

## THE FINE STRUCTURE OF THE CNS IN MULTIPLE SCLEROSIS. I. INTERPRETATION OF CYTOPLASMIC PAPOVAVIRUS-LIKE AND PARAMYXOVIRUS-LIKE INCLUSIONS

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### **The fine structure of the CNS in multiple sclerosis. I. Interpretation of cytoplasmic papovavirus-like and paramyxovirus-like inclusions**

During an electron microscopic study of the white matter in multiple sclerosis (MS), spheroidal reticular particles were found both in MS and in control brains. These particles have previously been described in the brain in MS and in brain-derived cell cultures in subacute sclerosing panencephalitis. In both cases they were interpreted as papovaviruses, but in size, morphology and distribution they are identical to the reticulosomes and related particles which occur as proteinaceous artefacts in a variety of tissues and in subcellular fractions. Inclusions in endothelial cell cytoplasm, previously reported from the CNS in MS as paramyxovirus similar to measles, have also been found in the present study. They were present both in MS and in control brains and are identified as 'rod-shaped tubular bodies', normally occurring organelles of endothelial cells. The necessity for a cautious interpretation of virus-like inclusions is emphasized.

### **Introduction**

Few neurological diseases have received such intensive electron microscopic (EM) study as has multiple sclerosis (MS) (see reviews by Andrews, 1972; Prineas, 1975; Mirra & Takei, 1976). Suggestions that a virus might be involved in its pathogenesis (e.g. Adams & Imagawa, 1962) have stimulated a search for viral particles during EM studies of MS brain at biopsy and necropsy, of cell cultures derived directly or indirectly from such brain, and of peripheral lymphocytes from MS patients. These searches have revealed a bewildering variety of 'virus like particles' and other unusual inclusions. Attention has centred mainly on the 'paramyxovirus-like' filaments found in the nuclei of unidentified perivascular cells (Prineas, 1972). Although this initial finding was confirmed, similar filaments were subsequently

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reported from a wide range of other, apparently unrelated diseases and it now seems likely that they are an unusual form of nuclear chromatin unrelated to viruses (Lampert & Lampert, 1975; Prineas, 1975; Mirra & Takei, 1976). Moreover, in a number of studies where identification of parainfluenza or measles virus was not in doubt (ter Meulen, Koprowski, Iwasaki, Käckell & Müller, 1972; Iwasaki, Koprowski, Müller, ter Meulen & Käckell, 1973; Field, Cowshall, Narang & Bell, 1972; Thorne & Dermott, 1976) the techniques used involved indirect 'viral rescue' in cell culture with its attendant well-recognized risk of contamination by non MS-associated virus (Field *et al.*, 1972).

Despite those reservations there remain a few unexplained reports of cytoplasmic 'paramyxovirus-like' filaments and tubules (Prineas, 1972; Dubois-Dalcq, Schumacher & Sever, 1973; Narang & Field 1973a; Tanaka, Iwasaki & Koprowski, 1974; 1975a; Pathak & Webb, 1976) and a second type of intranuclear filament (Tanaka *et al.*, 1974; 1975a, b; Tanaka, Santoli & Koprowski, 1976). Isolated reports of single MS cases containing particles resembling papovaviruses (Bauer, ter Meulen, Koprowski, Argyrakis & Orthner, 1975) and coronaviruses (Tanaka, Iwasaki & Koprowski, 1976) have also appeared and there are several reports of unspecified 'virus-like' inclusions (e.g. Boyazis, Martin, Bouteille, Guazzi & Manacorda 1967; Andrews & Andrews, 1973). That these may not always be what they seem is shown by the controversy over the 'budding' virus-like particles found in an MS biopsy by Narang & Field (1973b). These were subsequently reinterpreted by Hill (1973) as nuclear pores, a view not accepted by the original authors (Narang & Field 1973c).

The results of EM studies in MS, therefore provide no consistent or unequivocal evidence of virus involvement in the disease. In this respect MS differs from the confirmed virus diseases of the CNS such as subacute sclerosing panencephalitis (SSPE), progressive multifocal leucoencephalopathy and herpes encephalitis, in all of which the causal organisms have been convincingly demonstrated in a reasonable number of cases (see review by Mirra & Takei, 1976). However, the number of unexplained inclusions and 'possible viruses' in MS is still large and it remains to the electron microscopist to identify these as far as is possible within the limitations of present knowledge and understanding of normal and pathological subcellular structure.

