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SHORT COMMUNICATION

ELECTRON MICROSCOPY OF RUBELLA INFECTED BHK21 AND VERO CELLS

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Norrby et al. (1963) first reported on the morphology of rubella virus by negative staining. They observed particles with a strong resemblance to those of mouse leukemia virus. Structures resembling paramyxoviruses also seemed to be present in the virus (Philips et al., 1965).

Data in recent reports on the size and structure of the particles observed by electron microscopy have been in agreement, though differing from those reported by earlier authors. Best et al. (1967) observed negatively stained aggregates in preparations of virus infected tissue culture fluid and specific rabbit antiserum and reported that the virus particles ranged in size between 50 and 70 mμ. On the other hand Holmes et al. (1968) reported two types of virus particles in sections of rubella-infected RK13 and BHK21 cells, one being 60 mμ in size. Virus particles with the same morphology and size were also reported by Murphy et al. (1968) in rubella-infected BHK21 cells. McCombs et al. (1968) reported that the core of rubella virus is approximately 35 mμ in diameter and the entire virion 50 mμ in diameter and Hamvas et al. (1969) observed particles of 60–70 mμ in RK13, SIRC and BHK21 cells infected with the same agent. Independently from these studies we have observed the same particles of about 60 mμ in size in ultra-thin sections of rubella infected BHK21 and VERO cells. This report presents electron micrographs of our findings.

The Baylor strain of rubella virus was kindly supplied by Dr. Y. Minekawa. BHK21/WI-2 cells of the baby hamster kidney line and VERO cells of the African green monkey kidney line were used for experiments. The former was routinely cultivated in medium consisting of 90% Eagle's Minimum Essential Medium and 10% calf serum while the latter was grown in 199 medium supplemented with 3% calf serum. Rubella virus samples for infection of cells were prepared as follows. When maximal cytopathic effects were observed in infected cultures, cells and fluids were

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harvested and subjected to 3 cycles of freezing and thawing. Then the fluids were clarified by centrifugation at 2000 rev/min for 15 min and the resulting supernatant fluids were used.

For electron microscopic studies of rubella infected BHK cells, monolayer sheets grown in 30 ml Falcon plastic bottles were inoculated with 2 ml of viral sample having a titer of 16HA units. One week later when the cultures showed maximal cytopathic effects the cells were scraped off from the flask and the suspension was centrifuged gently to form pellets for electron microscopy. As controls, normal cell cultures were inoculated with the same amount of Minimum Essential Medium as that of the virus inoculum and then treated similarly to the infected cultures. Procedures for electron microscopy were essentially the same as those reported previously (Nii, 1969). Hitachi 11B and 11C electron microscopes were used for observation.

Fig. 1–11 show electron micrographs of rubella infected BHK21/WI-2 cells. In Fig. 1 many vacuolar structures are seen in the cytoplasm and one round and two oval particles are clearly observed in the vacuoles. One particle which is presumably oval is also seen in the right part of the picture. The outer envelopes of the particles appear to consist of the three layered structure characteristic of a unit membrane. Cores consisting of an electron dense shell and an inner part of low density are round or oval and their shapes may determine the general morphological appearance of the whole particles. A narrow relatively electron clear area is detectable between the envelope and the core of the particles. Fig. 2 also shows three particles located in vacuoles.

The diameter of round particles and length of the short axis of oval particles were found to be about 60 mμ while the size of round cores or shorter axis of oval forms of cores was about 30 mμ.

Fig. 3–6 illustrate the process of budding of particles. In one vacuole in the left of Fig. 3 two particles are in a budding enveloped with a vacuolar membrane. The budding process of one particle shown in Fig. 4 is almost complete. In Fig. 5 two particles are strung together by a common outer membrane giving a dumb-bell shaped particle. Part of the envelope of the left particle is still connected to the vacuolar membrane. Fig. 6 shows a single budding particle and an aberrant form in the middle of the picture. Fig. 7 shows two extracellular particles while in Fig. 8 one particle, indicated by an arrow, seems to be budding from a cytoplasmic process. Occasionally tubular forms were also encountered, as shown in the left upper corner of Fig. 9. A multiple layered membrane structure to which an array of granules are attached was observed but its significance is unknown (Fig. 10). Dilated cisternal profiles of the endoplasmic reticulum were occasionally observed like those reported in measles infected mouse brain by Wear et al. (1968) (Fig. 11).

In uninfected BHK cells no particles such as those described above have so far been observed. However, hamster cells sometimes harbor particles of 85 mμ diameter (Bernhard and Tournier, 1964). To avoid having to discriminate morphologically between forms of rubella virus particles and those of the passenger virus carried in hamster cells, VERO cells were used since no latent virus has been found in this cell line. VERO cell passaged rubella virus of the Baylor strain was used for infection of the cells. Particles of about 60 mμ diameter were also detected in infected VERO cells. In Fig. 12 several particles are seen budding in a cytoplasmic vacuole.

To study the development of rubella virus in relation to production of hemagglutinin and infectious virus and to the extent of cytopathic effects in rubella infected cultures a time sequential study was performed as follows. Replicate VERO cell monolayers of 3×10^6 cells each were inoculated separately with 0.5 ml of viral sample having a titer of 8 HA units. After a 2 hr adsorption period the inoculation medium was replaced by maintenance medium and 3, 4, 6, 9 and 11 days after infection four bottles were removed from the

incubator. Three were used for assay of infectivity and hemagglutinin in infected cells and the fluid phase of the cultures while the fourth was used for electron microscopy. Infectivity titrations were carried out in VERO cells by the endpoint method in tube titrations. Fifty per cent cytopathic doses were calculated after a 15 day period. The infective titer obtained by this method was approximately the same as that calculated by the 50% interference dose (InD50) using ECHO-11 as challenge virus. Assay of hemagglutinin was performed with pigeon erythrocytes by the method of Stewart et al. (1967) with a slight modification (Sasada, 1968).

