Chapter 7 Robert G. Milne

Immunoelectron Microscopy for Virus Identification

1 Introduction

I am going to discuss immunonegative staining—the immunoelectron microscopy (IEM) of virus (or other pathogen-related) particles in suspension—with only short excursions into the topic of immune reactions on or in thin sections, as these are considered elsewhere in this book. As the field has been thoroughly reviewed, what I shall say will be more an informal commentary than an exhaustive survey, and I shall not necessarily follow each statement by a precise citation, or attempt to mention all the interesting papers. In places I shall use the term "grid" to mean support film.

As all those know, who have worked with viruses both in negative stain and in thin sections, resolution is much higher in negative stain, and intensity and fidelity of immunolabeling is also greater; you would never work with sections again if they did not furnish positional information that is lost when you make an extract. This loss means that in negative stain you can only work with structures that can be recognized out of context—virus particles, subviral components, or certain virus-induced inclusions; perhaps also mycoplasmas or their fragments. However, sometimes the same structures can be recognized both in vitro and in situ; and with the advantage of immune labeling we can, with luck, identify an antigen in both contexts. In that case we can obtain both positional or contextual information as well as high-resolution details of structure or antigen location. An interesting technique that I will not discuss, but which may prove a valuable compromise, is immunonegative staining of thin cryosections.

1.1 The early days

As our symposium was held to celebrate 50 years of electron microscopy in plant pathology, and also in order to place today's methods in context, I would like to

Istituto di Fitovirologia Applicata CNR, Strada delle Cacce 73, I-10135 Torino, Italy

K. Mendgen et al. (eds.), *Electron Microscopy of Plant Pathogens*
© Springer-Verlag Berlin Heidelberg 1991

remind you of some historical IEM landmarks. These were often the occasion for interesting reviews such as those of Williams (1954) [77], Valentine (1961) [71], Kleczkowski (1961) [31], Horne and Wildy (1963) [27], Lafferty and Oertelis (1963) [35], Horne (1967) [26], Almeida and Waterson (1969) [1], Ball (1971) [3], Doane and Anderson (1977) [17], and Milne and Luisoni (1977) [53]. There are also more recent commentaries [5, 29, 32, 34, 36, 47, 48, 51, 61, 62, 74].

It all began when two independent teams [2, 64] examined mixtures of tobacco mosaic virus and its antiserum in the electron microscope. These preparations were without benefit of added contrast, and the microscopes were not very advanced, but the already known rod-shaped particles were seen to be specifically clumped and to be approximately doubled in thickness.

Eighteen years passed before reports appeared of imaging individual antibodies attached to virus particles. The preparations in question were contrasted by metal shadowing, and clearly this technique did not offer sufficiently rewarding results. Valentine in 1961 [71] discusses negative staining of viruses and mentions antibodies, but not until 1962 were negatively stained virus-antibody complexes reported [e.g., 28]. This kind of work culminated in the review of Almeida and Waterson [1].

2 Leaf-Dip Serology

Leaf-dip serology [3, 4] became popular with plant virologists because it was simple and reliably demonstrated serological reactions in the electron microscope by negative staining. However, the method was inherently flawed because it consisted in mixing together a sap extract from an infected leaf, the antiserum diluted in buffer, and the negative stain. Conditions were made worse because of two factors. First, the sap-antiserum mixture was dried on the grid before being negatively stained, and as we know now, negative stain helps to support structures as they dry, minimizing distortion and, especially, flattening. This is not to mention the sticky mess resulting from drying even diluted plant sap and serum down on the support film.

Secondly, the serological reaction was reported to work much better in phosphate buffer (titer four two-fold steps higher) than in ammonium acetate buffer, but the latter was used since it sublimes on drying, whereas phosphate leaves crystalline deposits. A third factor working against the system was the use of PTA (neutralized phosphotungstic acid), although this was mixed with vanadomolybdate. As was then already known in part [20], PTA disrupts the particles of some viruses, especially those held together by protein-nucleic acid interactions (examples: alfalfa mosaic, cucumber mosaic, geminiviruses) or those containing lipid (rhabdoviruses, spotted wilt). PTA may also give poor stain distribution and lower resolution than some other stains such as uranyl acetate [47, 61, 62].

However, Ball and Brakke were able to demonstrate relatively clear results; moreover they used the method to titrate antisera—the first quantitative use of electron microscopy in this way.

3 Immune Complexes from Ouchterlony Plates

Meanwhile, in another part of the forest, a small but interesting development took place. Agar gel diffusion has long played a major part in virus diagnostics and titration of antisera, and the technique of cutting out the precipitin band, crushing the agar in a little buffer, and negative staining the immune complexes released has been reinvented several times. The first to describe the method were Watson et al. in 1966 [76]. Examples from our laboratory were the finding that whole closterovirus particles (grapevine virus A, 800 nm modal length) could migrate through agar to form a clear band [12], and the separation of the spherical particles of white clover cryptic viruses 1 and 2 to form two distinct bands, using a mixed virus preparation and a mixed antiserum (Figs. 1–3); [E. Luisoni and R.G. Milne unpubl.; 8, 9].

3.1 Clumping

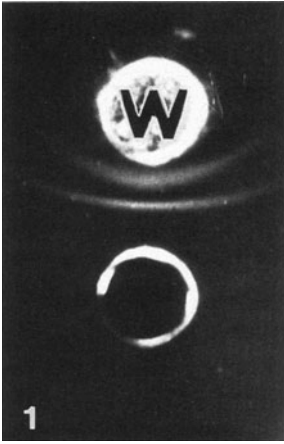
As we have seen, the classical approach to immunoelectron microscopy in vitro was to mix antigen and antibody, and detect the resulting clumps, which resemble those in Figs. 2 and 3 [see e.g., 50]. This method is little used now by plant virologists, as the clumping effect is sometimes nonspecific, especially if the preparation has been pelleted and resuspended. Moreover, it requires relatively large concentrations of virus to be effective.