In the course of a correlated light and EM study of the white matter from 8 confirmed MS brains obtained at early necropsy (3-9 hours after death) we have found a number of unusual inclusions. Because of their number and diversity it is convenient to consider them in two main categories namely intranuclear and extranuclear. The former group which comprises non-viral 'paramyxovirus-like filaments', fibrillar lattices, vesicular nuclear bodies and 'crystalloid honeycombs' will be described in a subsequent paper (Kirk, 1978c). The two kinds of cytoplasmic inclusions described in detail in the present paper have previously been seen in MS CNS tissue where they were reported as viruses (Bauer *et al.*, 1975; Pathak & Webb, 1976). One kind has also been reported in brain cell cultures derived from cases of SSPE where they were also identified as viruses (Oyanagi, ter Meulen, Müller, Katz & Koprowski, 1970; Koprowski, Barbanti-Brodano & Katz, 1970). We have had the opportunity of examining these inclusions more closely and in a variety of locations and have come to different con-

clusions as to their nature. The more difficult questions concerning the nature of dense cored particles and hollow-cored vesicles which were also found in this study, will be tackled in further papers in this series (Kirk, 1978a; 1978b).

## Materials and methods

Blocks (1–2 mm cubes; six to eight from each of seven to sixteen areas per case) for electron microscopy were taken from the white matter of the cerebral hemispheres and cerebellum of eight patients who had died with clinically diagnosed MS (Table 1). The diagnosis was confirmed histopathologically (Dr I.V. Allen). Control material was available from two sources. EM blocks were taken at necropsy from the

**Table 1.** Clinical and other details of MS cases

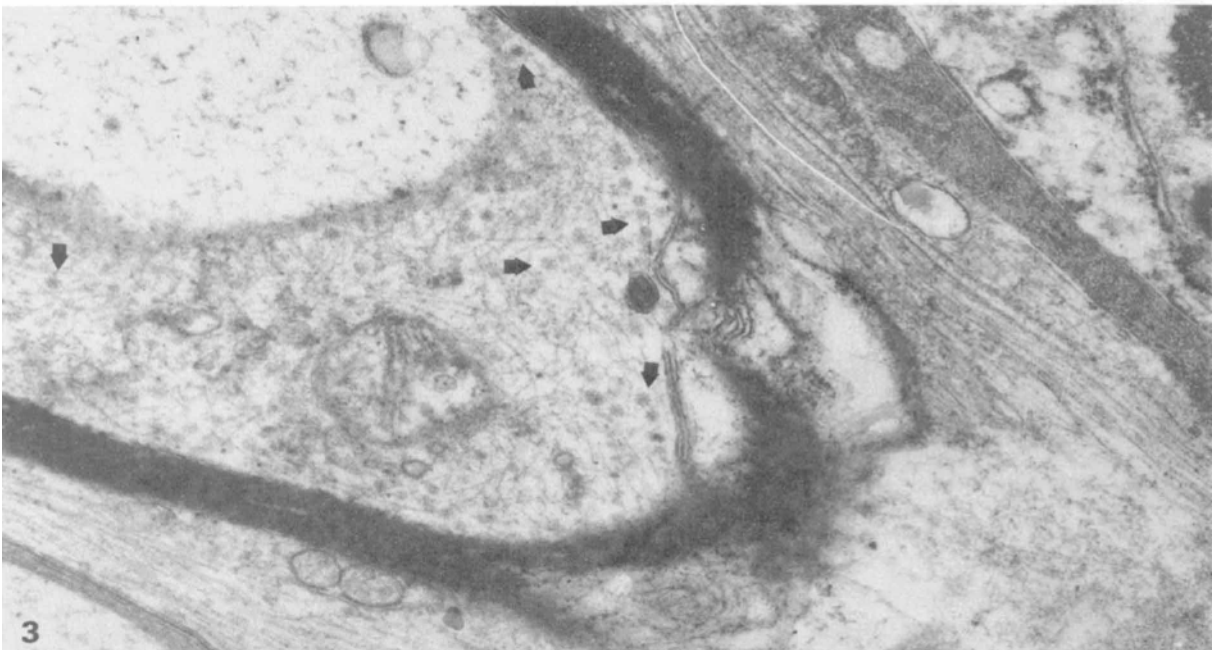
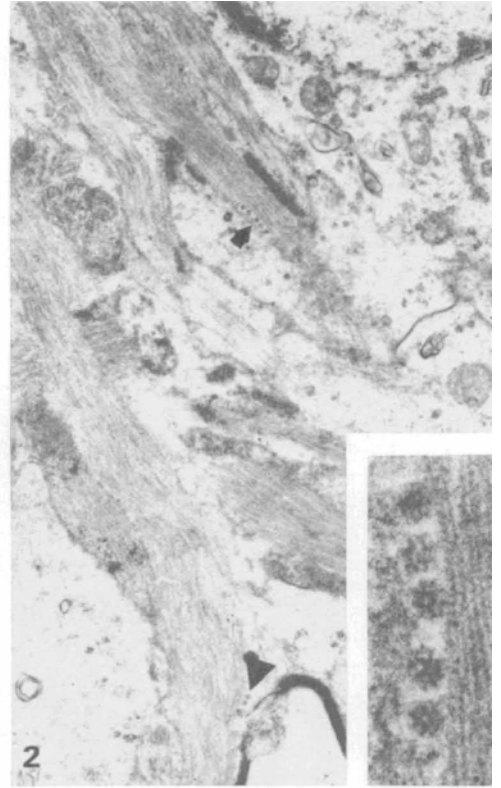
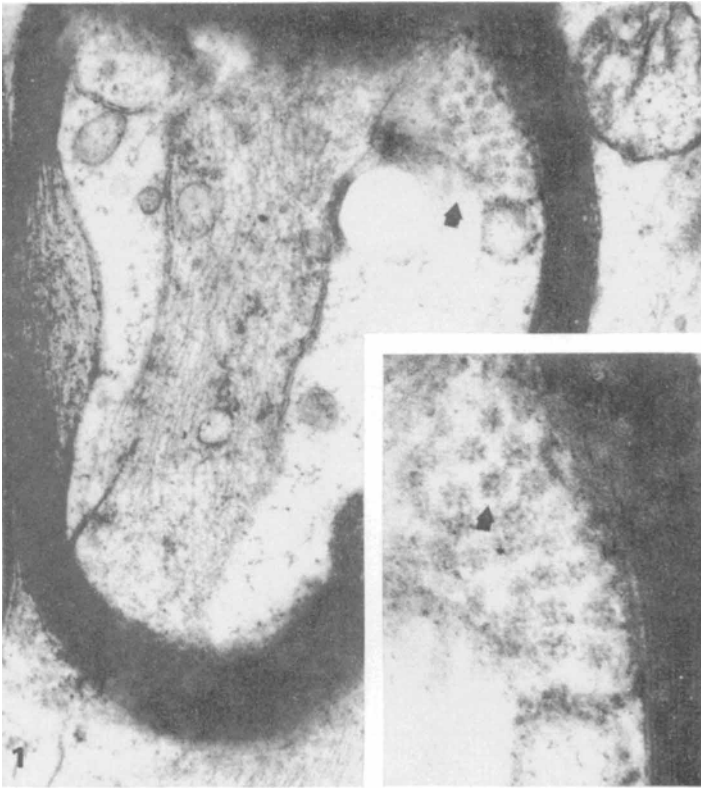
<i>Case no.</i>	<i>Sex</i>	<i>Age (yr)</i>	<i>History duration (yr)</i>	<i>Cause of death</i>	<i>Death–necropsy interval (h)</i>
1	F	29	10	peritonitis	3
2	F	60	24	bronchopneumonia	9
3	M	24	1½	bronchopneumonia	3½
4	F	72	33	bronchopneumonia	3
5	F	50	12	carcinoma; pancreas	3
6	F	63	27	ruptured abdominal aneurysm	4½
7	M	52	20	bronchopneumonia	4½
8	F	57	38	bronchopneumonia	5½