The experimental results are shown in Table 1. A few particles appeared in groups in cytoplasmic vacuoles or singly in extracellular spaces 4 days after infection and the date of their first appearance coincided with that of the first cytopathic effects observed in the infected cultures. Thereafter the number of detectable virus particles increased with time after infection.

All the viral forms observed in rubella infected BHK cells were also detected in VERO cells in this time sequential study. In samples at later stages of virus infection many aberrant forms were also observed. The arrow in Fig. 13 indicates a core adjacent to a vacuolar membrane. Many particles with aberrant forms are seen in a cytoplasmic vacuole and most of them are tubular. In the vacuoles shown in Fig. 14 there are some round particles of about 60 mμ diameter and many bigger viral particles with various aberrant forms. A few very big particles are also observed. Several cytoplasmic vacuoles shown in Fig. 15 contain numerous round particles.

Fig. 16 and 17 illustrate round extracellular particles. They are uniform in size with an average diameter of about 60 mμ.

Dilated cisternal profiles of the endoplasmic reticulum are also observed in infected VERO cells (Fig. 18) like those in infected BHK cells.

No viral particles were observed in uninfected VERO cells.

We have never observed crystalline structures such as those found by Kim and Boatman (1967) and by Holmes et al. (1968, 1969). The significance of such structures is uncertain. This problem will be discussed in a subsequent report.

In conclusion, electron microscopic observa-

TABLE 1. *Appearance of viral particles in rubella-infected VERO cells correlated with extent of CPE and titer of infectious virus and hemagglutinin*

Days after virus inoculation		3	4	6	9	11
Virus particle[a]		−	+	++	+++	++
CPE[b]		−	+	+	++	+++
HA	associated with cells	0	NT[c]	0	8	8
	in culture fluids	4	NT	4	16	8
$TCID_{50}$	associated with cells	3.5	NT	≧ 5.5	≧ 5.5	≧ 5.5
	in culture fluids	2.5	NT	3.5	5.3	≧ 5.5

Replicate VERO cell monolayers with 3×10^6 cells each were inoculated separately with 0.5 ml of viral sample, (titer, 8 HA units). After a 2 hr adsorption period the inoculation fluid was replaced by maintenance medium and 3, 4, 6, 9 and 11 days later four bottles were removed from the incubator. Three were used for assay of infectivity and hemagglutinin and the other for electron microscopy.

a Relative number of virus particles observed in sectioned cells of samples prepared on the date indicated.
b Extent of CPE revealed in the infected cultures.
c Not tested.

FIGURE 1. and 2. *Rubella virions in cytoplasmic vacuoles of BHK 21/WI-2 cells, one week after virus inoculation.*
FIGURE 1. ×85,000 FIGURE 2. ×78,000

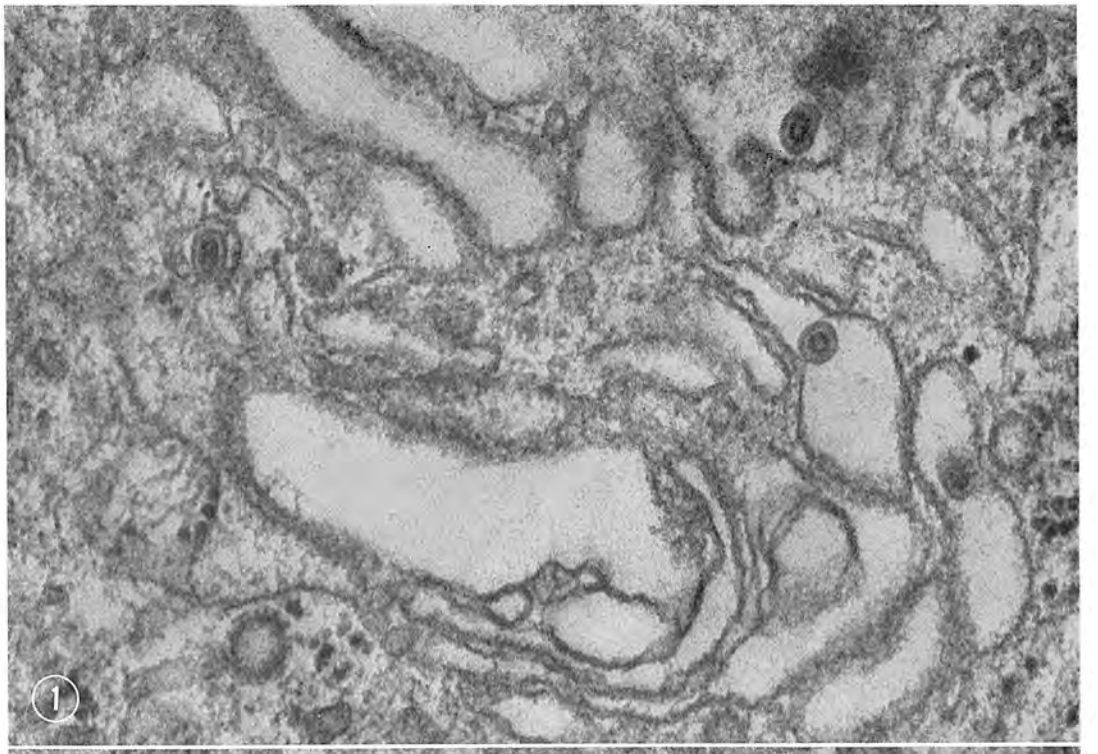
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FIGURE 3, 4, 5 and 6. *Budding process of rubella virus particles into cytoplasmic vacuoles of BHK21/WI-2 cells, one week after virus inoculation. In Fig.* 5 *one dumb-bell like particle is budding and in Fig.* 6 *one aberrant form is shown besides one particle with a single bud.*

FIGURE 3. ×74,000 FIGURE 4. ×64,500
FIGURE 5. ×75,000 FIGURE 6. ×71,000

FIGURE 7. *Two extracellular particles of rubella virus. BHK21/WI-2 cells, one week after virus inoculation.* ×57,000