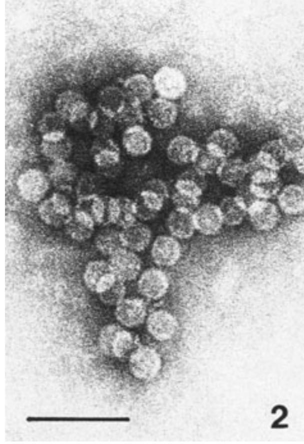
4 ISEM

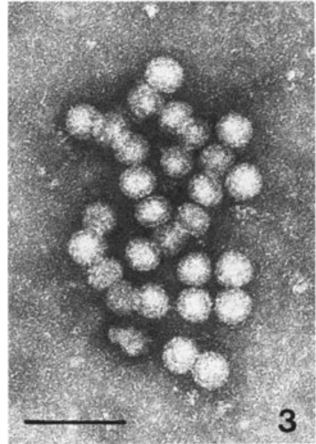
In 1973 K.S. Derrick published his "serologically specific electron microscopy" method [14; see also 6, 16, 24, 59], which was later simplified, improved, and renamed more appropriately as immunosorbent electron microscopy (ISEM) [38, 39, 46, 53, 57, 63].

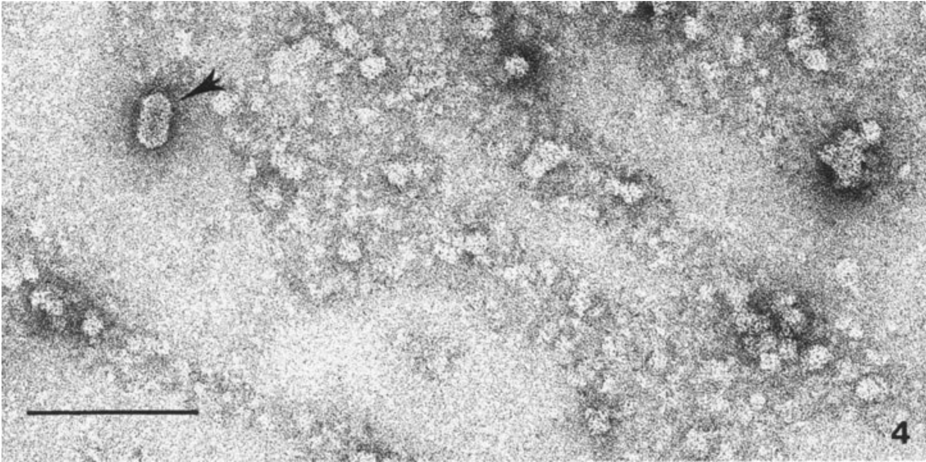
In this method, the grid is coated with a dilution of the antiserum, and rinsed; there follows a trapping phase in which the virus preparation is incubated with the grid, and virus particles are bound (Figs. 4 and 5). Even perhaps more important, [39], the layer of antibody and other serum constituents inhibits non-specific binding of structures, other than the viral antigen, that may be in the preparation. The result is selective immuno-purification of the virus on the grid.

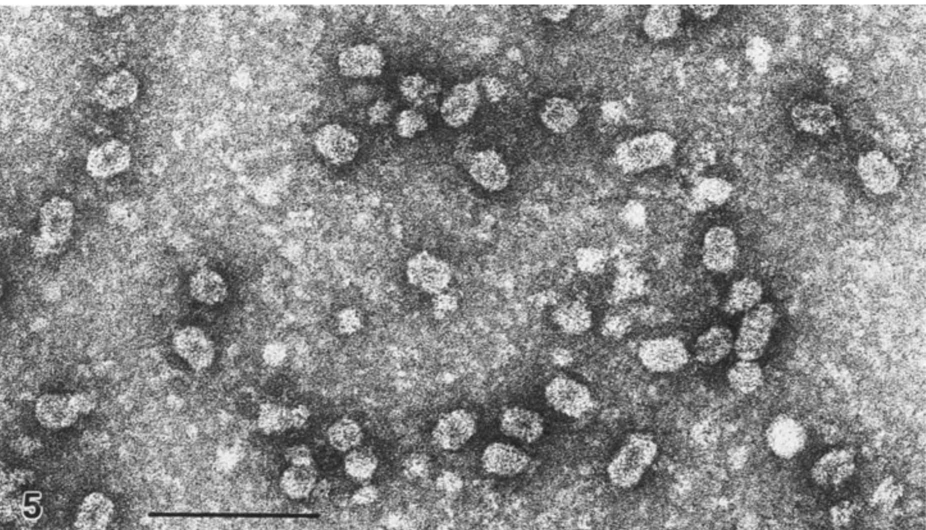
It was also made clear [39] that when crude virus preparations are absorbed to grids without the benefit of ISEM, host constituents generally compete effectively with virus particles for an anchored site on the support film, so that many virus particles in the preparation are not retained on the grid. For similar reasons, coating grids with undiluted antiserum or indeed serum diluted less than about 1/1000 can inhibit efficient trapping, probably because with less diluted serum, proteins other


W
1


2


3


4


5

than the trapping antibodies preferentially occupy available sites on the support film [50].

In ELISA, antiserum coating times are of the order of 60 min or more, but with the strictly equivalent grid coating of the ISEM procedure, coating times of 5 min at room temperature have been shown to bind nearly all the effective antibody that is going to become attached [see 49]. This curiosity has not adequately been explained. However, with virus adsorption, the kinetics are different, and significantly more virus may continue to be trapped for several hours. Trouble may come if the virus particles detach or become degraded during long incubations (say, over 4 h). We routinely trap virus for only 15 min, while realizing that more could be had with longer incubation, if necessary.

Standard conditions will often give a satisfactory response with ISEM, but to optimize the system, a number of buffers, ionic strengths, and pH values should be tested, for both the antibody coating and virus trapping steps [11]. Additives such as EDTA, reducing agents, polyethylene glycol, or polyvinylpyrrolidone should also be considered.

The sensitivity of ISEM is generally comparable to that of ELISA, and it may detect 0.1–10 ng/ml of virus in volumes of a few μl [see 61]. Where the particle is especially stable or conspicuous, or where ELISA backgrounds are troublesome, ISEM can be appreciably more sensitive than ELISA [see e.g., 45]; in other cases ISEM has been considerably less sensitive, probably because free viral coat protein in the preparation competes in binding to the antibody-coated grid [46, 74].

An undeniable problem with ISEM for mass screening is its labor-intensive nature compared with ELISA. An advantage that hardly needs promoting to this readership is: seeing is believing. Just a very few virus particles observed on the grid give a clear positive result; this avoids the ELISA dilemma of trying to decide whether a given low level of absorbance above background is positive or not, and also avoids the requirements for a number of controls necessary to the ELISA test.