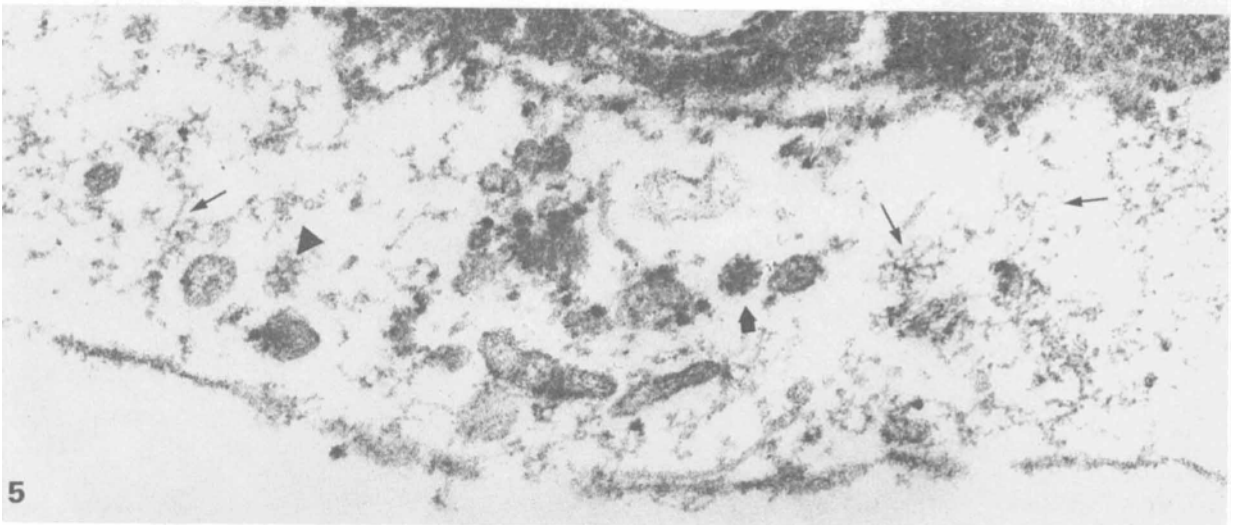
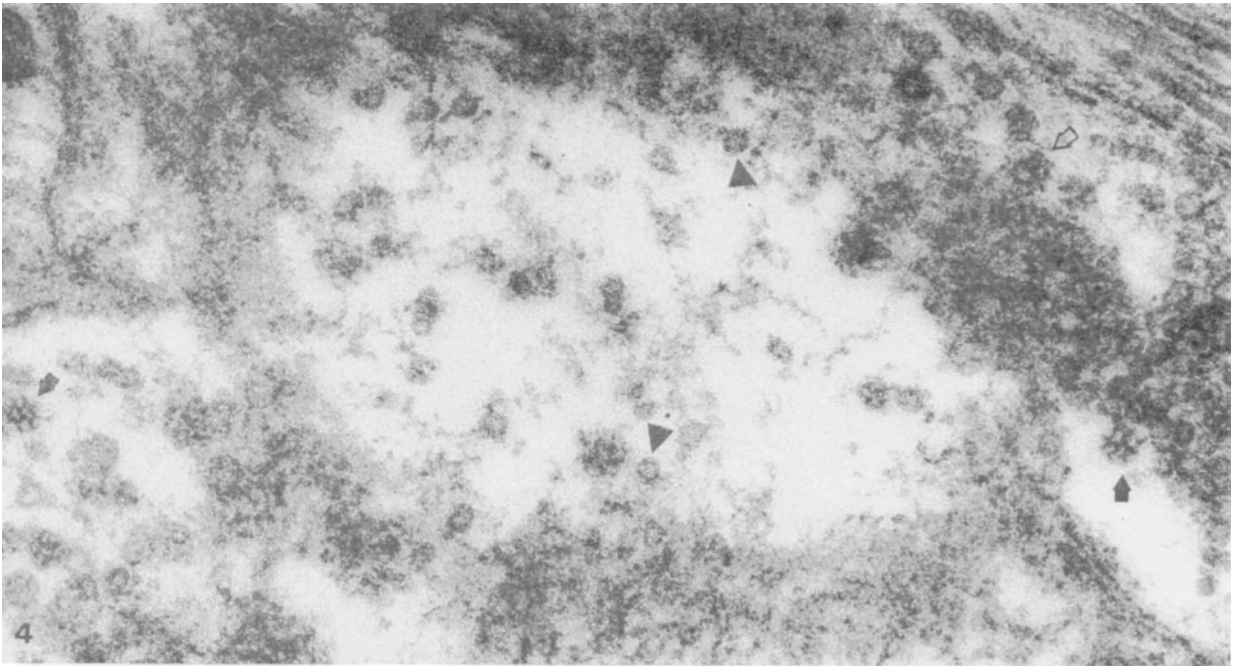
brains of eight patients dying with non-neurological conditions. Secondly, material was available from a number of cerebral biopsies, mostly done for suspected viral encephalitis but also from a case of oligodendroglioma, a case of sarcoidosis and several cases of dementia. Fixation of EM blocks was carried out by immersion for 4 h or overnight in chilled 2.5% glutaraldehyde in either 0.1 M s-collidine or 0.1 M phosphate buffer. After postfixation in 1% OsO<sub>4</sub> the blocks were dehydrated through graded alcohols and embedded in Epon 812. Semithin (1 µm) sections used for locating areas for EM viewing were stained with toluidine blue. Ultrathin (60 nm) sections of selected areas were stained with uranyl acetate and lead citrate and examined with an AEI EM801 electron microscope. Areas for viewing in the MS material were not initially selected for the purpose of finding inclusions. Rather, they were selected so as to be representative of the white matter in MS. Areas in which inclusions were found included supposedly normal white matter as well as a range of plaque lesions and their surroundings. The plaques in question ranged from presumed active to old gliosed.

