six linked microtubules, one of which is closely adjoined by a spherical vesicle (v). In this instance, adjacent arms project from the vesicle (arrow) and a microtubule (arrowhead), but no clear linkage is established. (Magn. × 400 000.)

Figure 15. Similar to the last micrograph, but illustrating a link between a single vesicle (v) and one of a group of axonal microtubules. (Magn. × 400 000.)

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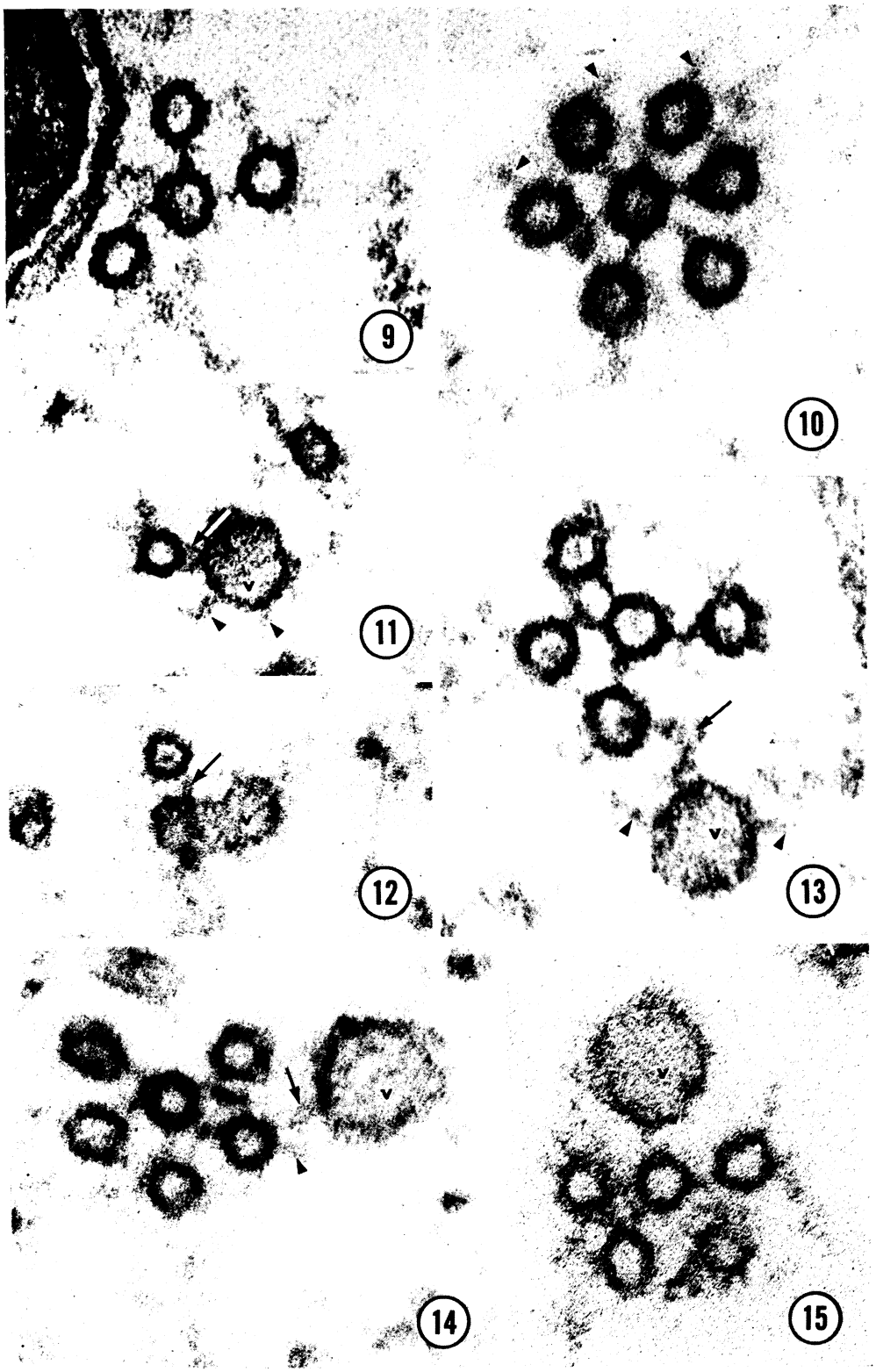

FIGURES 9–15. For legends see facing page.

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FIGURES 16 AND 17. Micrographs illustrating the disposition of non-spherical vesicles in axons of the lamprey spinal cord. In general, the distribution of these structures, perhaps containing chemical transmitters distinct from those of the spherical vesicles, parallels the latter. Synaptic foci (figure 16) of vesicles are grouped in a large presynaptic axon (1) adjoining small postsynaptic members (2). As elsewhere (cf. figure 4), vesicles are grouped around microtubules in the peripheral zone of the synaptic focus (arrows), and longitudinal sections of such axons reveal (figure 17) a similar association of vesicles (v) and tubules (arrows) (cf. figure 5). Bridges (arrow) between non-spherical vesicles (v) and microtubules (T) are resolved (inset; see also figure 12)—paralleling the situation in axons containing spherical vesicles. (Magnifications: figure 16, × 100 000; figure 17, × 150 000; inset, × 300 000.)

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and tubules occur in the vicinity of the synapse. In each instance, a gap of *ca.* 10 nm is interposed between vesicle and tubule surfaces. Figure 5 includes the corresponding longitudinal aspect of the vesicle-tubule association and its relationship to the presynaptic membrane. In one region of this field, vesicles closely packed around axoplasmic microtubules bypass the synapse; closer to the presynaptic membrane, microtubules are only partially vesicle-clad, and between these tubules and the axon membrane, large numbers of synaptic vesicles are randomly arranged in a typical focal cluster. The morphological picture is consistent with the suggestion that vesicles 'spill' or become detached from microtubules and move towards the adjacent plasma membrane region across which release of transmitter takes place. In earlier accounts of the organization of synaptic axoplasm in *Petromyzon* (Järlfors & Smith 1969; Smith *et al.* 1970), no evidence of physical links between vesicles and tubules was presented; but now cross-bridges linking these two structures have been recognized in heavily 'stained' sections. The field shown in figure 6 includes a group of spherical synaptic vesicles, some grouped around microtubules and the remainder unassociated—as in figure 4. Each vesicle is limited by a membrane *ca.* 5 nm in width, and staining with salts of uranium and lead, as in this as subsequent figures, emphasizes the presence of short projections from the vesicle surface. These are approximately 6 to 10 nm in length, *ca.* 5 nm in width, and generally appear to set at an angle to the vesicle radius.

As will be shown later (figures 9, 10) axonal microtubules, in common with these of the mitotic spindle and other intracellular systems, possess lateral side arms which are involved in linking microtubule groups. However, these may be distinct from the bridges involved in vesicle–tubule associations which appear to involve a component stemming from the vesicle. One such bridge is illustrated in figure 7, while figure 8 includes three vesicle–tubule links and, in addition, an unattached projection extending from a vesicle towards a microtubule.

Links between pairs and larger fascicles of axoplasmic microtubules have been described in the axon hillock and initial segment of neurons by Palay, Sotelo, Peters & Orkand (1968) and Peters, Proskauer & Kaiserman-Abramof (1968), and these are frequently observed in sections of the lamprey spinal cord, apparently throughout the axon. A group of four linked microtubules is seen in figure 9, while figure 10 includes a regular pattern of six microtubules evenly spaced around an axial tubule, together with 'open' bridges stemming from the outer surface of the microtubule ring. In each instance, radial bridges *ca.* 10 to 12 nm in length link adjoining microtubule surfaces.

'Synaptic vesicles' have generally been considered to be restricted to the vicinity of synaptic terminals, and while fast and slow centrifugal transport of materials and the axon has been well documented, the possibility that transmitter-charged vesicles are transported to synaptic foci from distant parts of the cell, perhaps including the perikaryon, has seldom featured in models of transmitter action. However, if the accumulation of tubule-linked vesicles near synaptic areas reflects a dynamic axoplasmic event, then similar vesicles may reasonably be sought for in non-synaptic regions of the nerve cell. Accordingly, the fine structure of areas of *Petromyzon* axoplasm *not* closely adjoining synapses was carefully examined. Such areas included (i) regions of small axon profiles in which synapses (judged by the criteria mentioned previously) were sparse, and (ii) central regions of the giant (Müller) fibres in the spinal cord (30 μm in diameter in the present material) in which vesicle clusters within the peripheral micron of axoplasm flank junctions with small postsynaptic units (Smith *et al.* 1970). The principal conclusions resulting from these observations are twofold: (i) both neurofilaments and microtubules occur in the cytoplasm of nerve processes throughout the wide size spectrum encountered in this spinal cord,

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but as has been discussed elsewhere (Wuerker & Palay 1969; Smith *et al.* 1970) there is an approximate reciprocity in the proportion of the two components, small profiles containing a higher proportion of microtubules than larger profiles. (ii) In these non-synaptic regions membrane-limited vesicles occur in association with microtubules, with low frequency. Where such associations occur, however, structural cross-bridges are resolved, similar to those found in the vicinity of synapses. Figure 11 includes a single microtubule and a single spherical vesicle, the latter bearing three surface projections, one of which appears to forge the link with the adjacent microtubule. In figure 13, a group of linked microtubules closely approach a single spherical vesicle; the latter bears projections, which do not directly traverse the gap to the microtubule surface, but one of which may be linked with a microtubule arm. Figure 14 includes closely adjoining vesicle and microtubule projections which do not show the evidence of linkage included in figure 13, while in figure 15 a spherical vesicle is linked to one of a five-membered microtubule group, across a narrow gap as in figure 11 or (figures 7, 8) in the vicinity of a synapse.