◄

Figs. 1–3. A gel double-diffusion plate (**1**, × 4) showing two bands resulting from interaction of a mixture (*upper well W*) of white clover cryptic viruses 1 and 2 (WCCV1, WCCV2) and an antiserum to both viruses (*lower well*). The bands from this plate were each cut out and crushed in buffer; material from the extracts was then adsorbed to grids and negatively stained in uranyl acetate. The lower band contained exclusively clumps of the more rapidly diffusing WCCV1 (ca, 30 nm in diameter **2**) and the upper band contained only clumps of WCCV2 (ca, 38 nm in diameter, **3**). Note that the relative positions of the bands also depends on the antigen/antibody ratios used in the test. Bars = 100 nm. **1** courtesy of Dr. E. Luisoni

Figs. 4 and **5.** Crude sap of a plant infected with Ourmia melon virus (OuMV) was adsorbed for 15 min to an untreated grid (**4**) or a grid precoated with OuMV antiserum diluted 1/1600, for 5 min (**5**). With limiting amounts of virus, the difference in particle counts was of the order of 10000 times [41]. The one particle found on a 400-mesh grid square (**41**) is *arrowed*. Bar = 100 nm

4.1 Protein A-ISEM

It had been known for some time that protein A had the specific capacity to bind to the Fc portion or "tail" of certain IgG's, but Shukla and Gough in 1979 [23, 65] were the first to use this property in the context of ISEM, and show that trapping efficiencies could be thereby improved. When a grid coated with protein A is incubated with antiserum, the IgG's are trapped with their active Fab portions exposed. Thus we have both selection and orientation. Experiments in several laboratories (see reviews cited above) have shown that in practice the increased sensitivity obtainable with protein A (increase in numbers of particles trapped from a given preparation by a given antiserum) is really significant only when the concentration of virus is not limiting.

However, using protein A we can largely overcome the inhibitory effect of using, for coating, antisera that have not been highly diluted. The ability to use less diluted sera means that those of low titer can be used more effectively. (A similar result is obtained if the IgG fraction is isolated and used for coating). A second benefit is that a wider spectrum of heterologous antigens can be detected by ISEM using protein A [38, 75].

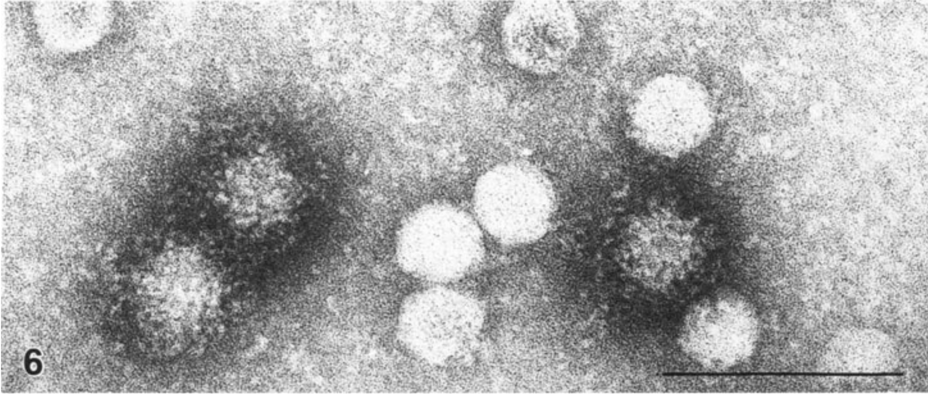
4.2 ISEM with dsRNA

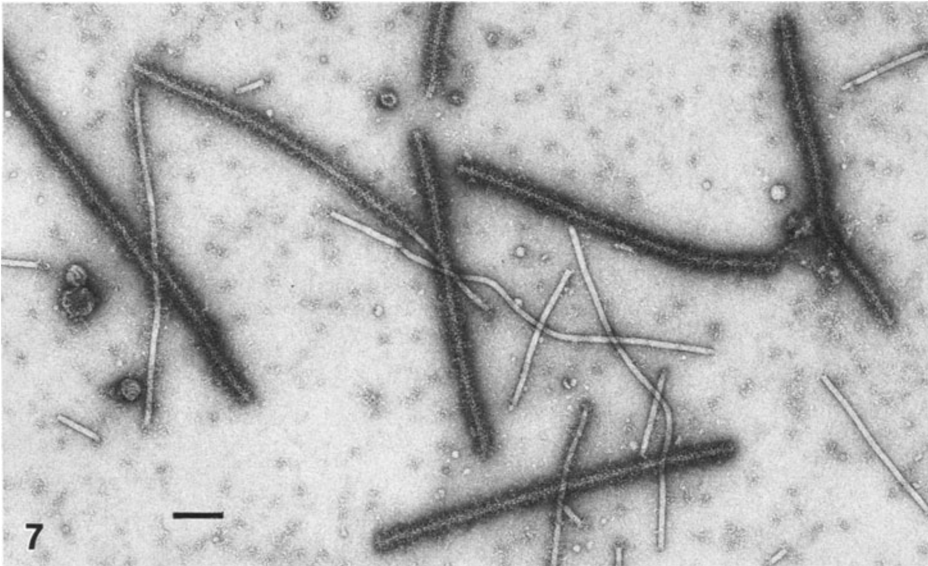
Derrick [15] extended the ISEM technique to the detection of double-stranded RNAs. The method has been used to detect circular dsRNAs in viroid-infected plants [21], and to trap a dsRNA-containing mycovirus from *Agaricus bisporus* [13]. In our laboratory, some preliminary attempts to trap dsRNA's were not encouraging, but further work should be done, as there is reason to believe the method can work.