FIGURE 8. *The arrow indicates budding of rubella virus from a cytoplasmic process of a BHK21/WI-2 cell, one week after virus inoculation.* ×47,000

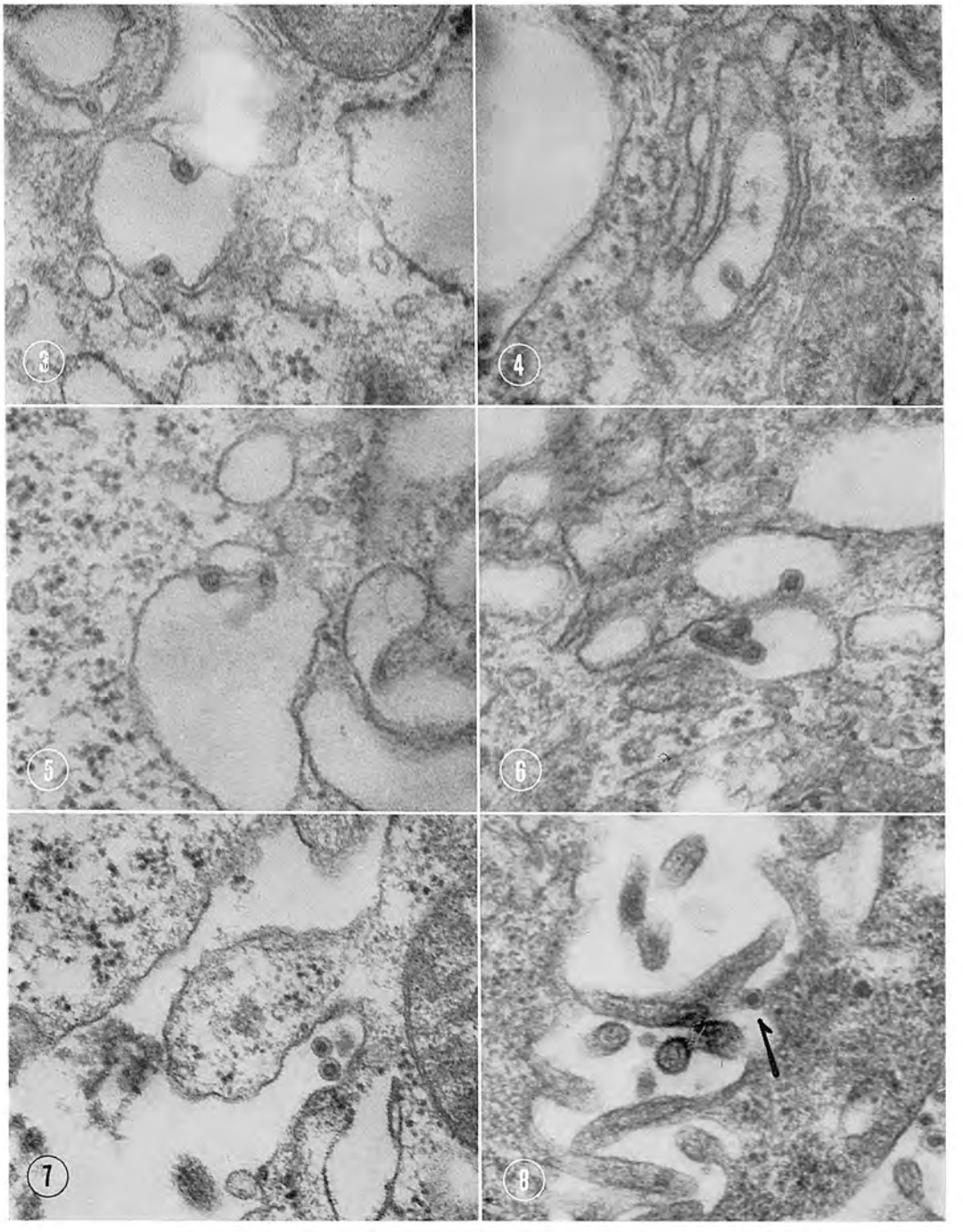
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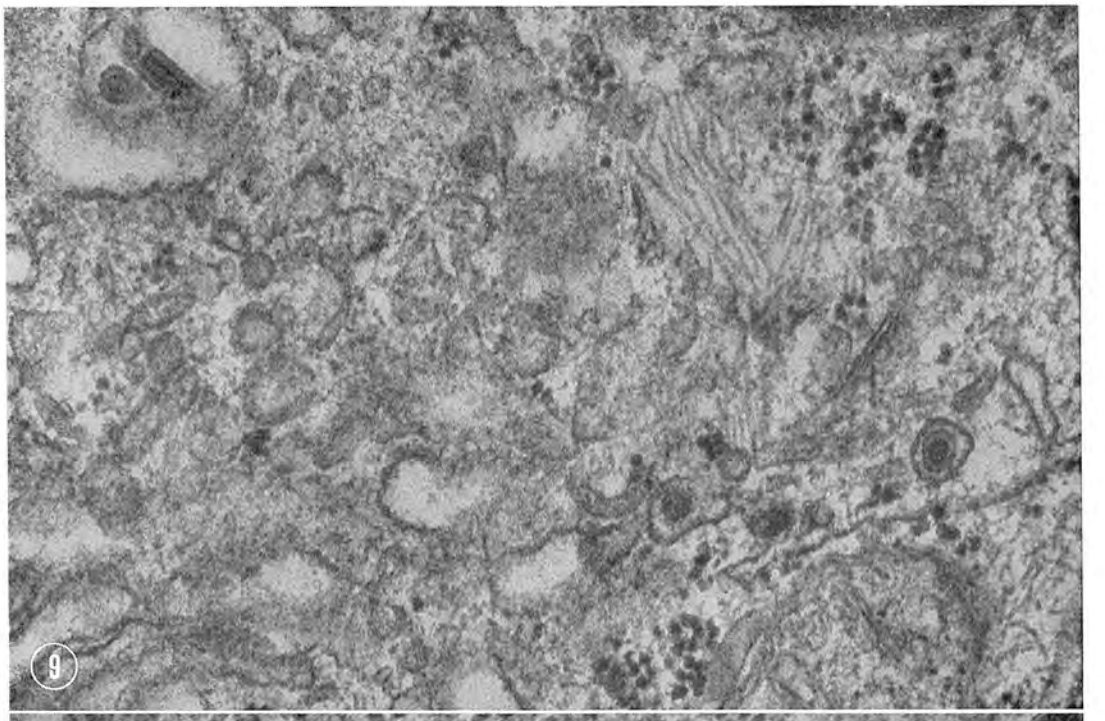
FIGURE 9. *A tubular form of rubella virus in the left upper corner of the picture. A BHK21/WI-2 cell, one week after virus inoculation.* ×72,500