Figure 12 includes a transverse profile of a microtubule linked by a *ca.* 9 nm bridge to a membrane-limited vesicle—a circumstance differing from that in figure 11 only in the non-spherical (flattened or ellipsoidal) vesicle profile. This field serves to introduce the remaining electron micrographs in this paper, and an important functional aspect of synaptic organization. Non-spherical vesicles have been described in synaptic foci in the nervous system of a variety of species, and evidence has been presented (see Discussion) suggesting that these structures contain transmitters different from those sequestered within spherical vesicles—in some instances, at least, reflecting inhibitory rather than excitatory function. Non-spherical vesicles are frequent in the ammocoete spinal cord but, as elsewhere, admixture of these with electron-lucent spherical vesicles within a single cell does not occur. Non-spherical vesicles similar to that in figure 12 are focally clustered around synaptic sites elsewhere in the cell profile from which this field was selected. This observation is of general occurrence, and it has been established that *isolated or sparsely distributed vesicles in non-synaptic areas of a nerve process conform to the shape of vesicles aggregated in synaptic foci of the same cell.*

A low-power micrograph of a transversely sectioned axon containing ellipsoidal vesicles is included in figure 16. As before (cf. figure 4) vesicles are associated in rosette patterns around microtubules, but while the corresponding 'complete' pattern of spherical vesicles is precise and five-membered, the grouping of non-spherical vesicles is more irregular. However, as in the case of spherical vesicles in a comparable situation, their ellipsoidal counterparts are similarly linked to microtubules by cross-bridges (inset, figure 16). As described previously for the first neuron group, the association between non-spherical vesicles and axoplasmic microtubules extends to a zone half a micrometre or so from the presynaptic surface, while immediately adjoining the plasma membrane the vesicles are unattached and randomly disposed. Figure 17 includes a field corresponding to that shown in figure 6, but shows in longitudinal aspect the close-packed arrangement of non-spherical vesicles around microtubules approaching a central synapse in the cord of *Petromyzon.*

The presence of structures with the physical appearance of 'synaptic vesicles' widely separated from the focal clusters is consistent with the hypothesis that *these foci are functionally dependent on other, and possibly distant, parts of the cell for the structural material of the vesicle and/or for components involved in chemical transmission.* The origin of these vesicles is as yet unknown, but if they are indeed translocated in association with microtubules as suggested here, the structurally

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complex perikaryon is an obvious candidate, and such an origin has been recognized for the products of 'neurosecretory' cells (see de Robertis 1964, for references). Preliminary studies on the cell bodies of lamprey spinal neurons have so far revealed no structural evidence to disqualify this region of the cell as the site of production of small electron-lucent vesicles similar in appearance to those present in axons.

Discussion

Our present understanding of neuronal function rests upon information from a variety of investigative techniques of electrophysiology, pharmacology, biochemistry and light- and electron microscopy. Studies along these diverse lines on a variety of animals suggest that evolution of nervous system form through the Phyla has been achieved conservatively—by extensive variation of cell numbers and interrelations of cellular units that vary within relatively narrow limits in their form, fine structure, and notably in the subcellular structures believed to be involved in controlled transfer of excitation from one cell to the next. This transfer is most generally effected across chemically mediated synapses, at which the rate of transmitter release into a narrow intercellular cleft is coupled with the arrival of excitation at the presynaptic side of the junction.

The integrative function of the nervous system requires the operation of synaptic terminals at distances from the cell body that are often vast, by usual intracellular standards. The maintenance of distant parts of the cell by the nucleus and complex zone of cytoplasm surrounding it presents a logistic problem that is evidently met not only by bulk centrifugal flow of axoplasm (Weiss 1967, 1969), but also by concurrent rapid translocation of specific materials (Dahlström 1965, 1967; Jasinski, Gorbman & Hara 1966; Kerkut, Shapira & Walker 1967; McEwen & Grafstein 1968; further references in Barondes 1969) along the axon. On the other hand, it is evident that certain synthetic processes may occur in the vicinity of the neuron terminals (see Barondes 1969, for references), and the possible role of the perikaryon in supplying materials involved in synaptic transmission in various neuron types has not yet been determined. Present concepts of transmitter release by exocytosis (Katz 1962) envisage momentary fusion between 'reactive sites' on the membrane limiting a quantal amount of transmitter within the synaptic vesicle, and corresponding reactive sites on the axon plasma membrane—a process occurring occasionally and spontaneously at all times, but with vastly increased frequency following depolarization of the terminal. It has generally been supposed that synaptic vesicles, which are present in concentrations of several thousand per cubic micrometre in neuromuscular junctions (Edwards, Ruska & de Harven 1958; Smith 1960) and in central synapses in the lamprey nerve cord (Smith *et al.* 1970) are virtually restricted to this region of the axoplasm, and that the synthesis of transmitter and/or its sequestration into membrane-limited quanta likewise occurs in the area of the synaptic focus.

Now, for the first time, evidence is available of a structural bridging between vesicles close to the presynaptic junctional surface and an axoplasmic structure. In the *Petromyzon* spinal cord, as elsewhere, both microtubules and neurofilaments are present, and it is exclusively with the former that the vesicle linkage occurs. Microtubules are strikingly absent from the synaptic focus, within which vesicles are randomly arranged and are considered to have the freedom of movement to the 'receptive sites' invoked in the hypothesis of release mentioned above.

Electron micrographs indicate that immediately inside the synaptic focus vesicles are not only ordered rather than randomly disposed, but that any vesicle movement in this part of the

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axoplasm is restricted to the direction of the microtubular long axis—that is, along the axon. Furthermore, the presence throughout the axoplasm of occasional vesicles with the spherical or flattened form characteristic of synaptic vesicles aggregated in the synaptic foci suggests that the vesicle-tubule association is quantitatively graded along the axon in recognition of synaptic sites, but that the synaptic zone is not qualitatively distinct from the rest of the axon except in possessing unassociated vesicles in the axoplasm immediately flanking the plasma membrane. It has been proposed (Smith *et al.* 1970) that these observations are consistent (i) with involvement of microtubules in transporting vesicles to synaptic sites, and (ii) with release of vesicles from microtubules opposite synaptic foci.

Little is known of the movements of synaptic vesicles *in vivo* and of the means by which they are mobilized within the vicinity of a synapse towards the axon membrane, before contact and discharge. Several studies have centred upon the electrical charge carried by synaptic vesicles (Hubbard & Willis 1962, 1968; Bass & Moore 1966; Landau & Kwanbunbumpen 1969), but so far no unified picture of this phase of the excitation-release coupling sequence has emerged. To this problem is now added the involvement of an ordered cytoplasmic system that may bring vesicles to within a few tenths of a micrometre from the synaptic surface—apparently to a zone where vesicles are disassociated from a transporting framework and move non-randomly into an axoplasmic zone in which the final coupling events occur. In a recent discussion of evidence for the 'vesicle hypothesis' of transmitter secretion, Hubbard & Kwanbunbumpen (1968) consider the involvement of synaptic vesicles in a 'feed-back mechanism regulating transmitter synthesis and mobilization to the demands of release. Increased release would...cause proportionate vesicle depletion which would automatically entail appropriate changes in transmitter synthesis and mobilisation'. Fine structural evidence from the lamprey material is not inconsistent with such delicately balanced regulation of synaptic function.

Arms projecting from microtubules were first recognized in the ciliary apparatus and subsequently identified as an ATPase protein (Gibbons & Rowe 1965). Subsequently, arms and intertubule cross-bridges have been described in a variety of situations, including the phragmoplast (Hepler & Jackson 1968) and mitotic spindle (Wilson 1969; Hepler, McIntosh & Cleland 1970) the distal process of insect mechanoreceptors (Smith 1969) and contractile ciliate axostyles (Grimstone & Cleveland 1965). McIntosh & Porter (1967) described cross-bridges between microtubules ensheathing the chick spermatid nucleus and proposed that bridges represent dynamic sites of tubule interaction, and that tubules slide over one another accommodating the elongating nucleus. In the axoplasm, the great majority of microtubules occur singly, but, as previously described in mammalian neurons (Palay *et al.* 1968; Peters *et al.* 1968) doublets and larger fascicles occur. As elsewhere, short arms extend from single tubules and as presumably 'available' projections from the outwardly facing surfaces of tubules linked into groups in lamprey nerve cell processes.

The most extensive consideration of mechanochemical implications of tubule-to-tubule linkage is based on the observed dual array of continuous spindle microtubules passing through the metaphase plate and of kinetochore tubules connected with the chromosomes (Hepler *et al.* 1970). In a theoretical model, McIntosh, Hepler & Van Wie (1970) account for the complex events of mitosis, including change of spindle shape and chromosome movements, in terms of interaction between the two sets of microtubules and of the equilibrium between microtubules and a pool of protein microtubule subunits. Although in this instance the movement of the 'particle' (the chromosome) is effected indirectly, these authors suggest that 'work performed by

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microtubule arms or bridges will account for most instances of cytoplasmic particle motion relative to an existing array of microtubules'. The proposed role of axonal microtubules is in accord with accumulating evidence concerning the mechanism of translocation of particulates within cells. Instances in which microtubules have been implicated in such movement include bidirectional migration of pigment granules in fish melanocytes (Bickle, Tilney & Porter 1966), of cytoplasmic particles along the arms of *Actinosphaerium* (Porter & Tilney 1965), movement of nuclei into a virus-induced syncytium (Holmes & Choppin 1968), of particles associated with digestion in tentacles of the suctorian ciliate *Tokophrya* (Rudzinska 1965, 1967), of particles associated with the mitotic spindle (Rebhun 1963) and of movement of ribosomes from trophocyte to oocyte in an insect (MacGregor & Stebbins 1970). In these examples, translocation is directional and typically occurs more rapidly than slow and non-specific axoplasmic flow.

The mechano-chemical function of bridges between tubules postulated in models of mitosis may, from the fine structural evidence, be attributed equally to those of the axon. *However, the function of such links in the axon is not clear, and it is the cross-bridges between microtubules (whether single or grouped into fascicles) and vesicles that lends itself most readily to functional interpretation.* It is noteworthy that projections from the vesicle surface appear to contribute to the cross-bridges, and that these projections are resolved among detached vesicles within the synaptic focus. It has not yet been clearly established whether each bridge involves an arm component from each member or whether the link is forged exclusively by one member or the other. Additional projections from an already 'cross-bridged' vesicle are often observed, though the number of projections on each vesicle is not known. It is conceivable that cross-bridges impel the vesicle along the surface of the tubule by interaction (linkage and disjunction) between the two, mediated by energy supplied by ATP, GTP or other chemical source (cf. Schmitt & Samson 1969). Alternatively, the vesicle-tubule bridge may be a more stable one—translocation being driven by tubule assembly at an initiating site (perhaps in the cell body) and concurrent depolymerization into tubule subunits elsewhere (perhaps at the cell terminal).