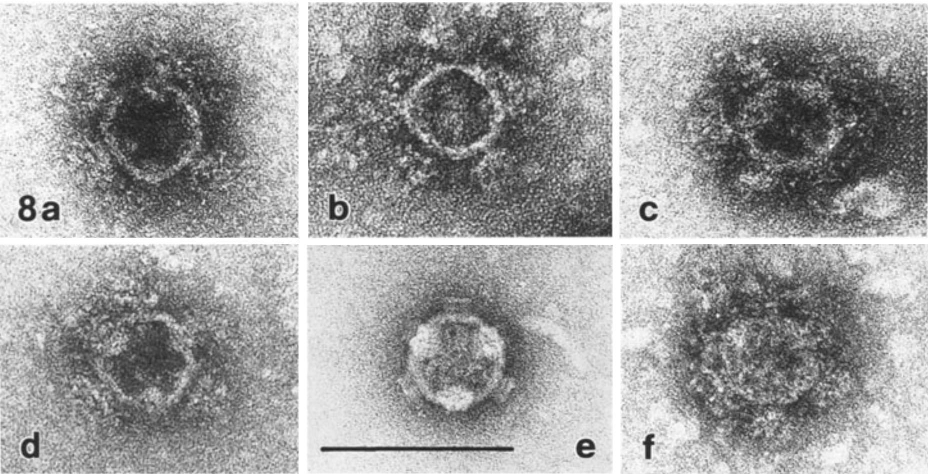
Fig. 6. Mixture of purified tobacco necrosis virus (TNV) and tomato bushy stunt virus (TBSV) absorbed to the grid, then treated with a saturating level of antibodies to TBSV. The TNV particles have remained clean, with sharp outlines, although antibody molecules are seen in the background. Bar = 100 nm

Fig. 7. A natural mixture of potyviruses from the wild European perennial cucurbit *Bryonia cretica*. The decorated particles have reacted with an antiserum to "white bryony mosaic virus"—supposed at that time to have been a carlavirus [see 55]. Note that one particle (near the center) is longer than normal and is free of antibody in part; such particles were not infrequent and probably arose by end-to-end aggregation of two virus particles from a mixed infection (or fragments of them) bearing different coat proteins. The phenomenon, detected only by EM decoration tests, also occurs with closteroviruses and may be responsible for anomalous transmission of one virus by vectors normally specific for another [see 56]. Bar = 100 nm

Fig. 8. a–f A panel of maize rough dwarf virus (Reoviridae) B-spiked subviral particles. All but the particle in **e**, which was untreated, were decorated with antiserum specific for the B spikes. Bar = 100 nm


6


7


8a
b
c
d
e
f

5 Decoration

The clear advantages but also the evident defects of leaf-dip serology led Milne and Luisoni [52, 53] to develop the decoration method, only to discover later that they had been elegantly preempted by two groups working on bacteriophage structural proteins [70, 78]. In this method, the virus particles (or other structures) are first absorbed to the grid under whatever conditions are optimal for that process, and the grid is rinsed (a) to remove unwanted salts, proteins, sugars, impurities or buffers, and (b) to set up conditions which are optimal for the next phase: attachment of antibody to the virus. After antibody attachment, the grid is again rinsed, and then stained, usually with uranyl acetate. By separating and optimizing each step, much clearer, more sensitive, and more consistent results are obtainable, and since each virus particle is, ideally, well separated from the others, interpretation of the image is simplified (Figs. 6–8).

Uranyl acetate has worked satisfactorily in our hands, although the pH (about 4.2) seems close to that which would cause antigen-antibody separation. However, fixing the reacted particles with glutaraldehyde before applying uranyl acetate does not make any difference. It is interesting that a final rinse of the particles, before staining, with very slightly acidified distilled water, will immediately remove the decorating antibody—which however remains in place if the decorated particles have been fixed.

Decoration has become a popular method of identifying plant viruses, mainly because the result is (or should be) unequivocal and direct (Figs. 6, 7). Of all serological methods it is one of the most easily and safely interpreted because you see the virus particle and you see the antibody attached to it—and where the antibody is attached. False positives and false negatives are rare, though with some viruses and especially with poor or preliminary antisera, clear interpretation may not be possible in the murky preparations that result.

The localization of proteins on the surface of virus particles has been elegantly demonstrated by decoration in a number of cases. Yanagida and Ahmad-Zadeh [78] determined the position of certain gene products on the capsid of phage T4, and similar beautiful work was done by Tosi and Anderson [70] with phage 29. Luisoni et al. [44], as part of the serological analysis of the capsids of Fijiviruses (Reoviridae), showed that the B spikes and the inner capsids could be decorated with specific antisera (Fig. 8). Fukuda et al. [22] demonstrated the initiation of TMV rod assembly near the 3′ terminus of the RNA, by beginning encapsidation with the protein of one strain and completing it with the serologically different protein of a second strain. A similar approach earlier allowed Otsuki and Takebe [58] to demonstrate mixedly coated virus particles in protoplasts doubly infected by strains of TMV.

5.1 ISEM Plus Decoration

A natural step, once ISEM and decoration were established techniques, was to combine them [53], since ISEM cleans and concentrates the virus, presenting an optimized field for the decoration step (Figs. 10–12). Even where the numbers of virus particles are not limiting, ISEM is a very useful preliminary to decoration [see for example 54].

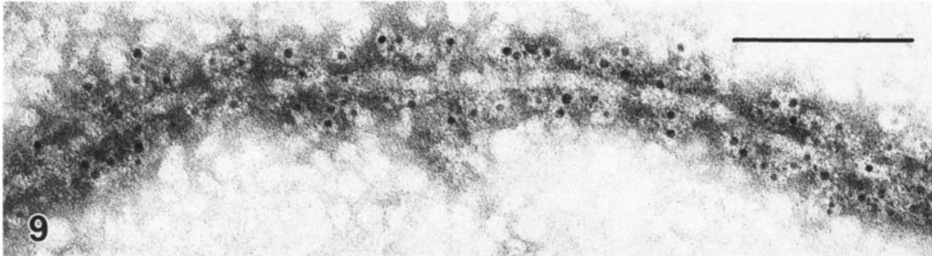
A further advantage of combining ISEM with decoration is that different antibodies can if desired be used for each phase. For example, a mixed antiserum to white clover cryptic viruses 1 and 2 was employed to trap both viruses from the crude sap of carrier plants, and antiserum to virus 2 was then used to decorate that virus but not the other, and show them to be distinct [8]. Similarly, a mixture of three cryptic viruses from hop trefoil (*Medicago lupulina*) was differentiated, although not all the viruses could be separated, and not all the relevant monovalent antisera were available [43]. Fukuda et al., referred to above [22], also trapped their mixedly coated TMV particles by ISEM before decorating them with the differentiating antisera.