FIGURE 10. *A multiple layered membrane structure to which an array of granules is attached. A BHK21/WI-2 cell, one week after virus inoculation.* ×83,000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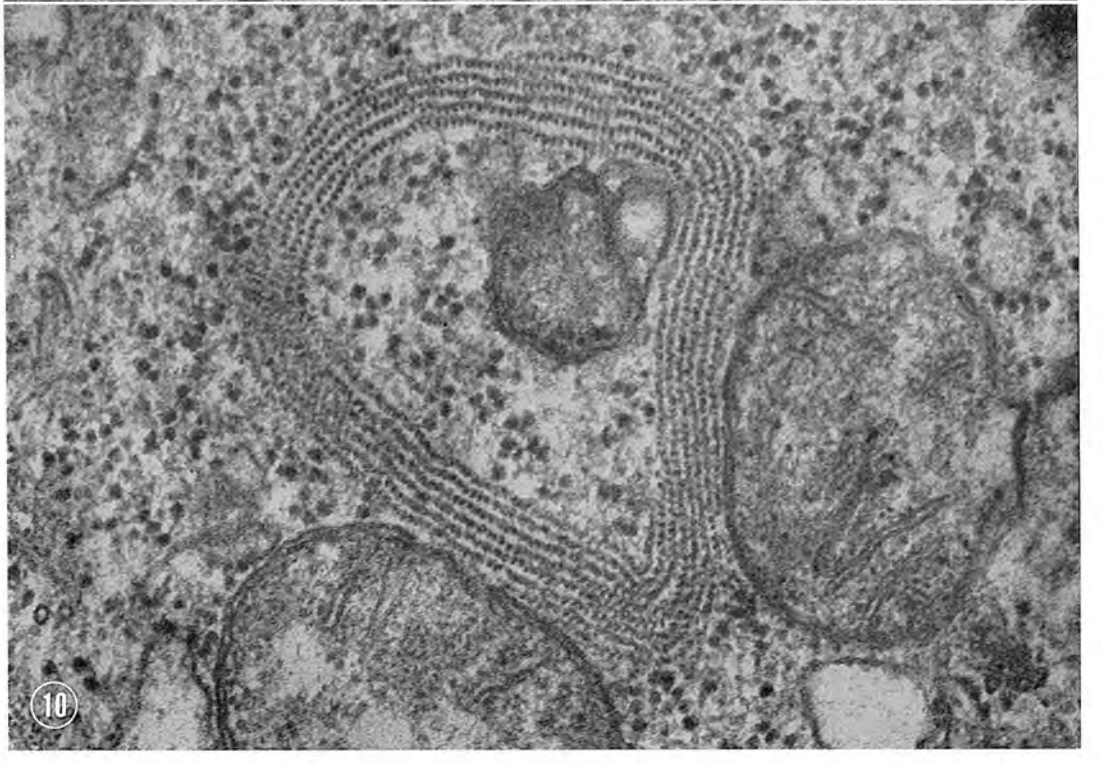
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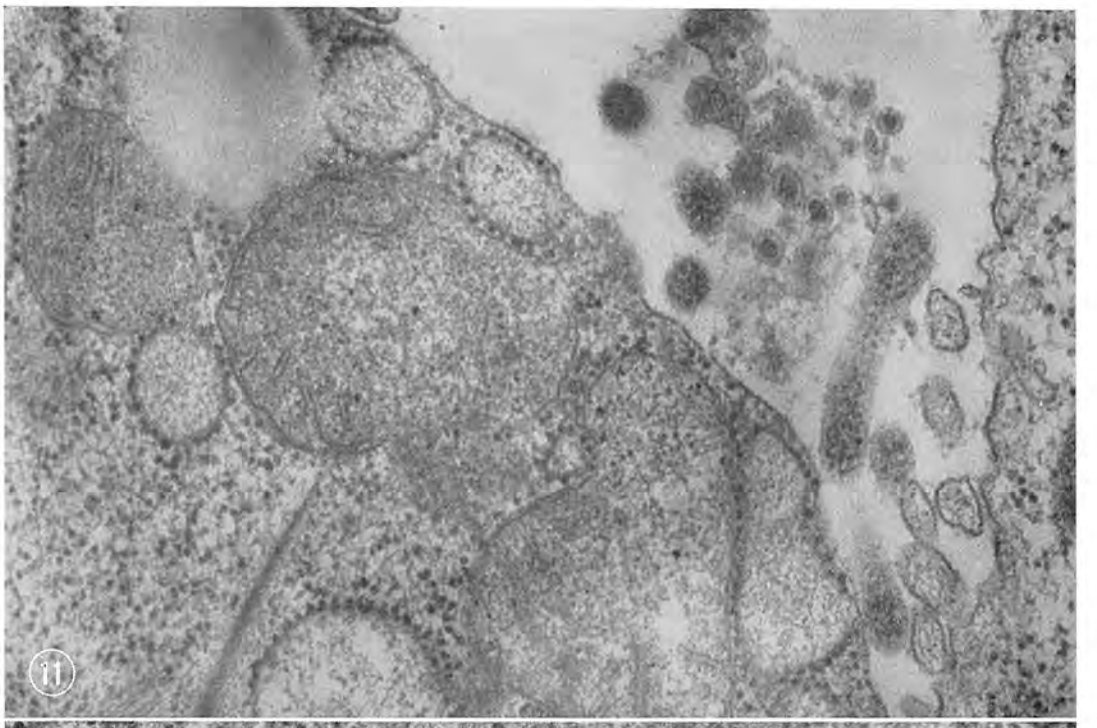
FIGURE 11. *Dilated cisternal profiles of the endoplasmic reticulum in a BHK21/WI-2 cell, one week after virus inoculation.* ×63,000