There is little doubt of the equivalence between axoplasmic microtubules and those of other cells. Borisy & Taylor (1967) have found that [^{3}H]colchicine complexes with a brain protein, and a protein in cilia and dividing cells. Shelanski & Taylor (1967) have identified this component of dividing cells as microtubule protein. Evidence in favour of the transporting function of axonal microtubules is provided by the observation that, in common with microtubules of other cells, these are depolymerized by colchicine and *Vinca* alkaloids (Fernandez 1970; H. Fernandez & F. E. Samson, personal communication)—substances that have been shown by these authors and by McEwen & Grafstein (1968) to inhibit axoplasmic translocation, in addition to mitosis and other translocatory processes involving microtubules (Robbins & Gonatas 1964; Freed 1965).

In the above discussion, no distinction has been made between spherical and non-spherical vesicles. In the lamprey spinal cord, the two vesicle populations appear to be identical *vis-à-vis* their linkage with microtubules and disjunction close to the synaptic membrane. While no information on chemical identity of a transmitter can yet be made by inspection of the shape of a synaptic vesicle, there is good evidence that transmitter diversity may be paralleled by vesicle morphology, as in the correlation between terminals containing non-spherical vesicles and inhibitory function, discussed by Uchizono (1965, 1967), Atwood & Jones (1967), and Nakajima (1970). The uniform disposition of spherical and non-spherical vesicles in lamprey axons is here interpreted as according with the evidence that a common release mechanism is

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involved in the secretion of chemically diverse transmitters—as suggested by the similarity of spontaneous miniature end-plate potentials in insect non-cholinergic junctions (Usherwood 1963) to these resulting from low-frequency release of quantal amounts of acetylcholine from motor nerve terminals on vertebrate skeletal muscle (Fatt & Katz 1952; del Castillo & Katz 1954; Katz 1962).

The most fully documented aspects of nerve excitation and synaptic transfer concern the ionic events of membrane excitation along the axon cylinder, and the coupling of excitation with rapid, massive and local secretion of transmitter molecules at specialized points along the axon, or at its terminal. The present preliminary observations suggest that these events, involving the limiting membrane of the cell and an extremely narrow layer of axoplasm immediately within it, are in turn coupled with a sequence of events taking place more deeply within the cell, in association with cytoplasm in which structure and function are interrelated in a considerably more complex manner than has hitherto been supposed.

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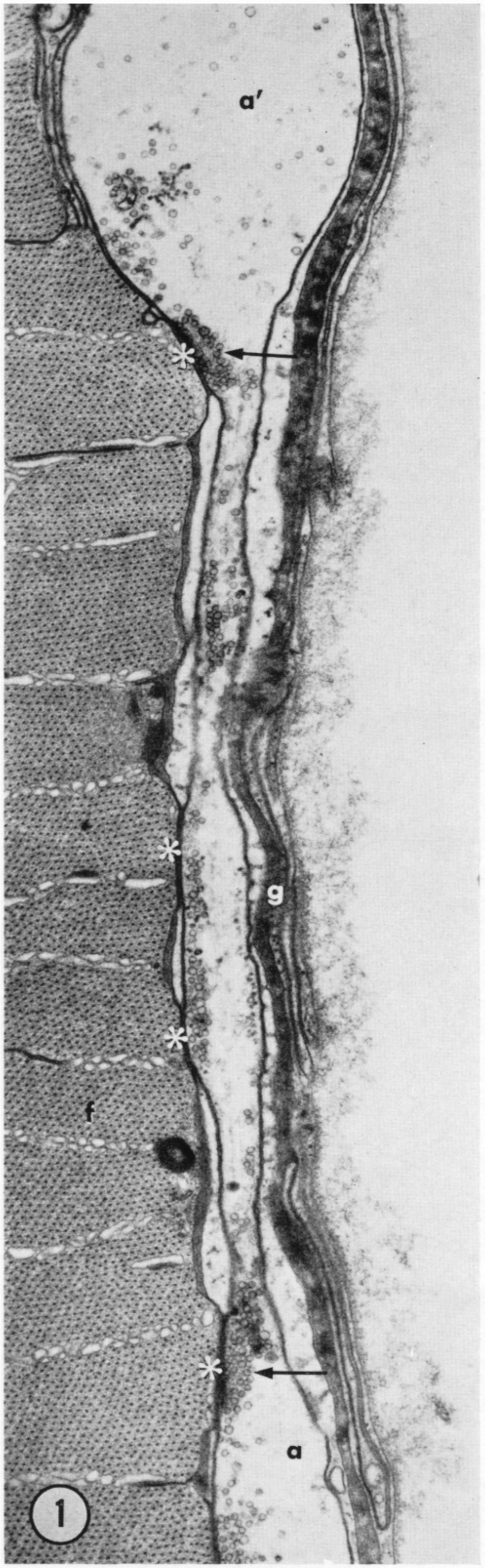

FIGURE 1. Electron micrograph of a field at the periphery of a skeletal muscle fibre (*f*) of the scorpion *Hadrurus hirsutus*. A motor axon (*a*) terminating in a bulbous expansion (*a′*) runs across the fibre surface and is flanked externally by a glial cell (*g*). At four points (*) nerve and muscle cell membranes are locally closely apposed: synaptic vesicles are scattered generally throughout the axoplasm of the terminal, and are focally clustered opposite at least two such regions of membrane junction (arrows). (Magn. ×25000.)

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FIGURE 2. *Hadrurus hirsutus*. A portion of the last field, at higher magnification. The terminal axoplasm contains numerous scattered synaptic vesicles 45 to 55 nm in diameter (arrowheads): the tight cluster of vesicles (black arrow) adjoining the axon membrane is believed to represent a focus of transmitter release. Note the narrow intercellular cleft (white arrow) between axon and muscle limiting membranes. (Magn. × 100 000.)

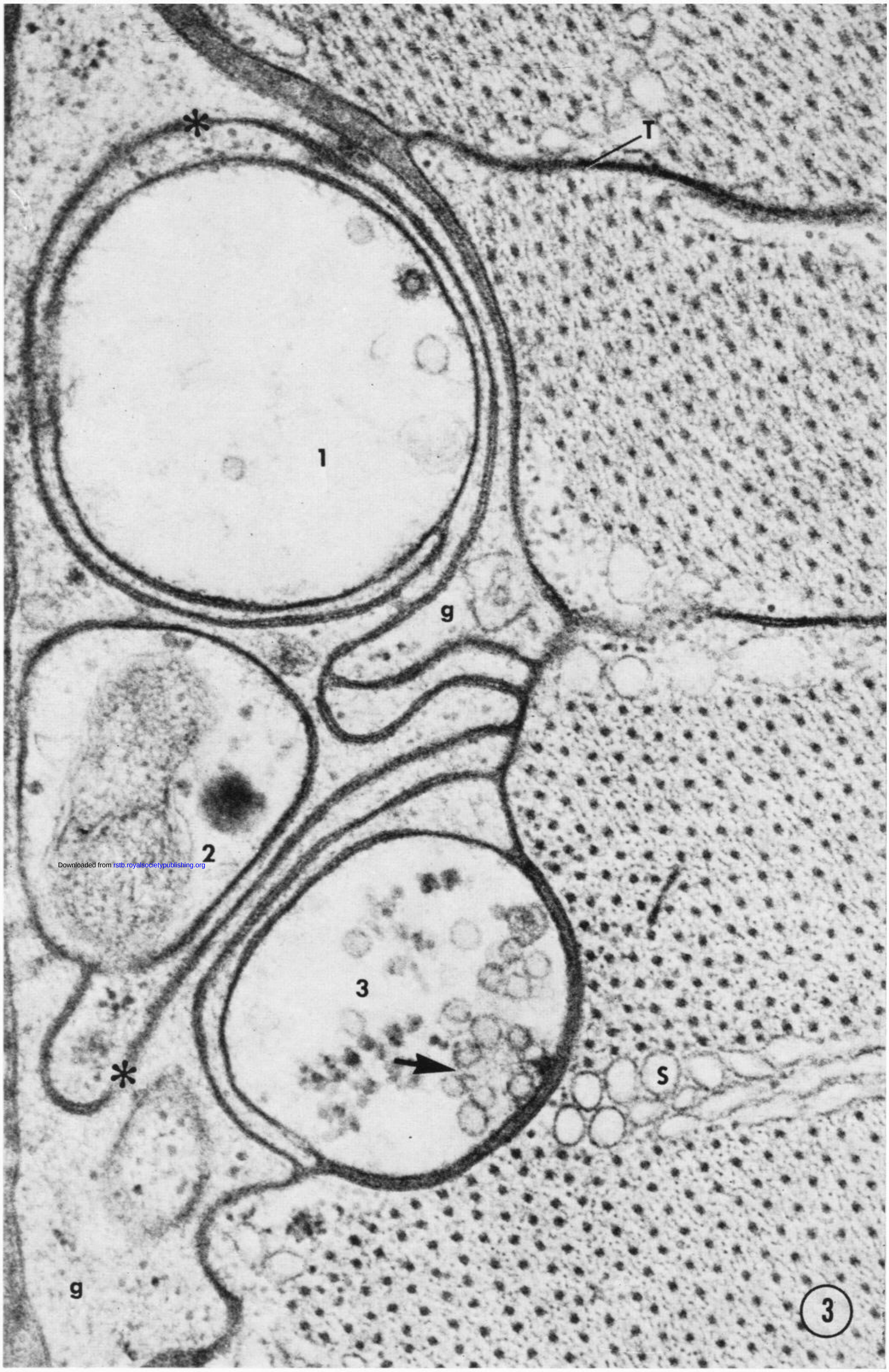

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FIGURE 3. *Hadrurus hirsutus*. A field comparable with figures 1 and 2, but in which three axons approaching the muscle surface are sectioned transversely. Axons 1 and 2 are still completely surrounded by the glial cytoplasm (g) and mesaxon membrane invaginations (*). Axon 3 is partially limited by glia, but where this sheath is absent close apposition of nerve and muscle plasma membrane occurs, and in this region a focal cluster of synaptic vesicles (arrows) is present. Note the transverse tubular (T) invaginations of the muscle surface membrane, and the intracellular membranes of the sarcoplasmic reticulum (S). (Magn. ×80000.)