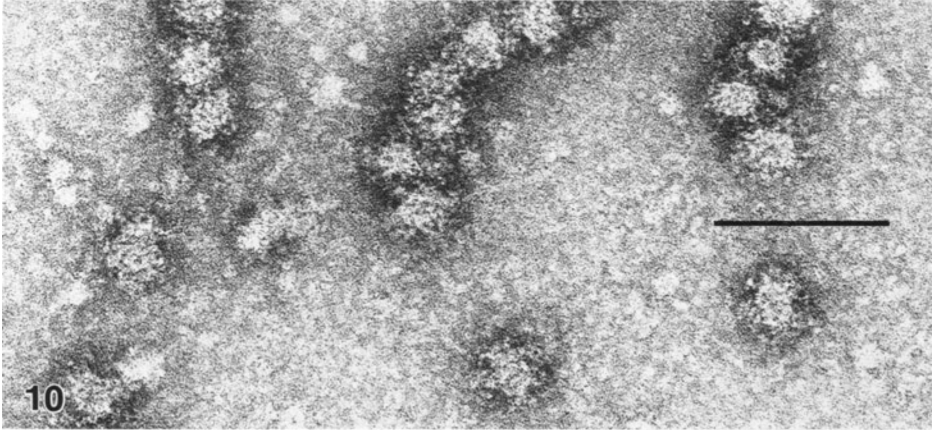
Despite the above, the decoration technique is often (even usually) done badly, and then results are both less beautiful and less informative. The main problems (apart from human factors) may be among the following: poor antisera, insufficiently thorough washing steps, poor support films (e.g., use of uncarboned plastic films), use of PTA, drying of the grid before negative staining, or even persistence in the use of the classical but outmoded leaf-dip serology. Roberts [61, 62] has good advice on how to avoid these and other pitfalls.

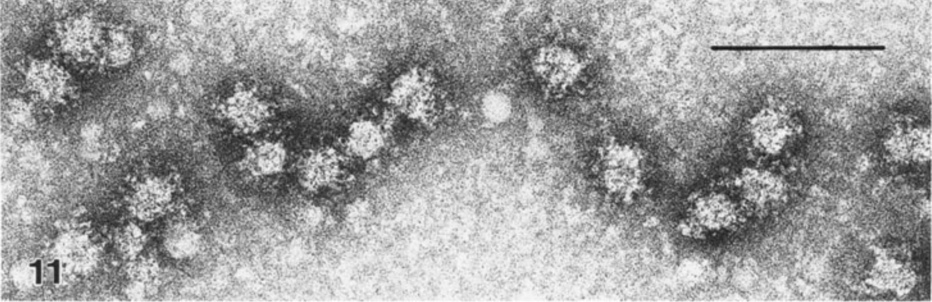
One problem common to all serological tests, but sometimes easy to overcome using glutaraldehyde fixation, is that of antigenic change in the virus particle. It has become increasingly clear that viruses, especially perhaps filamentous ones, may be subject to partial hydrolysis in vitro or even in vivo, with consequences for the preparation of antisera and for their subsequent use. Shukla et al. [66, 67] have described the all too easy removal of the antigenically specific N-terminal end of potyvirus coat proteins, whereas Koeing et al. [33] described similar hydrolysis of the coat protein of a potexvirus. In vivo coat protein hydrolysis can be responsible for failures and anomalous results in the detection of bean yellow mosaic potyvirus in gladiolus [69], and antigen breakdown has been described in a mite-transmitted potyvirus [37]. Such problems are more likely with long incubation times involving unrinsed sap preparations, and may be responsible for the patchy or inconsistent appearance of decoration sometimes reported.

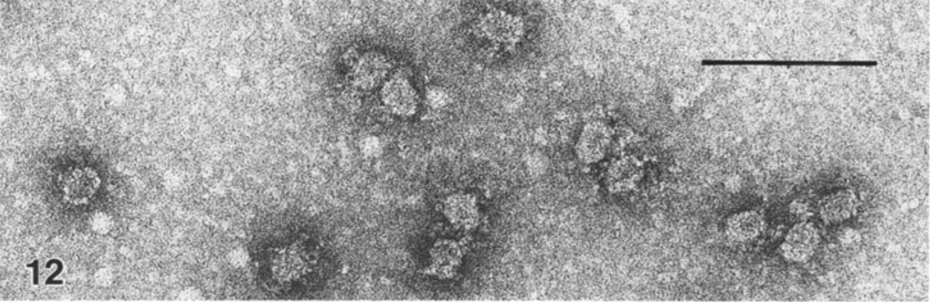
5.2 Quantitative Decoration

As we have seen, Ball and Brakke [3, 4] used leaf-dip serology to titrate antisera. The decoration method, being an improvement on leaf-dip serology, facilitates such


9


10


11


12

titration (Figs. 10–12), which can be done, from preparation to observation, easily within one hour [49]. Yet, unfortunately, the number of workers using decoration quantitatively are few whereas those using it as a yes/no measure of relationship are many.

5.3 Double Decoration

If the first decorating antibody (say, rabbit anti-plum pox virus) is followed by a second (this could be sheep anti-rabbit IgG), and the complex is negatively stained, then the particles, originally thin, pale and difficult to see at low magnification, become thickened and blackened so that they are easily noted, even at only 5000 × enlargement [30]. The stain penetrates among the attached antibodies and gives the virus particle a highly contrasted coat. The method is useful for rapid diagnosis, especially by unskilled electron microscope operators or those working with an old or low-performance instrument.

5.4 Gold Labeling

As noted by Cristoforo Colombo, "Gold is the most exquisite of things. Whoever possesses gold can acquire all that he desires in the world. Truly, for gold he can gain entrance for his soul into paradise". While electron microscopists might express themselves differently, we can see what he means. Some examples of decoration enhanced by the use of gold-labeled antibodies can be cited [5, 7, 18, 19, 25, 40, 42, 60, 72, 73].

Generally, the approach has been to decorate the antigen with the primary antibody (say, rabbit anti-virus) and follow this with either a second, gold-labeled antibody (for example, 5 nm gold-goat anti-rabbit) (Fig. 9), or with gold-labeled protein A. Louro and Lesemann [42] noted that gold labeling could carry the decoration titer four twofold dilution steps higher than was possible without the gold, although in our laboratory we would claim an increase of only two twofold steps.