FIGURE 12. *Budding of rubella virus particles in a cytoplasmic vacuole of a VERO cell, one week after virus inoculation.* ×68,000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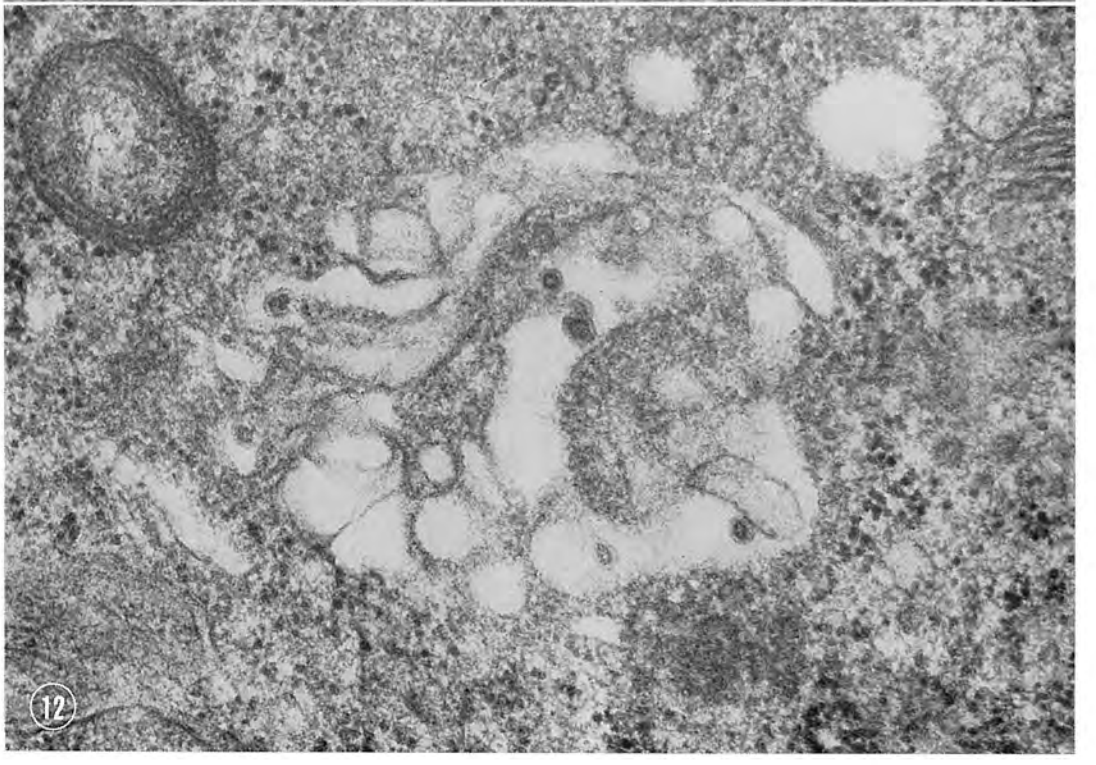
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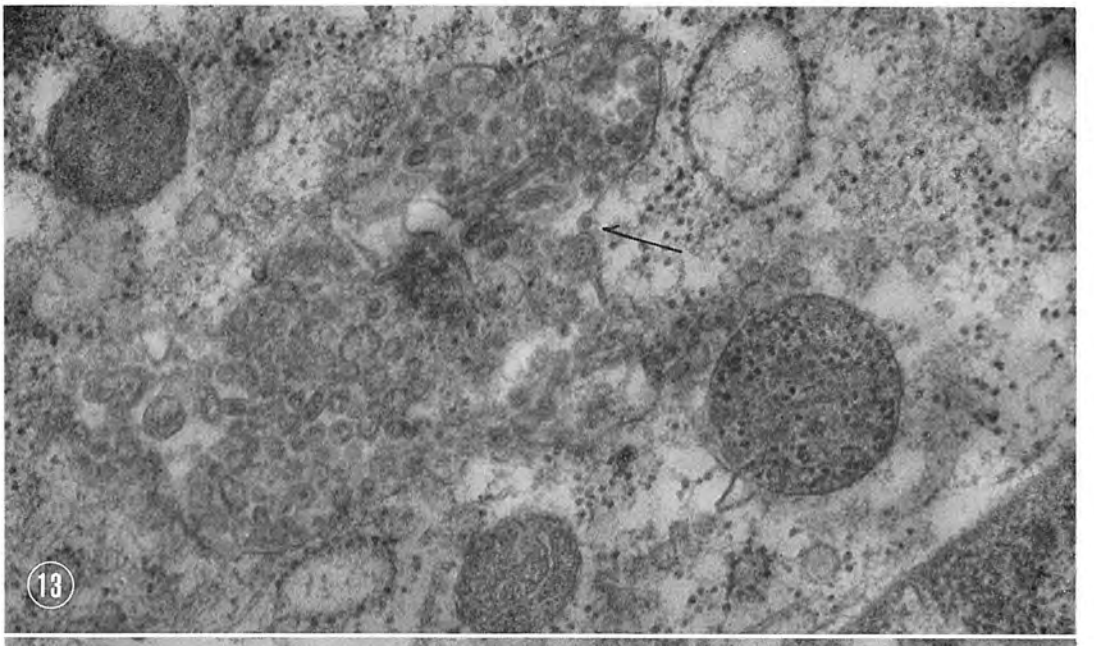
FIGURE 13 and 14. *Various forms of rubella virus in cytoplasmic vacuoles of a VERO cell, 9 days after virus inoculation. The arrow in Fig. 13 indicates a core adjacent to a vacuolar membrane.*