## Results

### *Reticular particles and networks*

In seven MS brains, in five out of eight control necropsy brains and in eleven out of sixteen brain biopsies, small (49–83 nm) variably electron dense spheroidal 'particles' were seen. In most cases they were not very prominent, but in a few, both MS and control, they were commonly observed. They were found in unidentified cell processes, within myelin sheaths (Figure 1), in fibrous astrocytic processes (Figure 2), in axons (Figure 3), in axon terminals along with normal smooth synaptic vesicles





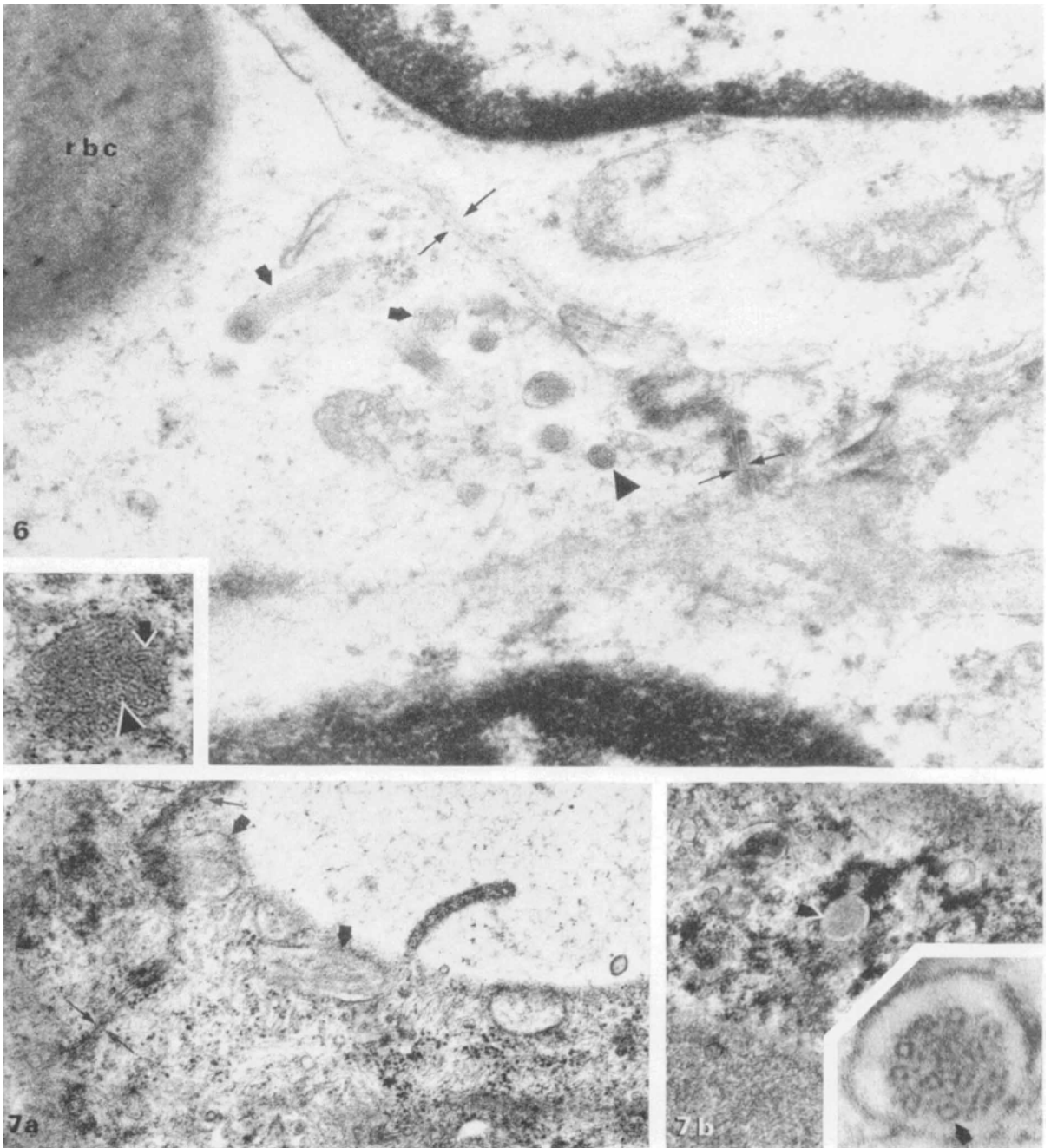
**Figure 1.** Myelinated axon with artefactually swollen sheath contains an array of reticular particles (*arrow*).  $\times 31\ 500$ . *Inset:* Detail shows electron-lucid halos around each particle and the central ring in one (*arrow*).  $\times 72\ 000$ .

**Figure 2.** Group of five particles (*arrow*) in linear array within a reactive astrocyte process. A pair of similar particles is also seen (*triangle*).  $\times 12\ 500$ . *Inset:* High magnification of particles demonstrates reticular form and electron-lucid halos. Compare the central particle with the arrowed particle in the previous figure.  $\times 72\ 000$ .

**Figure 3.** Myelinated axon in gliosed area contains numerous reticular particles (*arrows*) scattered amongst a mass of neurofilaments.  $\times 31\ 500$ .

**Figure 4.** Synapse containing reticular particles (*arrows*) in addition to smooth (*triangles*) and coated (*hollow arrow head*) synaptic vesicles.  $\times 72\ 000$ .

**Figure 5.** Poorly preserved glial cell cytoplasm contains a reticular particle (*large arrow*), and an array of hexagonal profiles (*triangle*). Note the angular filamentous network in the background (*small arrows*).  $\times 72\ 000$ .