Apart from the increase in sensitivity, however, the gold label has the great advantage of being easily and exactly identifiable, whereas what constitutes a trace

◄

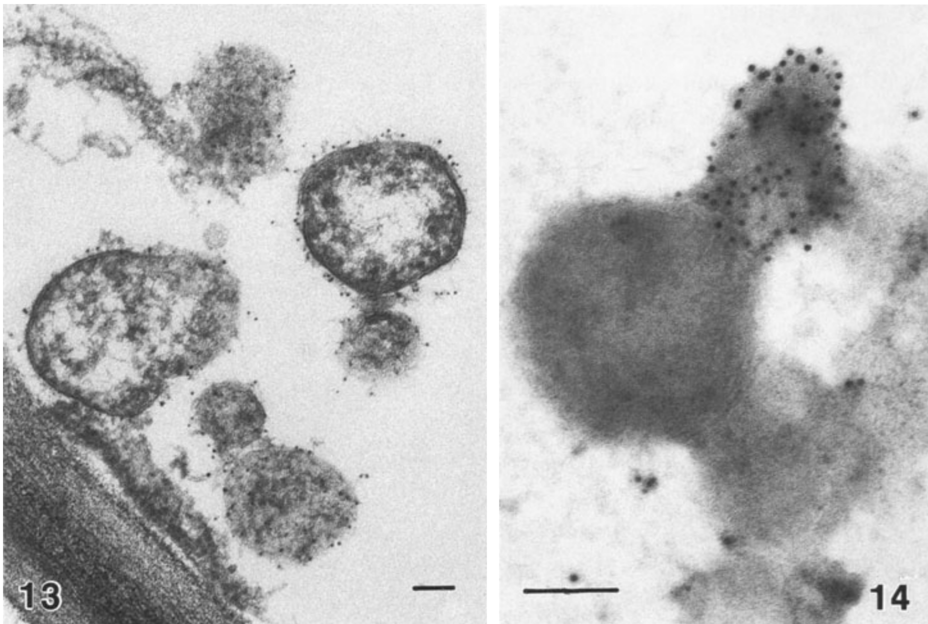
Fig. 9. A plum pox potyvirus particle adsorbed from infected apricot leaf sap (without ISEM) after extraction in buffer containing 2% polyvinylpyrrolidone (M_r 40 000) as protectant. The particles were decorated with a 1/2000 dilution of antiserum, followed by gold labeling using a 1/100 dilution of goat anti-rabbit 1gG-5nm gold (Janssen). Bar = 100 nm

Figs. 10–12. Preparation of OuMV particles trapped by ISEM as in Fig. 5, then decorated with different dilutions of the same antiserum: 1/32 (**10**), 1/256 (**11**) and 1/512 (**12**). The decoration titer of this antiserum was 1/1024. Bar = 100 nm

of unlabeled decorating antibody could be a matter of opinion, and certainly becomes so if the preparation and electron microscopy are not of a high standard. Where particular, localized epitopes are being identified, as, for example, those exposed at one end of TMV rods, only a very few antibody units (perhaps one) can attach to the site, and this is an ideal situation in which to exploit gold labeling [18]. GLAD (gold-labeled antibody decoration) has been effective in screening monoclonal antibody-secreting clones for antibodies to plum pox virus [25].

6 Mycoplasma-Like Organisms

Antibodies against MLO's, both monoclonal and polyclonal, are now becoming available, and methods are being developed to exploit such antibodies in taxonomy and diagnostics. Corn stunt spiroplasma has been detected by ISEM [16], and since then a number of MLO's have been revealed by this method [see 68]. We have used a



Figs. 13 and **14.** Gold labeling (5 nm gold-goat antimouse) of primula yellows MLO in thin sections and in vitro with a monoclonal antibody from M.F. Clark. **13** shows pre-embedding labeling of glutaraldehyde-fixed phloem tissue, followed by osmium fixation and classical Epon embedding. **14** shows a partially purified MLO preparation osmotically lyzed, trapped by ISEM, and decorated with gold. A highly labeled fragment (presumably MLO tissue) is seen together with unlabeled (presumed host) materials. 'Healthy' preparations gave no labeling above the rather low background. Bars = 100 nm

monoclonal from M. F. Clark [10] to label primula yellows MLO in thin sections (Fig. 13) using a pre-embedding labeling technique (R. G. Milne and R. Lenzi unpubl.) and have also labeled MLO fragments trapped by ISEM from partially purified primula yellows preparations (R. G. Milne and R. Lenzi unpubl.; Fig. 14).

Gold labeling of MLO's using embedding and sectioning methods is discussed elsewhere in this book. Our attempt to trap and label MLO fragments directly on grids was successful (Fig. 14), but the method requires further development, and we had less success in trapping intact MLO bodies. We need to be able to trap morphologically recognizable MLO's, not only fragments, and of course gold-label them convincingly. The technique should then become very useful, as it will be simple and not take more than an hour or two to perform.

7 Conclusion

This review has left many topics unexamined, but has attempted to touch on the major ones, and to discuss some areas where improvement is possible or progress is being made. My overall impression, however, is that immunoelectron microscopy of in vitro preparations (as opposed to thin sectioning) needs a new stimulus. It comprises a very useful, and indeed often used, collection of techniques, but they have become routine. I hope that this conference and similar ones will, as often happens, make contacts and produce stimuli that lead to something new.

Acknowledgment. I thank Vera Masenga and Riccardo Lenzi for technical assistance with some of the work described, and Dr. Michael Clark for MLO-infected plants and anti-MLO antibodies.

References

1. Almeida JD, Waterson AP (1969) The morphology of virus-antibody interaction. Adv Virus Res 15: 307–338
2. Anderson FA, Stanley WM (1941) A study by means of the electron microscope of the reaction between tobacco mosaic virus and its antiserum. J. Biol Chem 139: 339–344
3. Ball EM (1971) Leaf-dip serology. Methods Virol 5: 445–450
4. Ball EM, Brakke MK (1968) Leaf-dip serology for electron microscopic identification of plant viruses. Virology 36: 152–155
5. Beesley JE, Betts MP (1987) Colloidal gold probes for the identification of virus particles: an appraisal. Micron Microsc Acta 18: 299–305
6. Beier H, Shepherd RJ (1978) Serologically specific electron microscopy in the quantitative measurement of two isometric viruses. Phytopathology 68: 533–538
7. Bingren Wu, Mahoney J, Chernesky M (1989) Comparison of three protein A-gold immune electron microscopy methods for detecting rotaviruses. J Virol Methods 25: 109–118
8. Boccardo G, Milne RG, Luisoni E, Lisa V, Accotto GP (1985) Three seedborne cryptic viruses containing double-stranded RNA isolated from white clover. Virology 147: 29–40