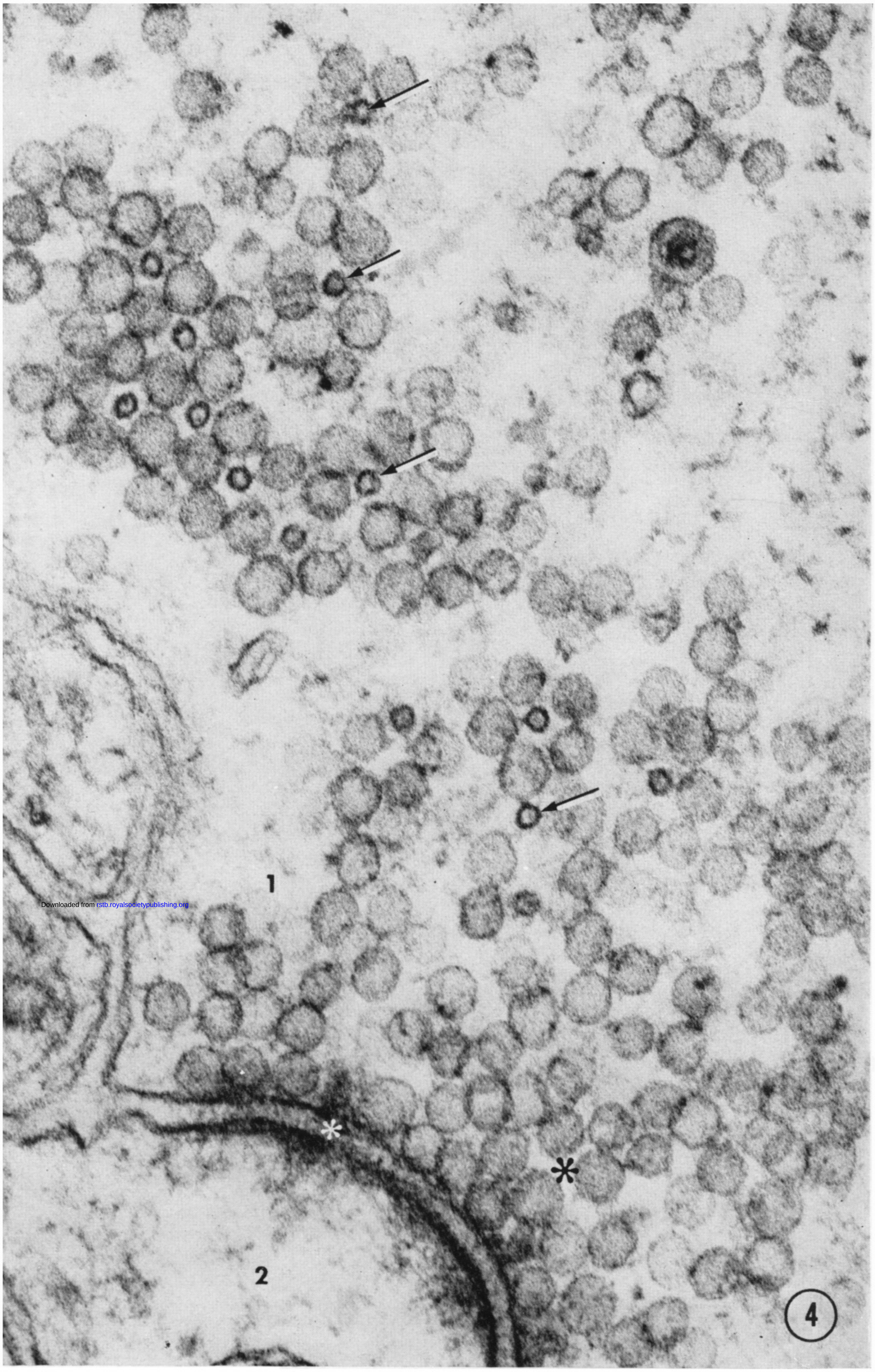

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FIGURE 4. Electron micrograph of a field within the spinal cord of the lamprey *Petromyzon marinus*, sectioned transversely to the long axis. A large presynaptic axon profile (1) adjoins a smaller postsynaptic unit (2), across a synaptic cleft (white asterisk) *ca.* 15 nm in width. Large numbers of spherical synaptic vesicles 45 to 55 nm in diameter are present in the presynaptic axoplasm: in the immediate vicinity of the axon membrane, the vesicles are randomly arranged (*) while in a zone of axoplasm commencing about one-third of a micrometre from the cell membrane, many synaptic vesicles are grouped in rosette fashion around microtubules (arrows). A 'complete' rosette involves five spherical vesicles, but incomplete groups and other configurations (cf. figures 6 to 8, 11, 13 to 15) occur in the lamprey axoplasm. (Magn. ×150000.)