FIGURE 13. ×53,000 FIGURE 14. ×54,000



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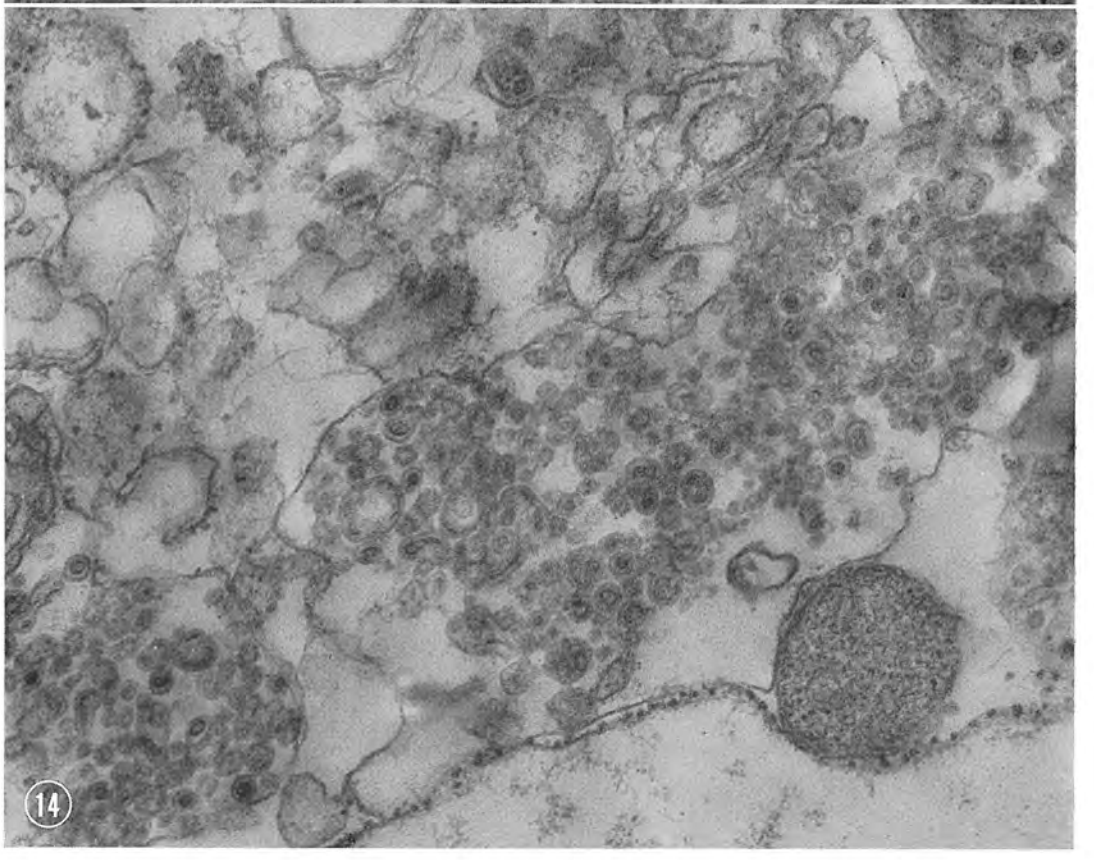

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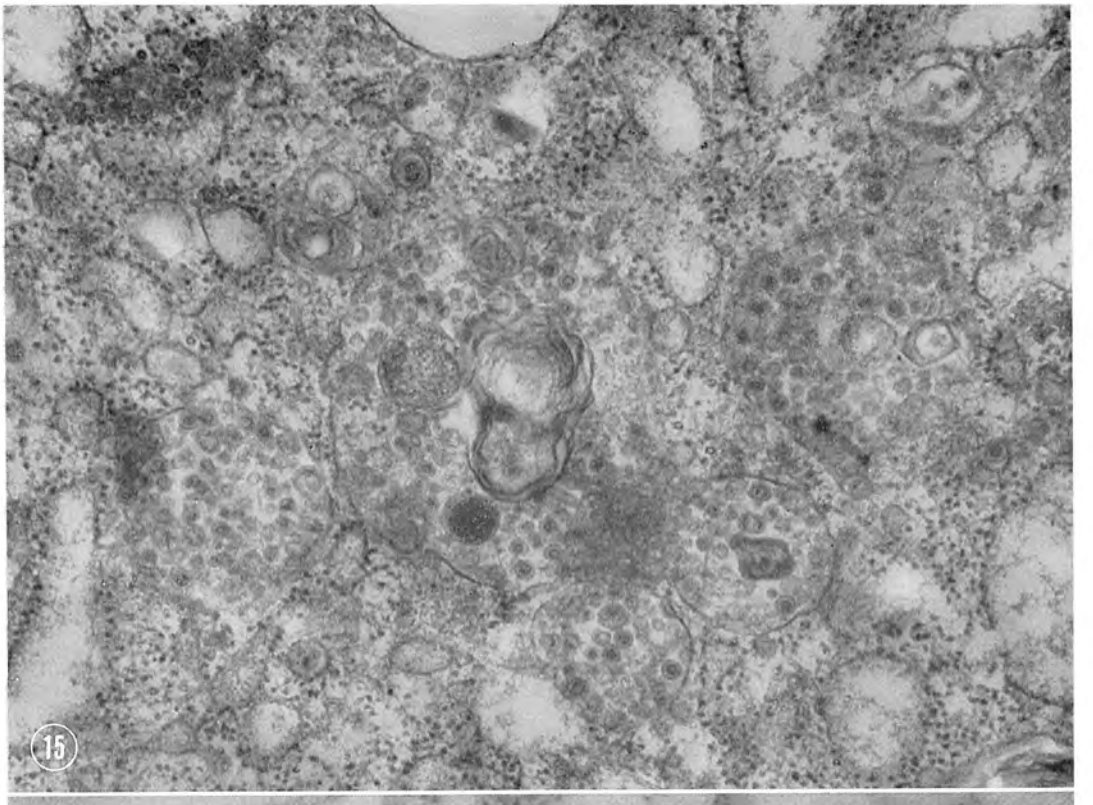
FIGURE 15. *Various forms of rubella virus in cytoplasmic vacuoles of a VERO cell, 9 days after virus inoculation.* ×44,000