9. Boccardo G, Lisa V, Luisoni E, Milne RG (1987) Cryptic plant viruses. Adv Virus Res 32: 171–214
10. Clark MF, Morton A, Buss SL (1989) Preparation of mycoplasma immunogens from plants and a comparison of polyclonal and monoclonal antibodies made against primula yellows MLO-associated antigens. Ann Appl Biol 114: 111–124
11. Cohen J, Loebenstein G, Milne RG (1982) Effect of pH and other conditions on immunosorbent electron microscopy of several plant viruses. J Virol Method 4: 323–330
12. Conti M, Milne RG, Luisoni E, Boccardo G (1980) A closterovirus from a stem-pitting diseased grapevine. Phytopathology 70: 394–399
13. Del Vecchio VG, Dixon C, Lemke PA (1979) Immune electron microscopy of virus-like particles of *Agaricus bisporus*. Exp Mycol 2: 138–144
14. Derrick KS (1973) Quantitative assay for plant viruses using serologically specific electron microscopy. Virology 56: 652–653
15. Derrick KS (1978) Double-stranded RNA is present in extracts of tobacco plants infected with tobacco mosaic virus. Science 199: 538–539
16. Derrick KS, Brlansky RH (1976) Assay for viruses and mycoplasmas using serologically specific electron microscopy. Phytopathology 66: 815–820
17. Doane FW, Anderson N (1977) Electron and immunoelectron microscopic procedures for diagnosis of viral infections. In: Kurstak E, Kurstak C (eds) Comparative diagnosis of viral diseases Vol 11. Academic Press, New York, pp 505
18. Dore I, Weiss E, Altschuh D, van Regenmortal MHV (1988) Visualization by elecrtron microscopy of the location of tobacco mosaic virus epitopes reacting with monoclonal antibodies in enzyme immunoassay. Virology 162: 279–289
19. El Ghorr AA, Snodgrass DR, Scott FMM (1988) Evaluation of an immunogold electron microscopy technique for detecting bovine coronavirus. J Virol Methods 19: 215–224
20. Francki RIB, Randles JW, Chambers TC, Wilson SB (1966) Some properties of purified cucumber mosaic virus (Q strain). Virology 28: 729–741
21. French RC, Price MA, Derrick KS (1982) Circular double-stranded RNA in potato spindle tuber viroid-infected tomatoes. Nature Lond 295: 259–260
22. Fukuda M, Okada Y, Otsuki Y, Takebe I (1980) The site of initiation of rod assembly on the RNA of a tobacco and a cowpea strain of tobacco mosaic virus. Virology 101: 493–502
23. Gough KH, Shukla DD (1980) Further studies on the use of protein A in immune electron microscopy for detecting virus particles. J Gen Virol 51: 415–419
24. Hamilton RI, Nichols C (1978) Serological methods for detection of pea seed-borne mosaic virus in leaves and seeds of *Pisum sativum*. Phytopathology 68: 539–543
25. Himmler G, Brix U, Steinkelner H, Laimer M, Mattanovich D, Katinger HWD (1988) Early screening for anti-plum pox virus monoclonal antibodies with different epitope specificities by means of gold-labelled immunosorbent electron microscopy. J Virol Methods 22: 351–358
26. Horne RW (1967) Electron microscopy of isolated virus particles and their components. Methods Virol 3: 521–574
27. Horne RW, Wildy P (1963) Virus structure revealed by negative staining. Adv Virus Res 10: 101–170
28. Hummeler K, Anderson TF, Brown R (1962) Identification of poliovirus particles of different antigenicity by specific agglutination as seen in the electron microscope. Virology 16: 84–90
29. Katz D, Kohn A (1984) Immunosorbent electron microscopy for detection of viruses. Adv Virus Res 29: 169–194
30. Kerlan C, Mille B, Dunez J (1981) Immunosorbent electron microscopy for detecting apple chlorotic leafspot and plum pox viruses. Phytopathology 71: 400–404
31. Kleczkowski A (1961) Serological behaviour of tobacco mosaic virus and of its protein fragments. Immunology 4: 130–141
32. Koenig R (1988) Serology and Immunochemistry. In: Milne RG (ed) The filamentous plant viruses. Plenum, New York, pp 111