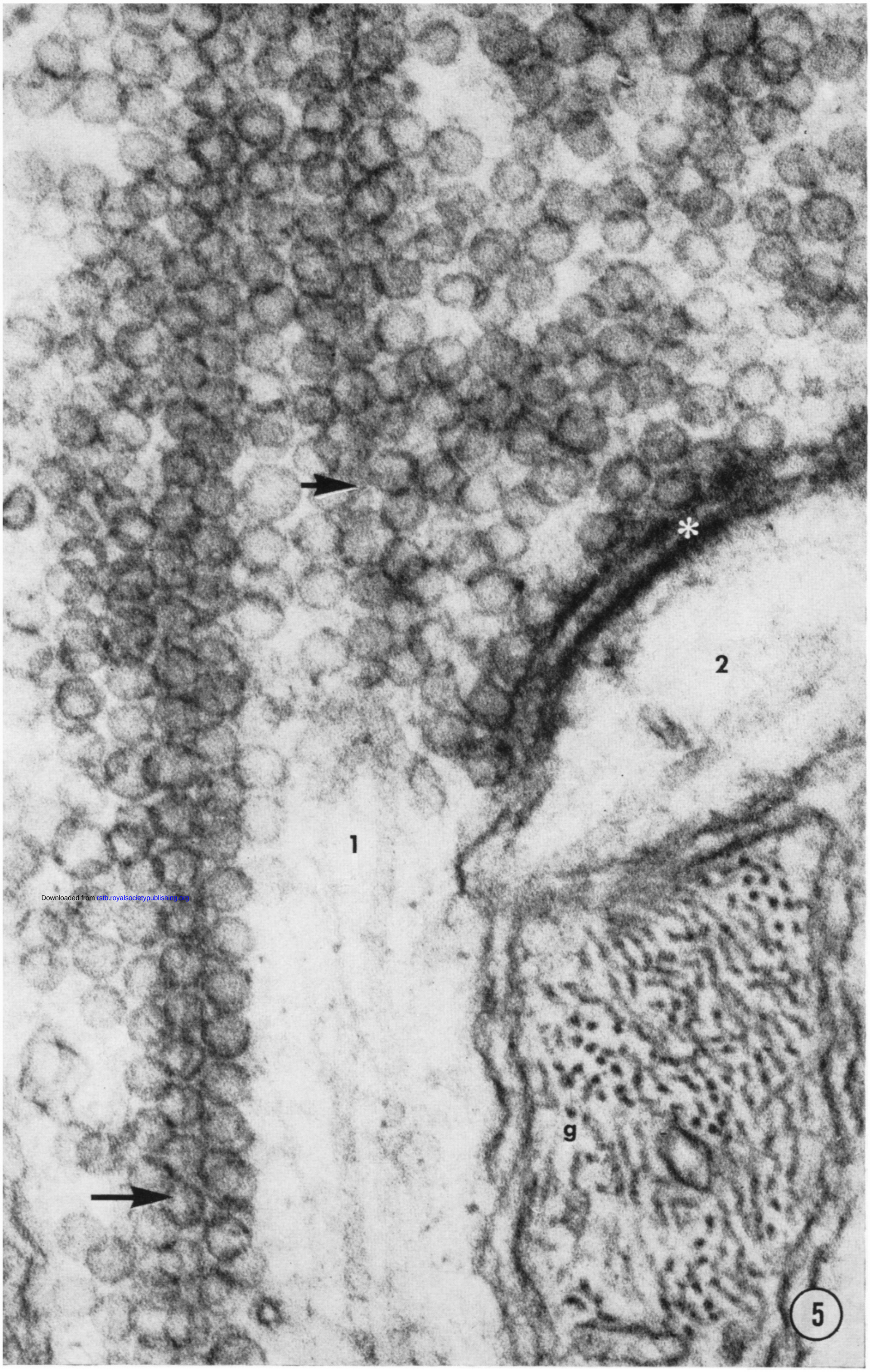

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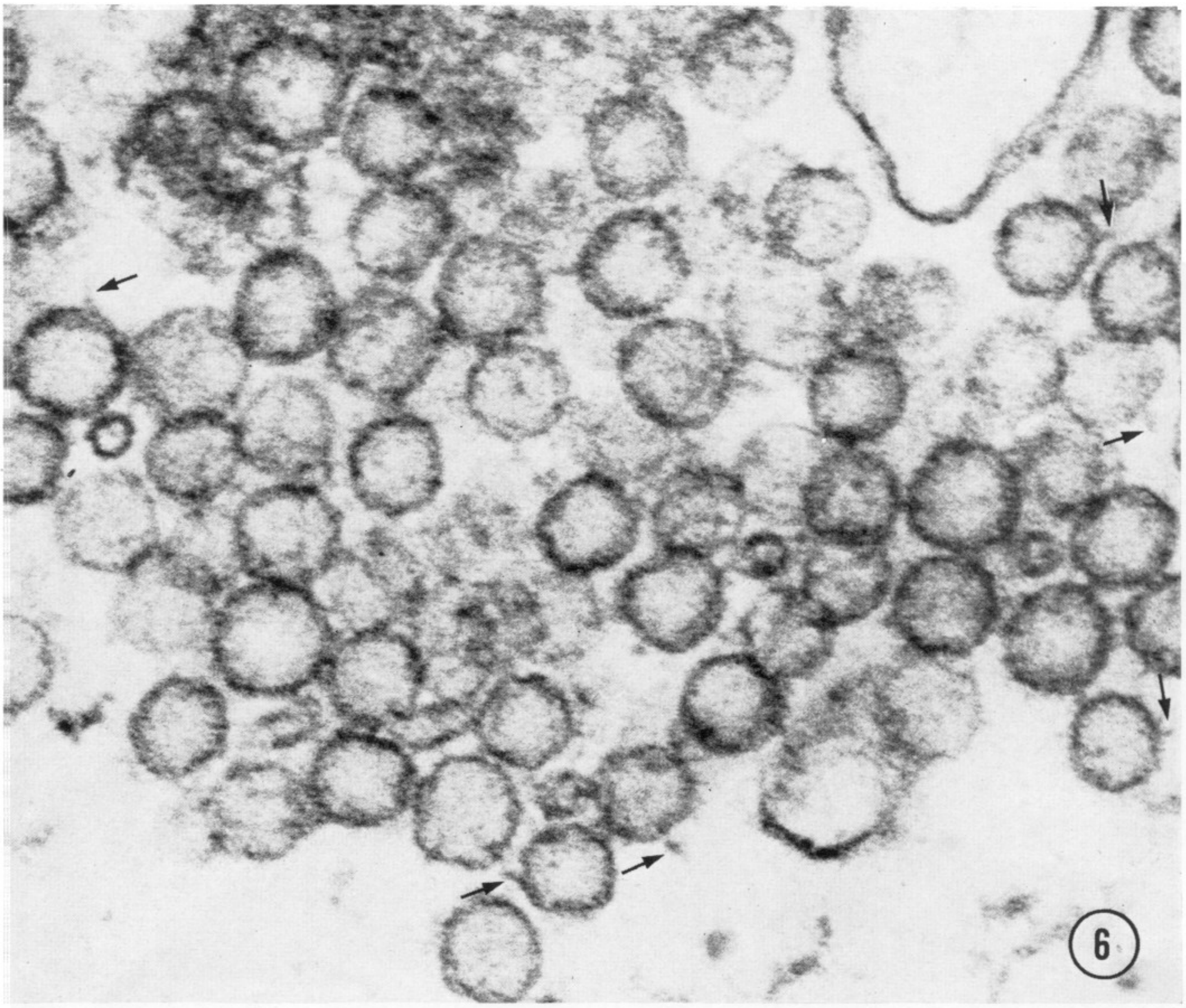
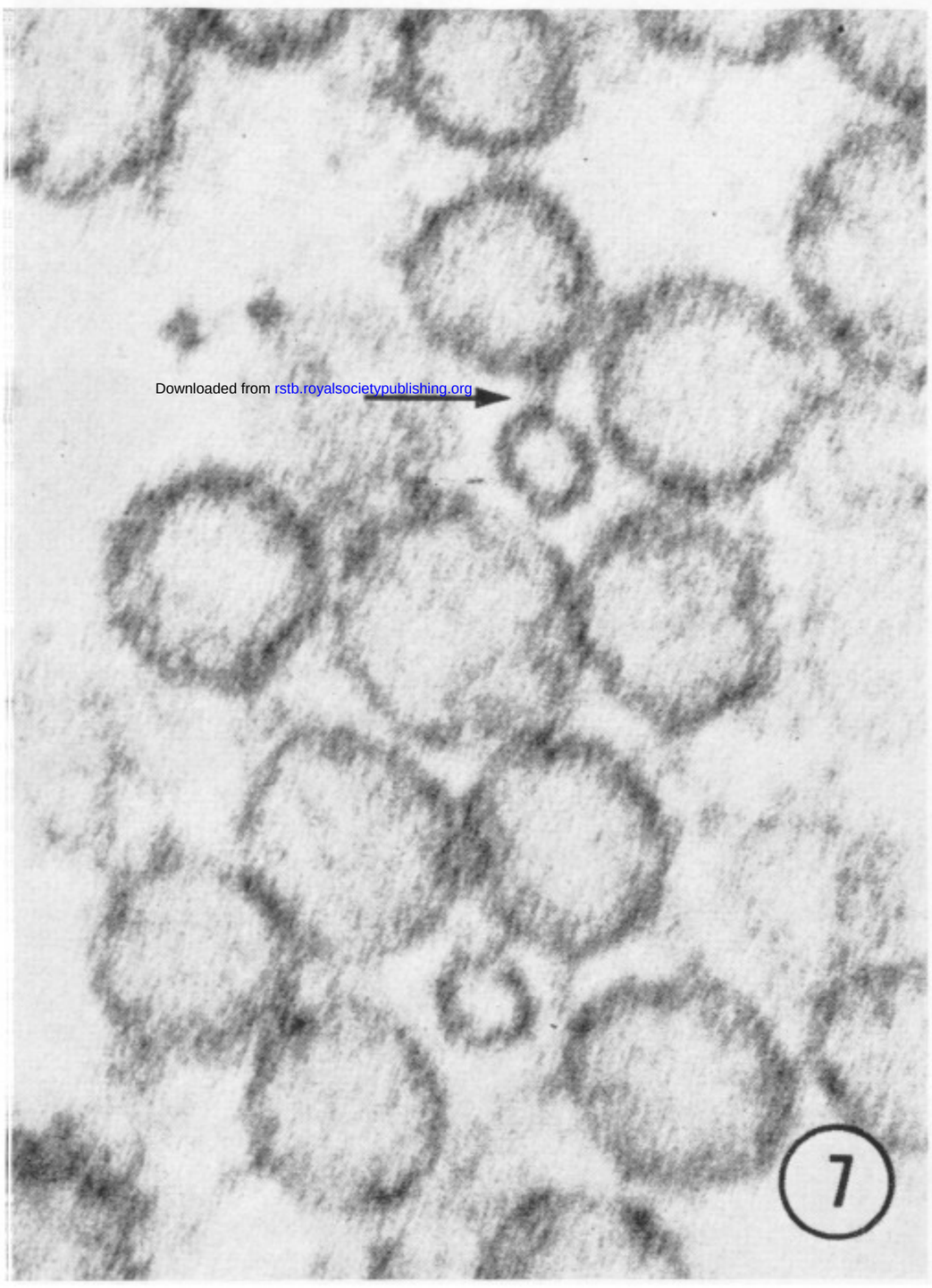
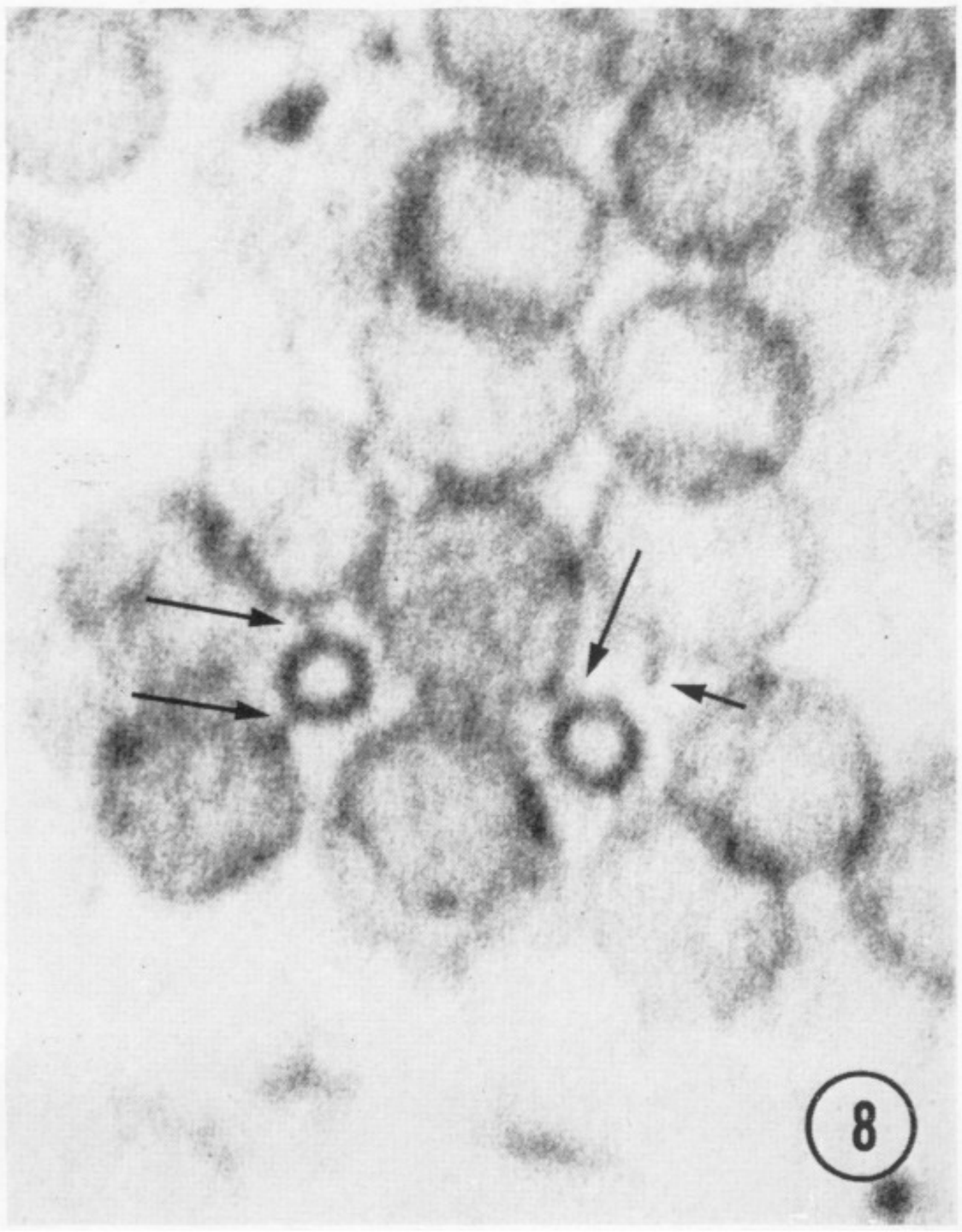
FIGURE 5. *Petromyzon marinus*. A micrograph corresponding to figure 4, but passing longitudinally through the presynaptic axon (1), close to a point of junction with a small postsynaptic unit (2). Note the opaque material associated with both apposed axon membranes, and the intervening synaptic cleft (*). A glial process containing cytoplasmic filaments is included at g. As in figure 4, synaptic vesicles immediately flanking the presynaptic membrane are unattached and randomly grouped; a short distance away, a passing microtubule (short arrow) is ensheathed with vesicles to the level of the synapse and a second (long arrow) nearby is uniformly surrounded by closely packed vesicles, similar to those in the synaptic focus. (From Smith, Järlfors & Beránek (1970). Reproduced by courtesy of the Rockefeller University Press.) (Magn. ×150000.)