**Figure 6.** Group of tubular bodies in an endothelial cell lying close to an intercellular junction (*paired arrows*) contain either parallel linear (*large arrows*) or circular (*triangle*) profiles.  $\times 41\ 500$ . *Inset:* A large number of circular (*triangle*) and short linear (*arrow*) profiles are seen together in this tubular body from another cell.  $\times 60\ 000$ . rbc—red blood cell.

**Figure 7. a** Two less compact (? mature) tubular bodies (*arrow*) adjoin the adluminal membrane of an endothelial cell. Note their proximity to an intercellular junction (*paired arrows*).  $\times 25\ 000$ .

**Figure 7. b** A typical tubular body bounded by a 'membrane ghost' which blends with the darkly clumped cytoplasm. Other membranes in this cell are also poorly preserved.  $\times 25\ 000$ . *Inset:* High magnification micrograph of a tubular body with a prominent internal electron-lucent halo and a well defined bounding membrane. The internal tubules which are set in an electron dense matrix have walls with a trilamellar appearance (*arrow*).  $\times 120\ 000$ .

(Figure 4) and in unidentified glial cell cytoplasm (Figure 5). Occasionally they presented as arrays (Figure 1) or in rows (Figures 2 & 3). The individual particles which were not bounded by membranes or walls were reticular in appearance. In most cases they were surrounded by electron-lucid halos which made them stand out more clearly against the background cytoplasm. Another kind of particle, almost identical in appearance but containing a small central vesicle (Figures 1, 2 & 4) was also seen and was found in all cases examined, both MS and control. Isolated single or grouped polygonal profiles (Figure 5) were also seen, but only rarely, in MS cases, in the post-mortem control tissue and in a number of the brain biopsies. In addition, but uncommonly, irregular reticular networks, composed of fine filaments were seen (Figure 5) both in MS and in control post-mortem brains.

### *Tubule-containing Bodies*

In addition to a normal complement of the larger membrane bound cytoplasmic organelles, viz. mitochondria, multivesicular bodies and other lysosomes, a small proportion of endothelial cell profiles contained membrane bound bodies which were not assignable to any of these categories. They were present both in the MS material and in the control material being more commonly seen in the former. Within the cell they were most frequently seen clustering near to the intercellular junctions (Figure 6). Morphologically the bodies were irregular cylinders bounded by a single membrane. Internally they contained a number of 20–23 nm tubules which presented as mainly circular profiles in transverse section and as parallel lines in longitudinal section. Occasional bodies cut in near transverse section contained mixtures of short linear and circular profiles (Figures 6 & 7b). At high magnification (Figure 7b) the walls of some tubules appeared trilamellar. The background matrix in which the tubules were set was variable in electron density, most commonly being rather dense (Figures 6 & 7b) but in some cases light (Figure 7a). The bodies with light background matrices were uncommon and when present were near the adluminal membrane of the endothelial cells. Under some circumstances, a narrow often indistinct electron lucid halo was seen underlying the bounding membrane. In some apparently poorly fixed material this halo was enlarged giving the body an uncharacteristic but none the less recognisable appearance (Figure 7b, inset). In occasional bodies the bounding membrane did not appear as a distinct dense line. Instead the electron lucid halo was sharply circumscribed by an irregularly thick electron-dense band which merged with the surrounding artifactually dense and clumped cytoplasm (Figure 7b).

Overall, there was a wide variation in the appearance of these bodies resulting from the interaction of plane of section, fixation quality and morphology.

### **Discussion**

Although the true nature and significance of all 'virus-like' inclusions reported from MS material is not yet fully understood, it is possible to exclude a proportion of these from the possible virus category. This may be of some value in exposing 'red herrings'

which may currently or in the future result in misdirection of some MS research effort. At the same time those inclusions which are suspected of being infectious particles can be highlighted and brought under closer scrutiny.

#### *Reticulosomes and coated vesicles*

Vesicle centred particles identical to those illustrated in Figure 1, 2 & 4 have previously been described as alveolate vesicles, ringed or annular vesicles, (dense) coated vesicles, pinocytotic vesicles with spiky fringes, dense rimmed vesicles, complex vesicles and vesicles in baskets (see review by Kanaseki & Kadota, 1969). Those without central vesicles (Figure 1-5) have variously been described as baskets (Kanaseki & Kadota, 1969) or reticulosomes (Gray, 1972). Both kinds have been described in normal animal nervous tissue (e.g. Gray, 1972; Peters, Palay & Webster, 1976) and in subcellular fractions (Kanaseki & Kadota, 1969; Blitz, Fine & Toselli 1977) their exact appearance depending on their location and on the quality of fixation. For the purposes of discussion in this paper we shall use the descriptive terms, coated vesicles and reticulosomes respectively.