33. Koenig R, Tremaine JH, Shepard F (1978) In situ degradation of the protein chain of potato virus X at the N- and C-termini. J Gen Virol 38: 329–337
34. Kojima M, Tyng-guang Chou, Shikata E (1978) Rapid diagnosis of potato leaf roll virus by immune electron microscopy. Ann Phytopathol Soc Jpn 44: 585–590
35. Lafferty KJ, Oertelis S (1963) The interaction between virus and antibody III. Examination of virus-antibody complexes with the electron microscope. Virology 21: 91–99
36. Lange L (1986) The practical application of new developments in test procedures for the detection of viruses in seed. In: Jones RAC, Torrance L (eds) Developments and Applications in Virus Testing. Association of Applied Biologists, Wellesbourne, Warwick, pp 269
37. Langenberg WG (1989) Rapid antigenic modification of wheat streak mosaic virus in vitro is prevented in glutaraldehyde-fixed tissue. J Gen Virol 70: 969–973
38. Lessmann DE, Paul HL (1980) Conditions for the use of protein A in combination with the Derrick method of immuno electron microscopy. Acta Hortic 110: 119–128
39. Lesemann DE, Bozarth RF, Koenig R (1980) The trapping of tymovirus particles on electron microscope grids by adsorption and serological binding. J Gen Virol 48: 257–264
40. Lin NS (1984) Gold-IgG complexes improve the detection and identification of viruses in leaf dip preparations. Methods Virol 8: 181–190
41. Lisa V, Milne RG, Accotto GP, Boccardo G, Caciagli P (1988) Ourmia melon virus, a virus from Iran with novel properties. Ann Appl Biol 112: 291–302
42. Louro D, Lesemann DE (1984) Use of protein A-gold complex for specific labelling of antibodies bound to plant viruses I. Viral antigens in suspensions. J Virol Methods 9: 107–122
43. Luisoni E, Milne RG, Accotto GP, Boccardo G (1987) Cryptic viruses in hop trefoil (*Medicago lupulina*) and their relationship to other cryptic viruses in legumes. Intervirology 28: 144–156
44. Luisoni E, Milne RG, Boccardo G (1975) The maize rough dwarf virion II. Serological analysis. Virology 68: 86–96
45. Luisoni E, Milne RG, Roggero P (1982) Diagnosis of rice ragged stunt virus by enzyme-linked immunosorbent assay and immunosorbent electron microscopy. Plant Dis 66: 929–932
46. Milne RG (1980) Some observations and experiments on immunosorbent electron microscopy of plant viruses. Acta Hortic 110: 129–135
47. Milne RG (1984) Electron microscopy for the identification of plant viruses in in vitro preparations. Methods Virol 7: 87–120
48. Milne RG (1986) New developments in electron microscope serology and their possible applications. In: Jones RAC, Torrance L (eds) Developments and applications in virus testing. Association of Applied Biologists, Wellesbourne, Warwick, pp 179
49. Milne RG (1988) Quantitative use of the electron microscope decoration technique for plant virus diagnostics. Acta Hortic 234: 321–329
50. Milne RG, Lesemann DE (1978) An immunoelectron microscopic investigation of oat sterile dwarf and related viruses. Virology 90: 299–304
51. Milne RG, Lesemann DE (1984) Immunosorbent electron microscopy in plant virus studies. Methods Virol 8: 85–101
52. Milne RG, Luisoni E (1975) Rapid high-resolution immune electron microscopy of plant viruses. Virology 68: 270–274
53. Milne RG, Luisoni E (1977) Rapid immune electron microscopy of virus preparations. Methods Virol 6: 265–281
54. Milne RG, Masenga V, Conti M (1986) Serological relationships between planthopper-vectored cereal rhabdoviruses. Intervirology 25: 83–87
55. Milne RG, Masenga V, Lovisolo O (1980) Viruses associated with white bryony (*Bryonia cretica* L.) mosaic in northern Italy. Phytopathol Mediterr 19: 115–120
56. Murant AF, Raccah B, Pirone TP (1988) Transmission by vectors. In: Milne RG (ed) The filamentous plant viruses. Plenum, New York, pp 237

57. Nicolaïeff A, van Regenmortel MHV (1980) Specificity of trapping of plant viruses on antibody-coated electron microscope grids. Ann Virol (Inst Pasteur) 131E: 95–110
58. Otsuki Y, Takebe I (1978) Production of mixedly coated particles in tobacco mesophyll protoplasts doubly infected by strains of tobacco mosaic virus. Virology 84: 162–171
59. Paliwal YC (1977) Rapid diagnosis of barley yellow dwarf virus in plants using serologically specific electron microscopy. Phytopathology Z 89: 25–36
60. Pares RD, Whitecross MI (1982) Gold-labelled antibody decoration (GLAD) in the diagnosis of plant viruses by immunoelectron microscopy. J Immunol Methods 51: 23–28
61. Roberts IM (1986a) Immunoelectron microscopy of extracts of virus-infected plants. In: Harris JR, Horne RW (eds) Electron microscopy of proteins, Vol 5, Viral structure. Academic Press, New York, pp 292
62. Roberts IM (1986b) Practical aspects of handling, preparing and staining samples containing plant virus particles for electron microscopy. In: Jones RAC, Torrance L (eds) Developments and applications in virus testing, Association of Applied Biologists, Wellesbourne, Warwick, pp 213
63. Roberts IM, Harrison BD (1979) Detection of potato leafroll and potato mop-top viruses by immunosorbent electron microscopy. Ann Appl Biol 93: 289–297
64. Schramm G, Friedrich-Freksa N (1941) Die Präcipitinreaktion des Tabakmosaikvirus mit Kaninchen und Schweineantiserum. Z Physiol Chem 270: 233–246
65. Shukla DD, Gough KH (1979) The use of protein *A* from *Staphylococcus aureus* in immune electron microscopy for detecting plant virus particles. J Gen Virol 45: 533–536
66. Shukla DD, Ward CW (1989) Structure of potyvirus coat proteins and its application in the taxonomy of the potyvirus group. Adv Virus Res 36: 273–314
67. Shukla DD, Jilka J, Tosic M, Ford RE (1989) A novel approach to the serology of potyviruses involving affinity-purified polyclonal antibodies directed towards virus-specific N-termini of coat proteins. J Gen Virol 70: 13–23
68. Sinha RC (1988) Serological detection of mycoplasmalike organisms from plants affected with yellows diseases. In: Hiruki C (ed) Tree mycoplasmas and mycoplasma diseases. University of Alberta Press, Edmonton, pp 143
69. Stein A, Salomon R, Cohen J, Loebenstein G (1986) Detection and characterization of bean yellow mosaic virus in corms of *Gladiolus grandiflorus*. Ann Appl Biol 109: 147–154
70. Tosi M, Anderson DL (1973) Antigenic properties of bacteriophage ϕ29 structural proteins. J Virol 12: 1548–1559
71. Valentine RC (1961) Contrast enhancement in the electron microscopy of viruses. Adv Virus Res 8: 287–318
72. Van Lent JWM, Verduin BJM (1985) Specific labelling of antibodies bound to plant viruses in mixed suspensions. Neth J Plant Pathol 91: 205–213
73. Van Lent JWM, Verduin BJM (1986) Detection of viral protein and particles in thin sections of infected plant tissue using immunogold labelling. In: Jones RAC, Torrance L (eds) Developments and applications in virus testing. Association of Applied Biologists, Wellesbourne, Warwick, pp 193
74. van Regenmortel MHV (1982) Serology and Immunochemistry of Plant Viruses, Academic Press, New York
75. van Regenmortel MHV, Nicolaïeff A, Burckard J (1980) Detection of a wide spectrum of virus strains by indirect ELISA and serological trapping electron microscopy (STREM). Acta Hortic 110: 107–115
76. Watson DH, Le Bouvier GL, Tomlinson JA, Walkey DG (1966) Electron microscopy and antigen precipitates extracted from gel diffusion plates. Immunology 10: 305
77. Williams RC (1954) Electron microscopy of viruses. Adv Virus Res 2: 183–239
78. Yanagida M, Ahmad-Zadeh C (1970) Determination of gene product position in bacteriophage T4 by specific antibody association. J Mol Biol 51: 411–421