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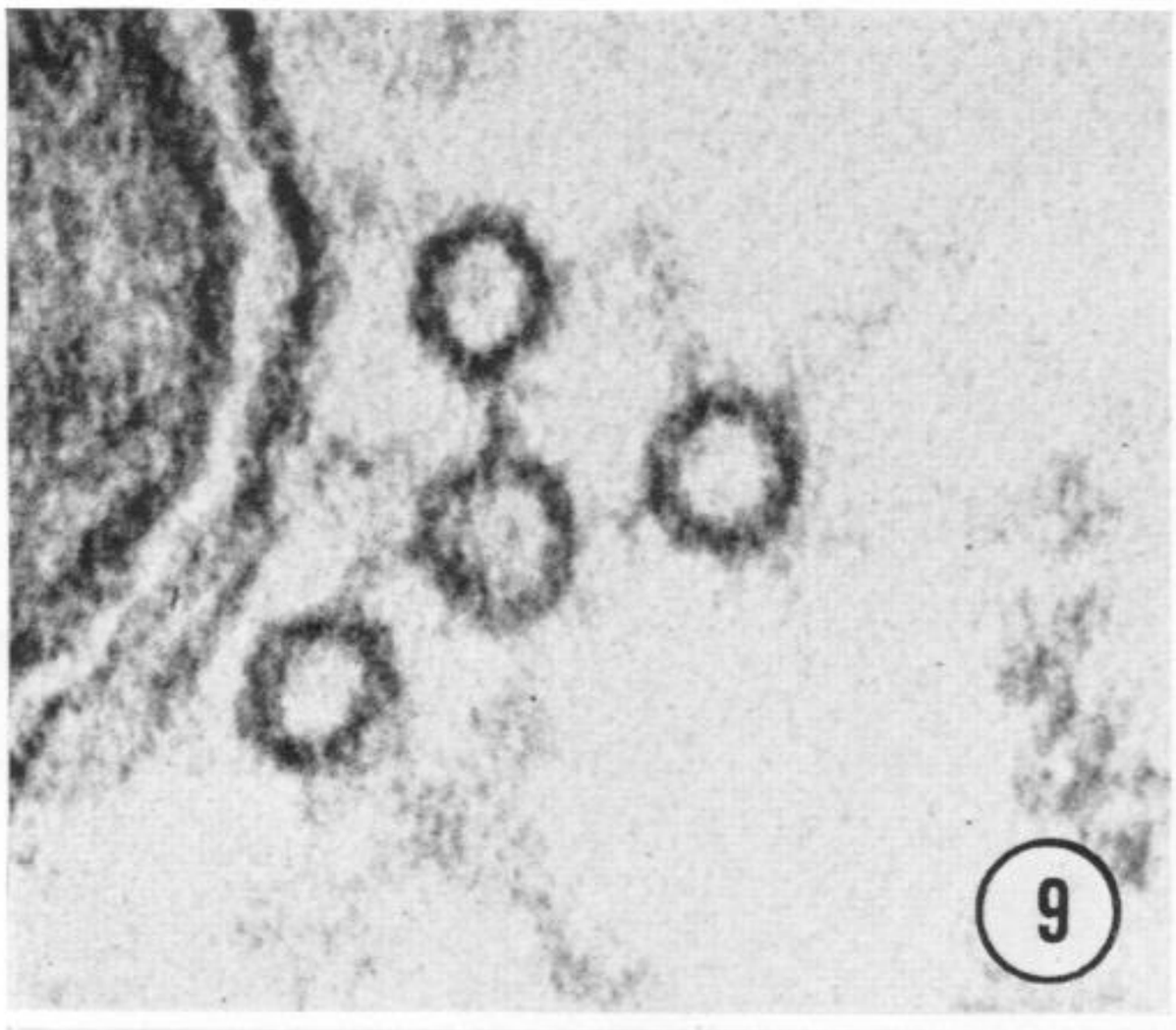
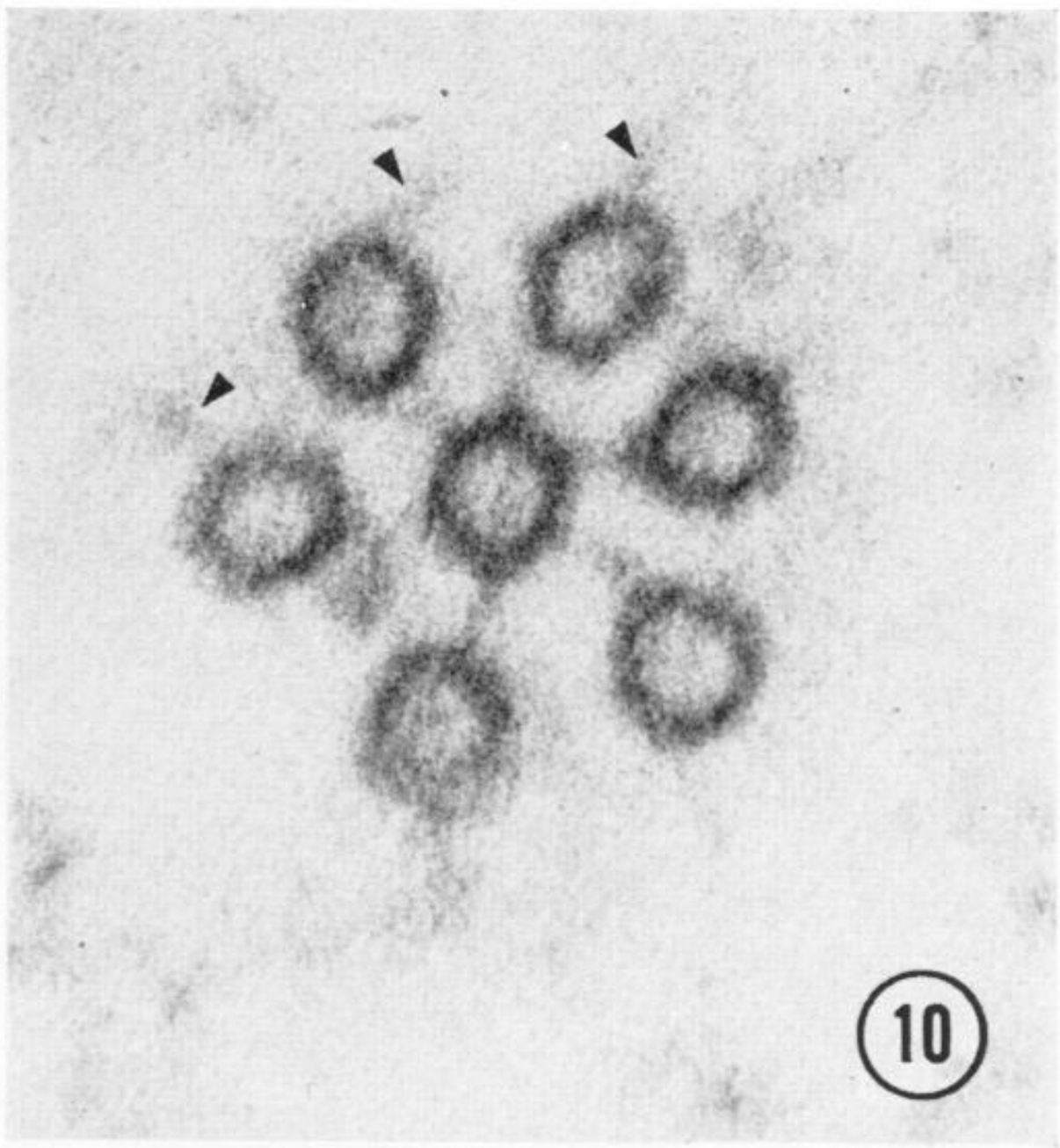
FIGURES 6 TO 8. Micrographs illustrating structural linkage between microtubules and vesicles in the vicinity of synapses in the spinal cord of *Petromyzon*. They suggest that a bridge component is provided by the vesicles.

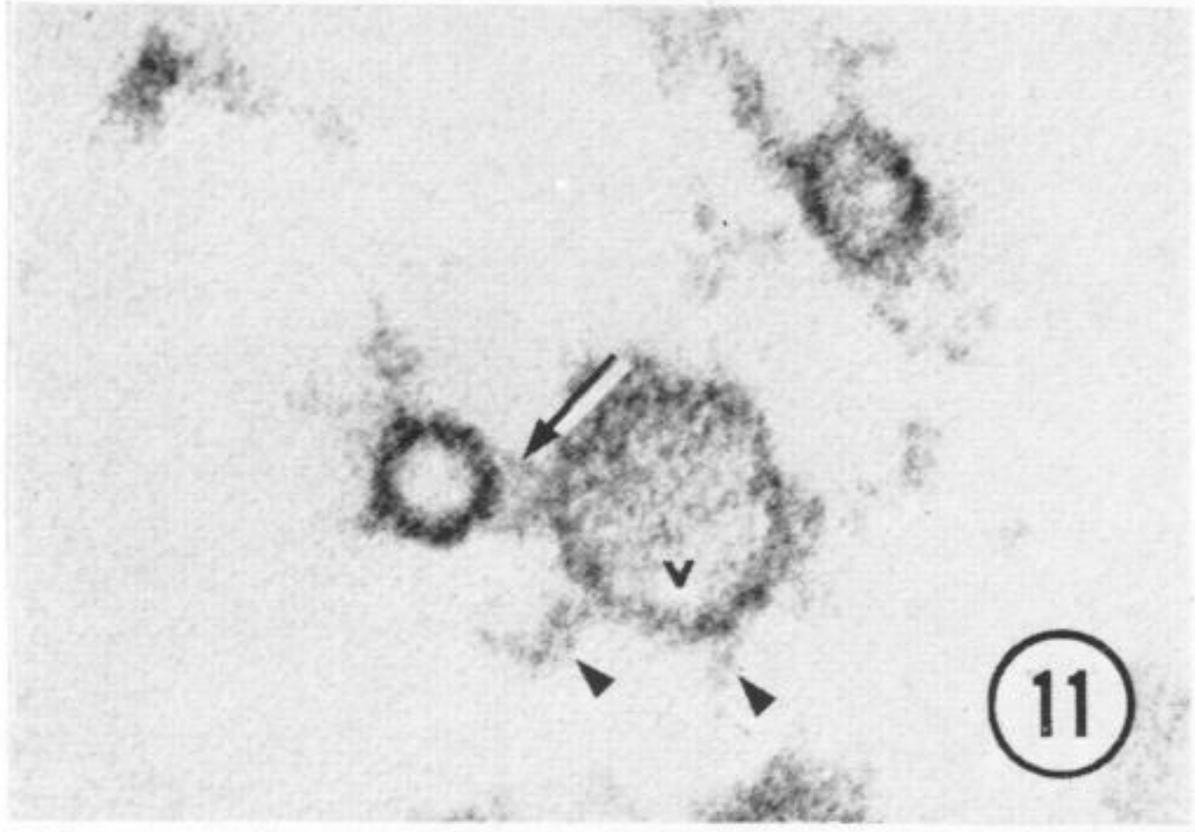
FIGURE 6. The synaptic vesicle membranes bear short (*ca.* 10 nm) projections (arrows) generally set at an angle with respect to the vesicle radii. (Magn. × 220 000.)