While it is generally accepted that coated vesicles and reticulosomes do appear in electron micrographs of nervous system and other tissues, interpretation of their structure and possible function has proved difficult and controversial. The generally accepted view (Peters *et al.*, 1976; Kanaseki & Kadota, 1969) has been that reticulosomes are the empty shells or baskets of coated vesicles. These coats or baskets were believed to be formed around the vesicles either at the cell membrane or at the forming facing of the golgi apparatus. They were subsequently lost as the vesicle became (for example) a normal uncoated synaptic vesicle. However, more recently, Gray (1972; 1973; 1975) has formulated and developed an alternative interpretation which has profound implications for those seeking to interpret electron micrographs of animal tissues. He now believes them to be one manifestation of an intracellular 'stereoframework' which forms as a denaturation and precipitation artifact in areas rich in protein complexes. A stereoframework appears as a reticular structure forming a three-dimensional framework around polygonal lucunae. Gray proposed that it has no direct relationship to the molecular architecture of the particular protein complex *in vivo*. Intracellularly it may appear for example as the coat on a coated vesicle, as the 'microfilamentous network' of growth cones, as the presynaptic 'dense projections', as the post-synaptic dense material, and as the 'pore complexes' in the inner and outer orifices of the nuclear pores. Nuclei, too, may contain stereoframeworks, but their composition is still a matter for debate. Gray (1975) for example has suggested that chromatin may appear as one, but the 'nuclear matrix framework' (Berezney & Coffey, 1977) which remains intact after removal of almost all chromatin, fits the description better. Some further light on the nature of stereoframeworks has been shed by recent work on clathrin, the coat protein of coated vesicles. First isolated and biochemically characterized by Pearse (1976), clathrin was described as a unique protein with a molecular weight of 180 000. Schook, Oress & Pusz (1977) have since demonstrated that clathrin can form a micro-filamentous net *in vitro*. Negatively-



stained preparations of this net have shown it to have a 'stereoframework' structure, while fixation in glutaraldehyde causes the filaments to disassemble. Schook and his colleagues have proposed that synaptic vesicles may exist *in vivo* enmeshed in a cytonet (a kind of cytoplasmic stereoframework) and that coated vesicles may originate from the fragmentation of such structures. Whether stereoframeworks actually exist *in vivo* as a coherent physical structure as this work might suggest or whether they are formed *de novo* during fixation from a matrix of colloidal proteins as Gray (1975) suggested, are questions which remain to be answered. Whatever the outcome, however, the work of both schools supports the notion that both reticulosomes and the coats of coated vesicles are artefactual, resulting from the clumping of protein material into spheroids or onto the surface of vesicles respectively.

Bauer *et al.* (1975) reported the finding of 'particles identical in form and size with papovavirus' in an axon from the cerebellar white matter of a case of MS taken at autopsy. However, their illustration is of reticular particles with electron-lucid halos which are clearly reticulosomes, indistinguishable from our Figure 1. They bear only a superficial resemblance to the crystalloid aggregates of papova virus which are seen for example in progressive multifocal leucoencephalopathy (Zu Rhein, 1969; Mazlo & Herndon, 1977) and in other conditions (Jordan & Doughty, 1969). The absence of intranuclear particles might also be considered atypical for papovavirus. MS is not the only condition in which confusion has arisen due to the similarity of reticulosomes and papovaviruses. Oyanagi *et al.* (1970) and Koprowski *et al.* (1970) for example illustrate 60 nm 'papovavirus-like' particles which they found in the cytoplasm in brain cell cultures derived from a biopsy in a case of SSPE. Their illustrations, however, are of reticular particles with halos, identical in morphology and size to reticulosomes. Moreover, some of their illustrations bear a striking resemblance to Hagenau's (1973) illustration of 'nuclear pores simulating viruses', a finding that incidentally supports Gray's stereoframework hypothesis. Whether or not papovavirus is actually present in SSPE is not within our scope to discuss. However, morphological evidence of the kind described above is clearly inadequate and misleading.

The filamentous reticular network seen in the poorly fixed glial cell (Figure 5) provides a further example of a stereoframework artifact. Such appearances, more extensively illustrated in Gray (1972; 1973; 1975) should be borne in mind when seeking to interpret unusual reticular intracytoplasmic filaments in pathological material. Such filaments have previously been reported from astrocytes in three MS cases (Tanaka *et al.*, 1975a) and the suggestion was made of an association with viruses, although no positive identification was possible.

In summary then, as reticulosomes, coated vesicles and allied structures are not viruses and are widely occurring, if artefactual, entities they are unlikely to be of any significance in the etiology of either MS or SSPE.