FIGURE 16. *Extracellular particles of rubella virus, VERO cells, 9 days after virus inoculation.* ×53,000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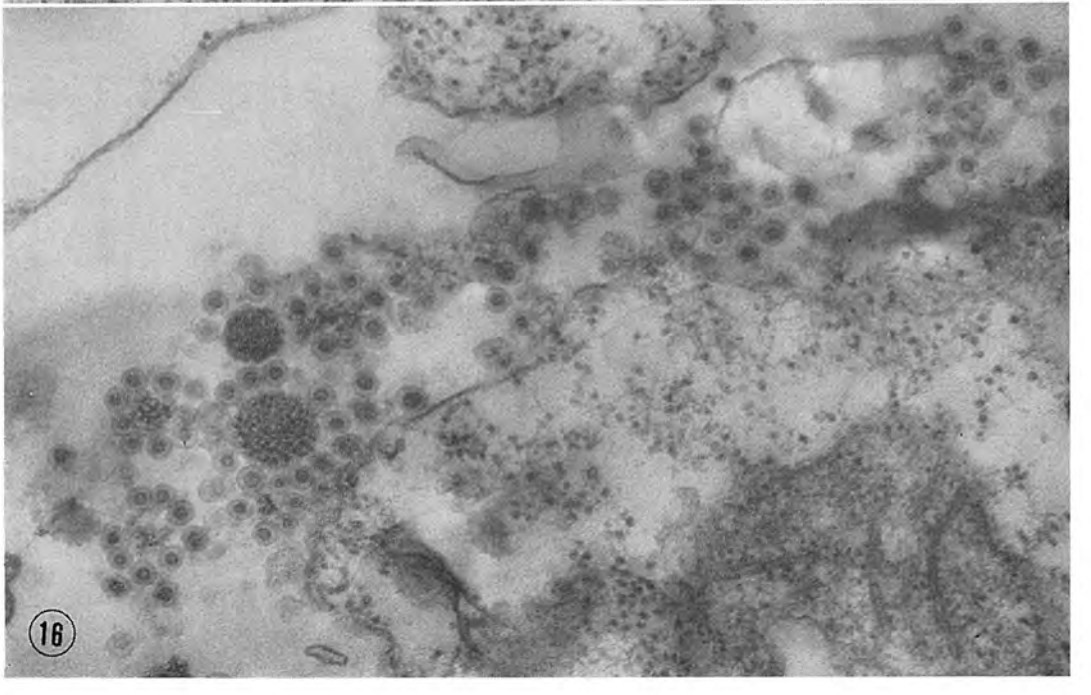
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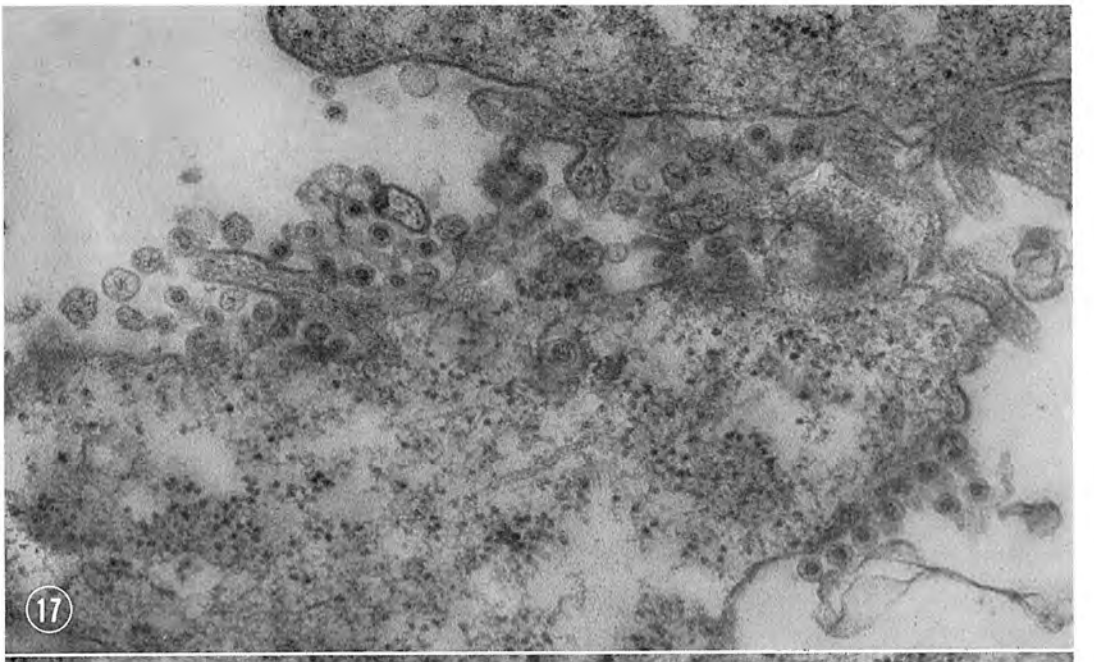
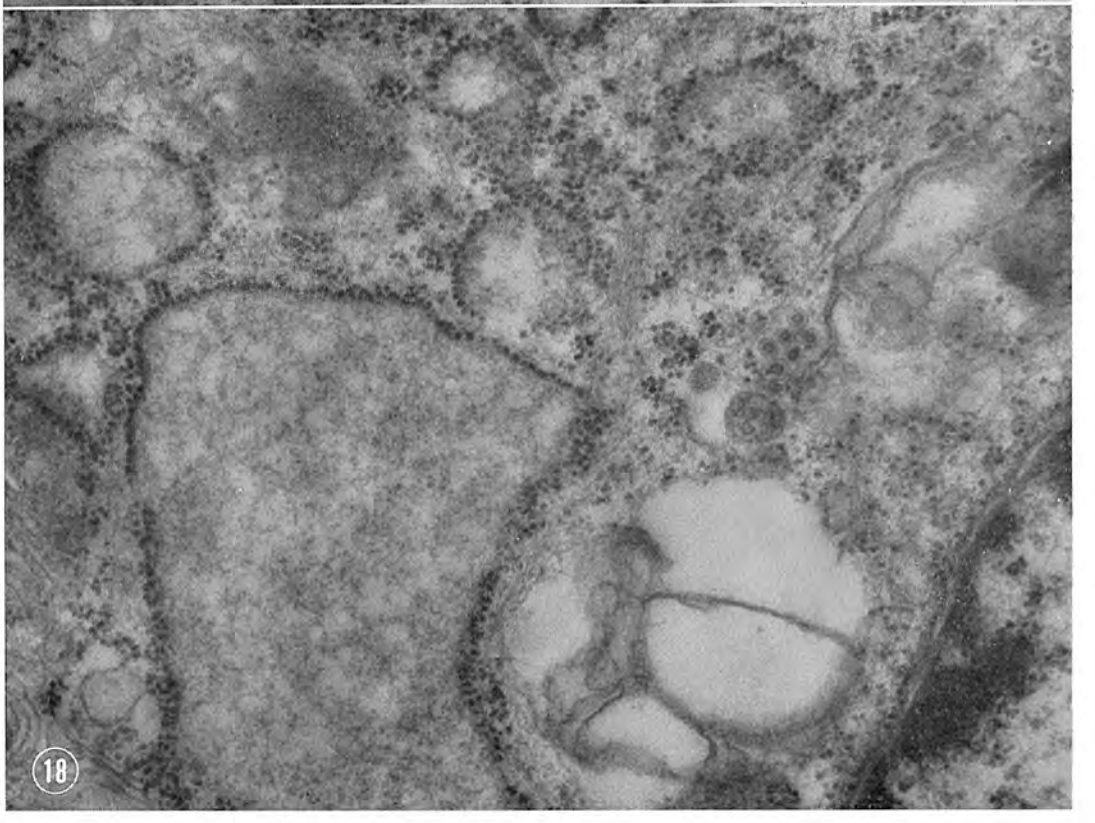
FIGURE 17. *Extracellular particles of rubella vivus, VERO cells, 9 days after virus inoculation.* ×55,000