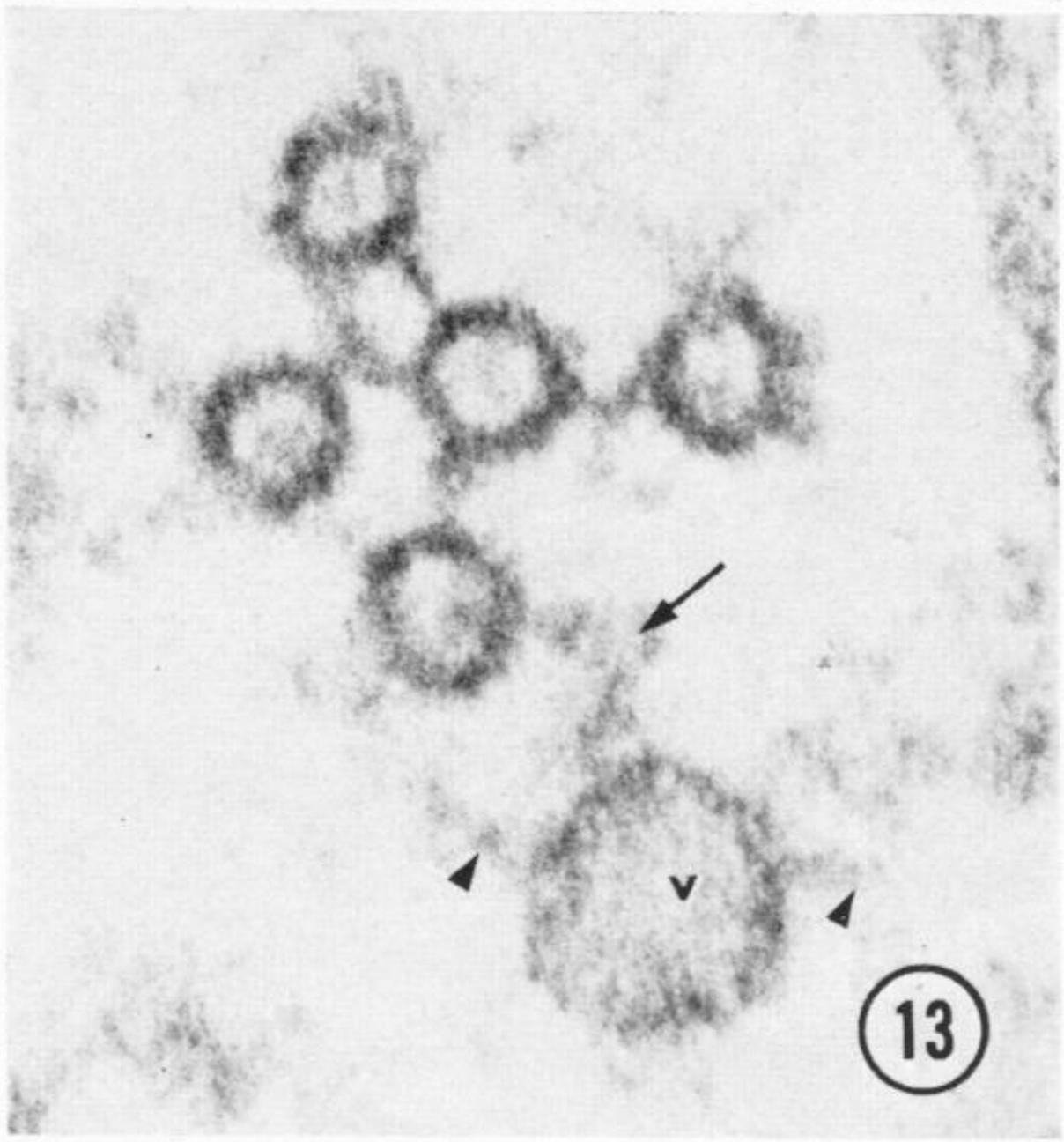
FIGURE 7. A structural link (arrow) between a microtubule and a member of a synaptic vesicle rosette (cf. figure 4). (Magn. × 300 000.)

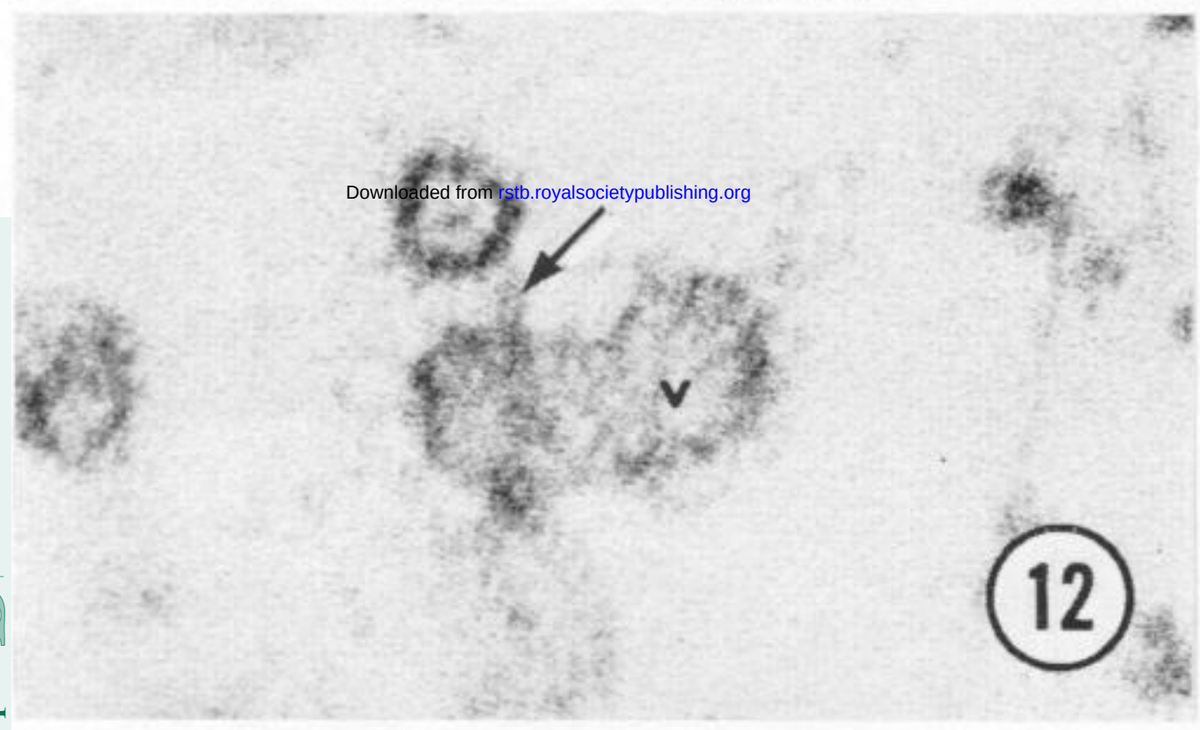
FIGURE 8. An axoplasmic field bordering a synapse illustrating (as in figure 7) structural bridges (long arrows) between microtubules and synaptic vesicles. In addition, a projection from the surface of a vesicle is resolved (short arrow). (Magn. × 300 000.)