#### *Rod-shaped tubular bodies (RTB)*

The tubule-containing pleomorphic bodies found in MS and control material in the

present study differ from other subcellular organelles and correspond exactly to descriptions of a common, but incompletely understood, endothelial cell organelle the 'rod-shaped tubulated body' (Weibel & Palade, 1964), or 'tubular body' (Ohsugi & Hirano 1977). RTB are normally occurring though often inconspicuous components of vascular endothelial cells. They were first reported in various organs of rats (Weibel & Palade, 1964) and have subsequently been found in a number of species including rabbits, frogs and humans (see Ghadially, 1975; Ohsugi & Hirano, 1977). However, to our knowledge, there are no reports of their occurrence in normal intracerebral vessels of rats. This may explain why they are not mentioned in the latest edition of the standard work on CNS fine structure (Peters *et al.* 1976).

They have however been found in normal human CNS although there is disagreement as to their frequency and prominence (Herlinger, Anzil, Blinzinger & Kronschi, 1974; Hirano, 1974; Hirano, Ghatak, Becker & Zimmerman, 1974; Ohsugi & Hirano, 1977). In pathological human CNS tissue, in particular in and around tumours, they are frequently increased in number, more pleomorphic and more conspicuous (Ohsugi & Hirano, 1977). The present report is the first recognition of their occurrence in MS brain. However, in 1976 Pathak & Webb described 'spherical masses 250–300 nm in diameter consisting of many twisted tubular structures 16–17 nm in diameter'. These were seen 'generally in the cytoplasm, particularly of the endothelial cells of blood vessels'. They did not perceive a clear limiting membrane round the bodies rather they saw either dense material or a clear space. They concluded that these spherical masses comprised clumps of 'paramyxovirus nucleocapsids . . . probably measles'. Having had the opportunity to inspect the originals of the published micrographs (courtesy of Drs Pathak & Webb) and to compare these with our own we conclude that the former are more likely to be cross sections of tubular bodies *sensu stricta*. There are three reasons for this conclusion. In the first place the measurements given of tubule size appear to be an underestimate if one is considering overall outer diameter. This would put the tubules outside the size range of the paramyxoviruses and into that of the microtubules found in 'tubular bodies'. Secondly, the absence of a 'clear' limiting membrane is, we believe, probably artefactual. There is in fact a sharp discontinuity in electron density at the outer margin of their 'clear space' and as in our Figure 7b the densely clumped cytoplasmic material grades into the dense, but fuzzy, membrane material. In the micrographs of Pathak & Webb the same effect is seen in parts of the plasmalemma and in the outer membrane of the nuclear envelope. The final piece of evidence is that the appearance, distribution and intracellular location of their bodies is in keeping with what we would expect if they were tubular bodies, i.e. in endothelial cells and near to inter-endothelial cell junctions. The significance of Pathak & Webb's intranuclear findings remains unclear.

Rod-shaped tubular bodies are believed to develop from saccules or cisternae of the golgi apparatus (Matsuda & Sugiura, 1970; Sengel & Stroebner, 1970; Hirano & Matsui, 1975; Ghadially, 1975) and it has been suggested that under some circumstances they may discharge their tubules into either the adluminal or the abluminal extracellular space (Kawamura & Kamijyo, 1976). In the endothelial cells of meningiomas, vacuolar RTB (like our Figure 7a) have been seen to fuse with each other and

with the plasma membrane to give rise to pores (Ohsugi & Hirano, 1977). Although the function of RTB is not known, there is some evidence implicating them in adrenalin-dependent blood coagulation enhancement (Burri & Weibel, 1968). Evidence to date does not support the view that RTB are generally increased in size or number in MS brains and in view of their normal occurrence it is unlikely that they are of any etiological significance in this disease.

## **Conclusions**

Careful examination of the evidence relating to the two basic kinds of inclusion described in this paper has revealed that they can be explained in terms of normal cell structure, albeit pathologically or artefactually altered. Thus we conclude that reticulosomes, previously reported from both MS and SSPE as papovavirions are actually 'stereoframework' artefacts formed from cytoplasmic proteins. We also suggest that cytoplasmic reticular filamentous aggregates previously described in MS and thought to resemble some small paramyxovirus nucleocapsids may be another example of a stereoframework artefact. Tubular bodies in endothelial cells, previously mistaken for paramyxoviruses are normally occurring subcellular organelles.

The results of this study and the history of EM research on MS to date prompt us to make some general remarks which may prove helpful. In view of the insensitivity of the electron microscope as a tool for searching for virus particles (Tanaka *et al.*, 1976; Koprowski, 1976) it is particularly important that when 'unusual' inclusions are found they should be recognized for what they are. The need for a sound, up to date and broad knowledge of normal and pathological cell structure cannot be over emphasized in this type of work. Account should also be taken of the possible effects on fine structure of postmortem change and the consequent sub-optimal fixation quality.

To avoid misleading other scientists, the medical profession in general and the general public, viral interpretations should only be seriously advocated when all other possibilities have received the fullest possible consideration. It should also be remembered that demonstration of a virus in MS brain would not in itself amount to a demonstration of a causal relationship with the disease (Gonatas *et al.*, 1967; Andrews & Andrews, 1973; Koprowski, 1976).

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