FIGURE 18. *Dilated cisternal profiles of the endoplasmic reticulum in a rubella infected VERO cell, 6 days after virus inoculation.* ×40,000





tions of rubella infected BHK21/WI-2 cells and VERO cells revealed round virus particles. The entire virion had a diameter of about 60mμ and the core a diameter of about 30 mμ. Various aberrant forms of particles were also observed, especially in samples obtained at late stages of infection. Dilated cisternal profiles of the endoplasmic reticulum were noted as one morphological change of infected cells.

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REFERENCES

Bernhard, W. and P. Tournier. 1964. Infection virale inapparente de cellules de hamsters décelée par la microscopie électronique. Ann. Inst. Pasteur 107 : 447–452.

Best, J. M., J. E. Banatvala, J. D. Almeida and A. P. Waterson. 1967. Morphological characteristics of rubella virus. Lancet 2 : 237–239.

Hamvas, J. J., S. Ugovsek, S. Iwakata and N. A. Labzoffsky. 1969. Virus particles in rubella infected tissue cultures. Arch. ges. Virusforsch. 26 : 287–294.

Holmes, I. H., M. C. Wark, I. Jack and J. Grutzner. 1968. Identification of two possible types of virus particle in rubella-infected cells. J. Gen. Virol. 2 : 37–42.

Holmes, I. H., M. C. Wark and M. F. Warburton. 1969. Is rubella an arbovirus? II. Ultrastructural morphology and development. Virology 37 : 15–25.

Kim, K. S. W. and E. S. Boatman. 1967. Electron microscopy of monkey kidney cell cultures infected with rubella virus. J. Virol. 1 : 205–214.

McCombs, R. M., J. P. Brunschwig and W. E. Rawls. 1968. Morphology of rubella virus. Exp. Mol. Path. 9 : 27–33.

Murphy, F. A., P. E. Halonen and A. K. Harrison. 1968. Electron microscopy of the development of rubella virus in BHK-21 cells. J. Virol. 2 : 1223–1227.

Nii, S. 1969. Persistent infection with herpes simplex virus in vitro. I. Establishment and characteristics of persistent herpes simplex virus infection in Earle's L Cells. Biken J. 12 : 45–67.

Norrby, E., P. Magnusson, B. Friding and S. Gard. 1969. A note on the morphology of rubella virus. Arch. ges. Virusforsch. 13 : 421–424.

Philips, C. A., A. M. Behbehani, L. W. Johnson and J. L. Melnick. 1965. Isolation of rubella virus. An epidemic characterized by rash and arthritis. J. Amer. Med. Ass. 191 : 615–618.

Sasada, T. 1968. Serological diagnosis of rubella by hemagglutination inhibition test. (in Japanese) Report of the Japanese research staff of the Virus Research Institute in Bangkok (Overseas Technical Cooperation Agency) : 3–4.

Stewart, G. L., P. D. Parkman, H. E. Hopps, R. D. Douglas, J. P. Hamilton and H. M. Meyer. 1967. Rubella-virus hemagglutination inhibition test. New Engl. J. Med. 276 : 554–557.

Wear, D. J., E. R. Rabin, L. S. Richardson and F. Rapp. 1958. Virus replication and ultrastructural changes after induction of encephalitis in mice by measles virus. Exp. Mol. Pathol. 9 : 405–417.