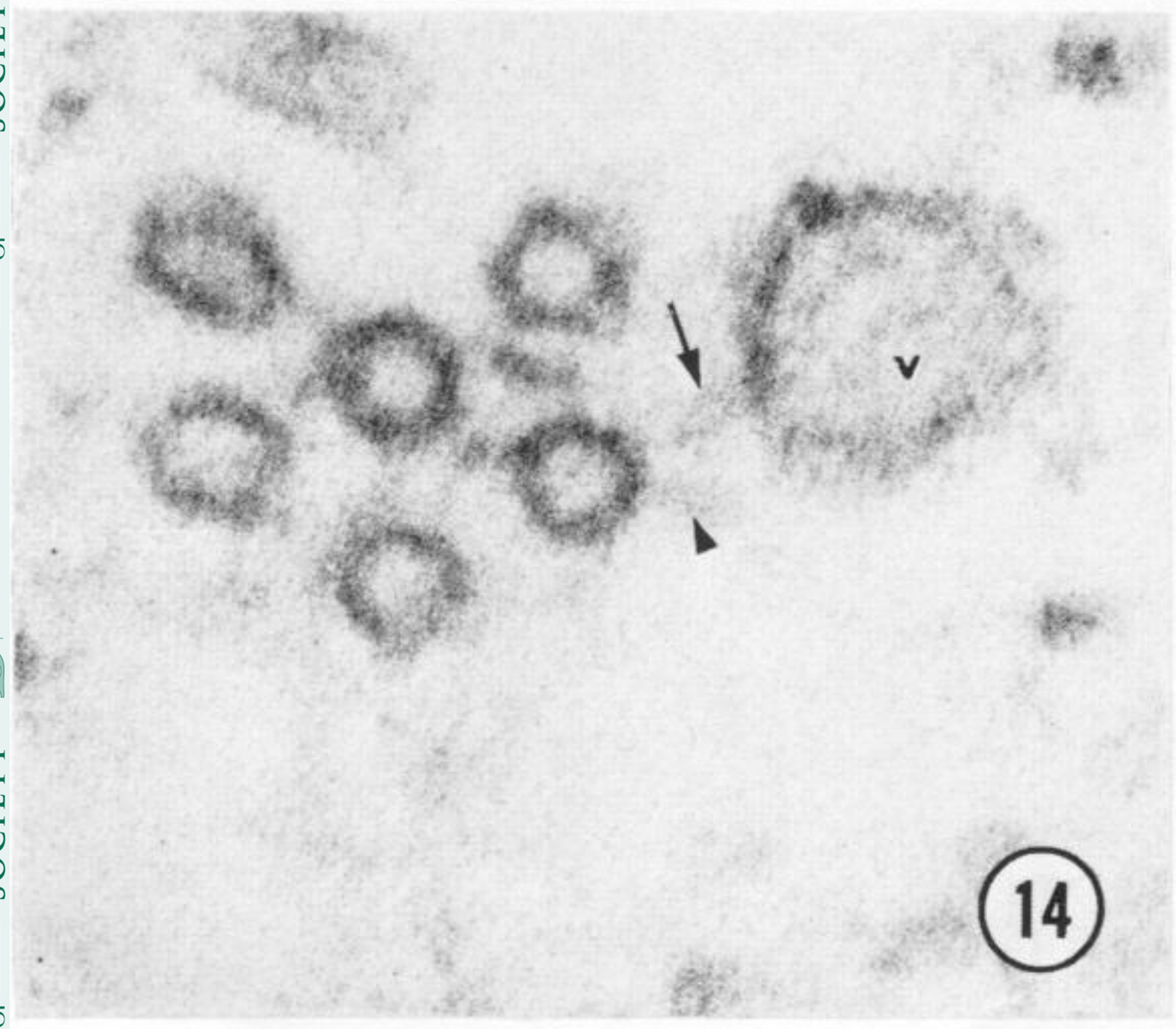


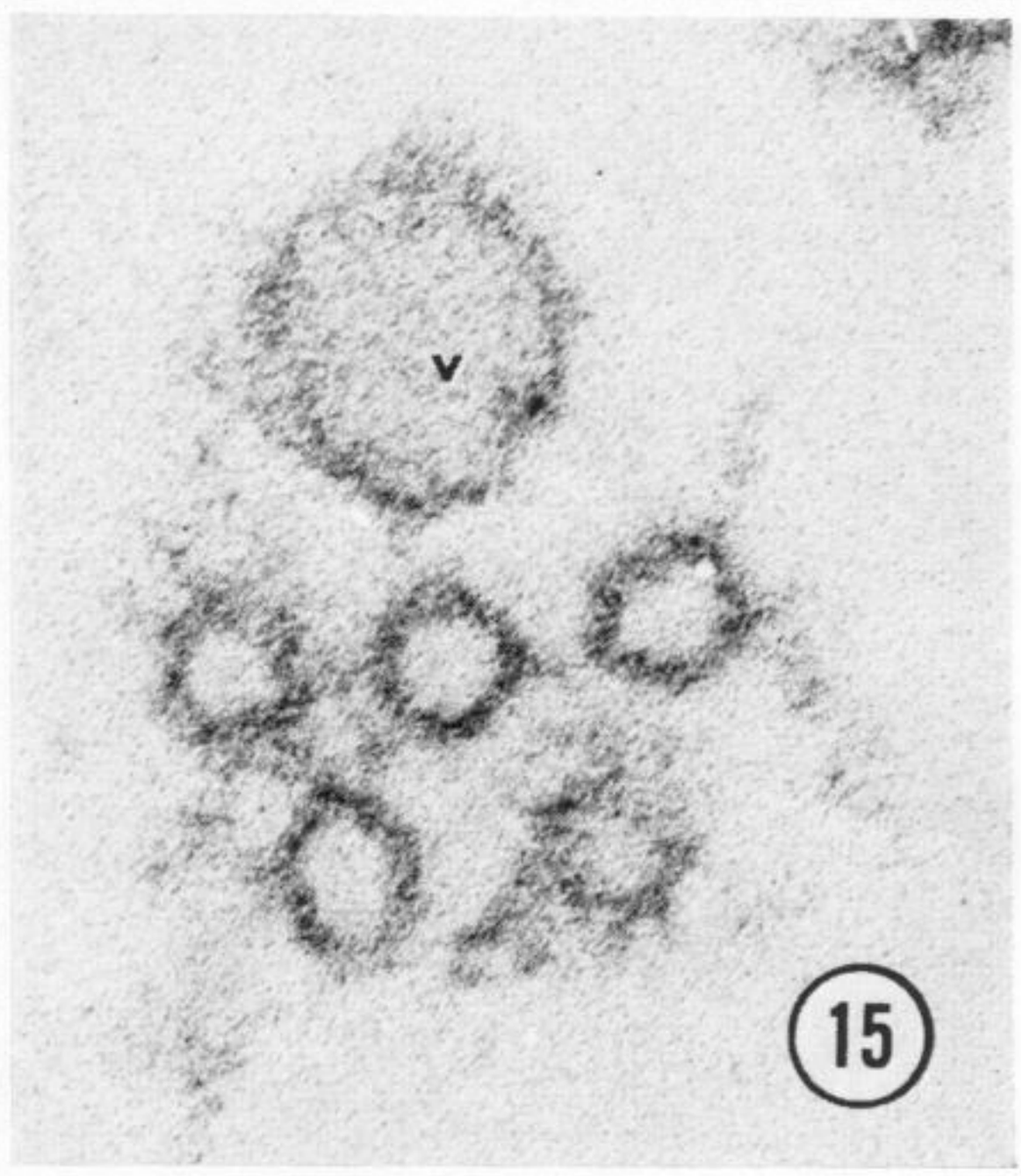












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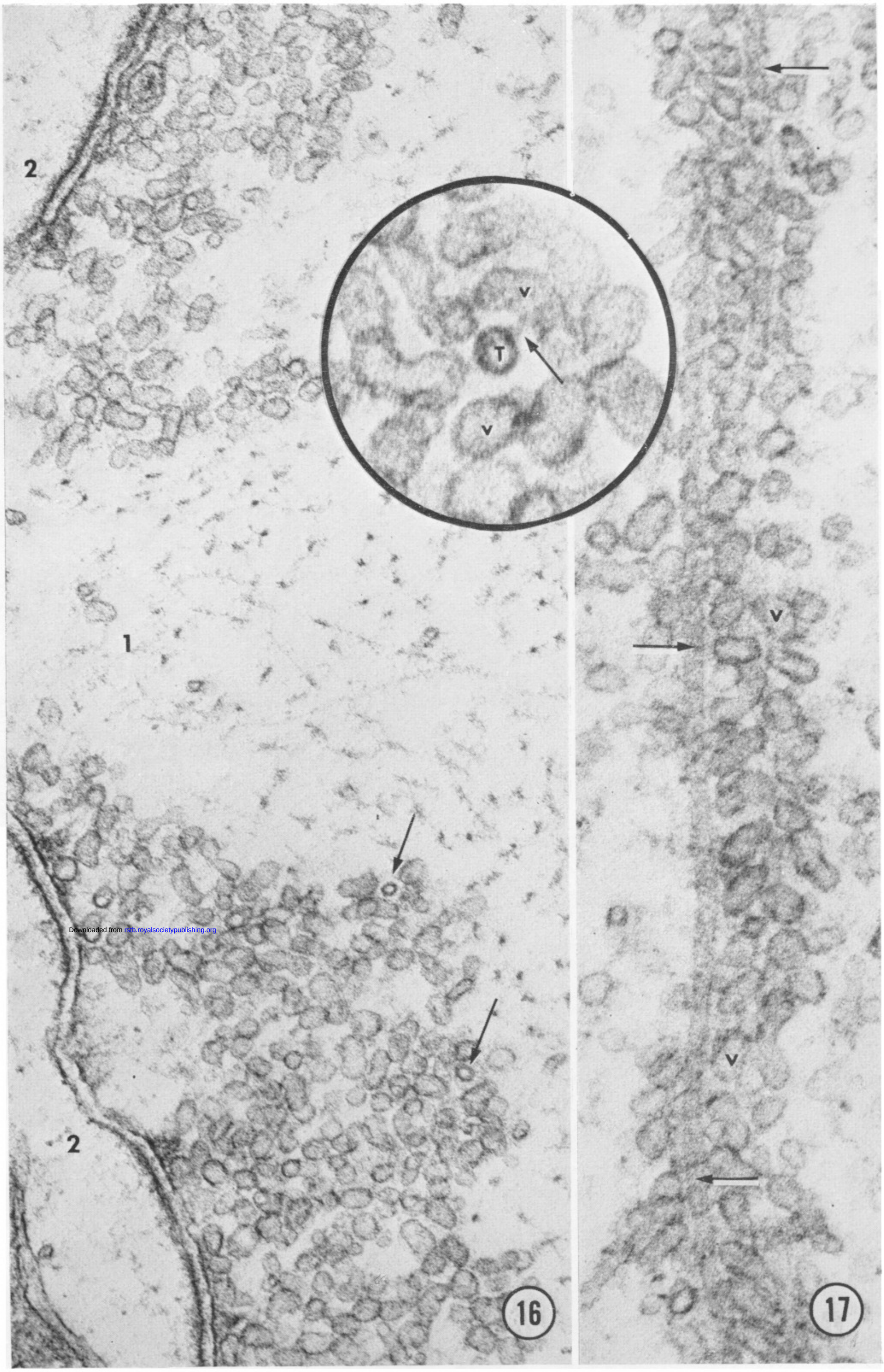
FIGURES 9–15. For legends see facing page.



FIGURES 16 AND 17. Micrographs illustrating the disposition of non-spherical vesicles in axons of the lamprey spinal cord. In general, the distribution of these structures, perhaps containing chemical transmitters distinct from those of the spherical vesicles, parallels the latter. Synaptic foci (figure 16) of vesicles are grouped in a large presynaptic axon (1) adjoining small postsynaptic members (2). As elsewhere (cf. figure 4), vesicles are grouped around microtubules in the peripheral zone of the synaptic focus (arrows), and longitudinal sections of such axons reveal (figure 17) a similar association of vesicles (v) and tubules (arrows) (cf. figure 5). Bridges (arrow) between non-spherical vesicles (v) and microtubules (T) are resolved (inset; see also figure 12)—paralleling the situation in axons containing spherical vesicles. (Magnifications: figure 16, $\times 100\,000$; figure 17, $\times 150\,000$; inset, $\times 300\,000$.)

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