J. gen. Virol. (1970), **7**, 143–151
Printed in Great Britain

Light and Electron Microscopy of Cells Infected with Tobacco Necrosis and Satellite Viruses

By B. KASSANIS, DOREEN A. VINCE AND R. D. WOODS
Rothamsted Experimental Station, Harpenden, Herts.

(*Accepted* 6 *January* 1970)

SUMMARY

French-bean leaves inoculated with the stipple streak strain of tobacco necrosis virus alone or mixed with the satellite virus were examined microscopically. Light microscopy showed two types of inclusion in some living epidermal cells, one hyaline, the other granular and containing small crystals. Some cells also contained crystals different in form from those in the inclusions. Light microscopy of parenchymatous cells fixed and stained for electron microscopy showed only one type of inclusion, with smooth appearance. The crystals seen in the living cells were not found, but some cells from leaves infected with the mixture had areas containing both heavily stained amorphous material and bodies 1 to 2 μm. across.

Electron microscopy of thin sections of the same fixed and stained material showed some cells to contain a structureless, electron-dense material, prominent cytoplasmic membranes and many vesicles, especially from leaves inoculated with the mixture. The electron-dense material often contained satellite virus in crystalline arrays and sometimes rhombic plates 1 to 2 μm. long. Whenever satellite virus was identified, tobacco necrosis virus occurred near to it. Cells infected with tobacco necrosis virus alone had well-defined areas in which the virus was concentrated and apparently uncontaminated. These areas corresponded to the inclusions seen in the light microscope. The relative frequency with which particles of the two viruses were detected by electron microscopy of leaf sections differed from the relative concentration of the two estimated by serological assays on sap extracted from leaves inoculated with the mixture.

INTRODUCTION

Satellite virus multiplies only in leaves infected with tobacco necrosis virus, so it is of interest to know the spatial relationship of the two viruses in infected cells. Also, when concentrated enough, the two viruses readily crystallize during purification, even from impure preparations, and we wanted to know whether they also form crystals in infected cells. For these reasons, thin sections of fixed and embedded pieces of inoculated French-bean leaves were examined by both light and electron microscopy, and living epidermal cells of the same leaves by light microscopy. Most strains of tobacco necrosis virus alone or in mixture with satellite virus cause only necrotic local lesions or, in a few species, some necrotic systemic symptoms. The bean stipple streak strain is exceptional and in French bean (*Phaseolus vulgaris* L., var. Prince) it causes a systemic mottle with only a few necroses, usually along the minor veins of infected leaves (van der Want, 1948). Most of our observations were made with this strain-host combination.

METHODS

The primary leaves of French-bean plants, kept in a glass-house with an average temperature of 20°, were infected by rubbing with sap from plants with either the stipple strain of tobacco necrosis virus alone or with the natural mixture of tobacco necrosis virus and satellite virus. The leaves were usually sampled a week later. The strain of satellite virus in the natural mixture is SV_1, and the original inoculum was kindly provided by Dr D. Noordam in 1961 (Babos & Kassanis, 1963). Concentrated inocula sometimes cause the inoculated leaf to wilt, but this did not happen with the diluted sap inoculum.

For electron microscopy, pieces of leaf lamina, selected from an area of the inoculated leaf that showed mottle and least necrosis, were infiltrated in 2·5% (v/v) glutaraldehyde in 0·1 M-phosphate buffer (pH 7) for 2 hr. The leaf pieces were then washed in a mixture of 0·25 M-sucrose and 0·2 M-phosphate buffer at pH 7, before fixing for 2 hr in 1% (w/v) osmium tetroxide in the buffer-sucrose mixture. After washing for 15 min. in the buffer-sucrose mixture, the pieces were dehydrated in ethanol and embedded in Epon.

Crystals of satellite virus and tobacco necrosis virus were pelleted by centrifugation and the small pellets fixed and prepared for sectioning in a similar manner.

Sections were cut with a Reichert OmU2 microtome and mounted on copper grids. They were stained in 2% (w/v) uranyl acetate followed by Reynold's lead citrate and examined in a Siemens Elmiskop IA.

RESULTS

Virus concentration

Satellite virus inoculated together with tobacco necrosis virus inhibits multiplication of tobacco necrosis virus. The degree of inhibition increases as the relative concentration of satellite virus in the inoculum increases (Kassanis, 1962). Also, some strains of tobacco necrosis virus are inhibited more than others (Babos & Kassanis, 1963). We used a natural mixture and found that sap extracted from inoculated leaves usually contained more satellite virus than tobacco necrosis virus when estimated serologically. Sap from systemically infected trifoliate leaves did not react with antiserum to satellite virus but contained as much tobacco necrosis virus as the inoculated leaves. By inoculating bean plants in series and taking the inoculum only from young trifoliate leaves immediately after symptoms appeared, we obtained a culture of stipple streak strain free from satellite virus. Considering the relative size of the particles of the two viruses and that twice as much satellite virus as tobacco necrosis virus is needed to give a visible precipitate with antiserum, at least twice as many particles of satellite virus as tobacco necrosis virus might have been expected in infected cells. However, electron microscopy showed many more tobacco necrosis virus than satellite virus particles.

Light microscopy

Epidermal strips, taken off the midrib of the lower leaf surface with forceps, were examined in a drop of water. In some areas of the tissue nearly all cells contained one or more inclusions usually lying against the cell wall. The inclusions appeared either granular, which contained many small crystals, or hyaline (Fig. 1 *a*, *b*). The inclusions were in cells from leaves inoculated with tobacco necrosis virus alone or with the mixture, but not in leaves from uninoculated plants. The hyaline inclusions, when examined in phase contrast, seemed lobed and were as refractive as the cell wall, contrasting with the nuclei which were less bright (Fig. 1 *c*). Both types of inclusion ranged in size and many were as large as the nucleus. Similar inclusions

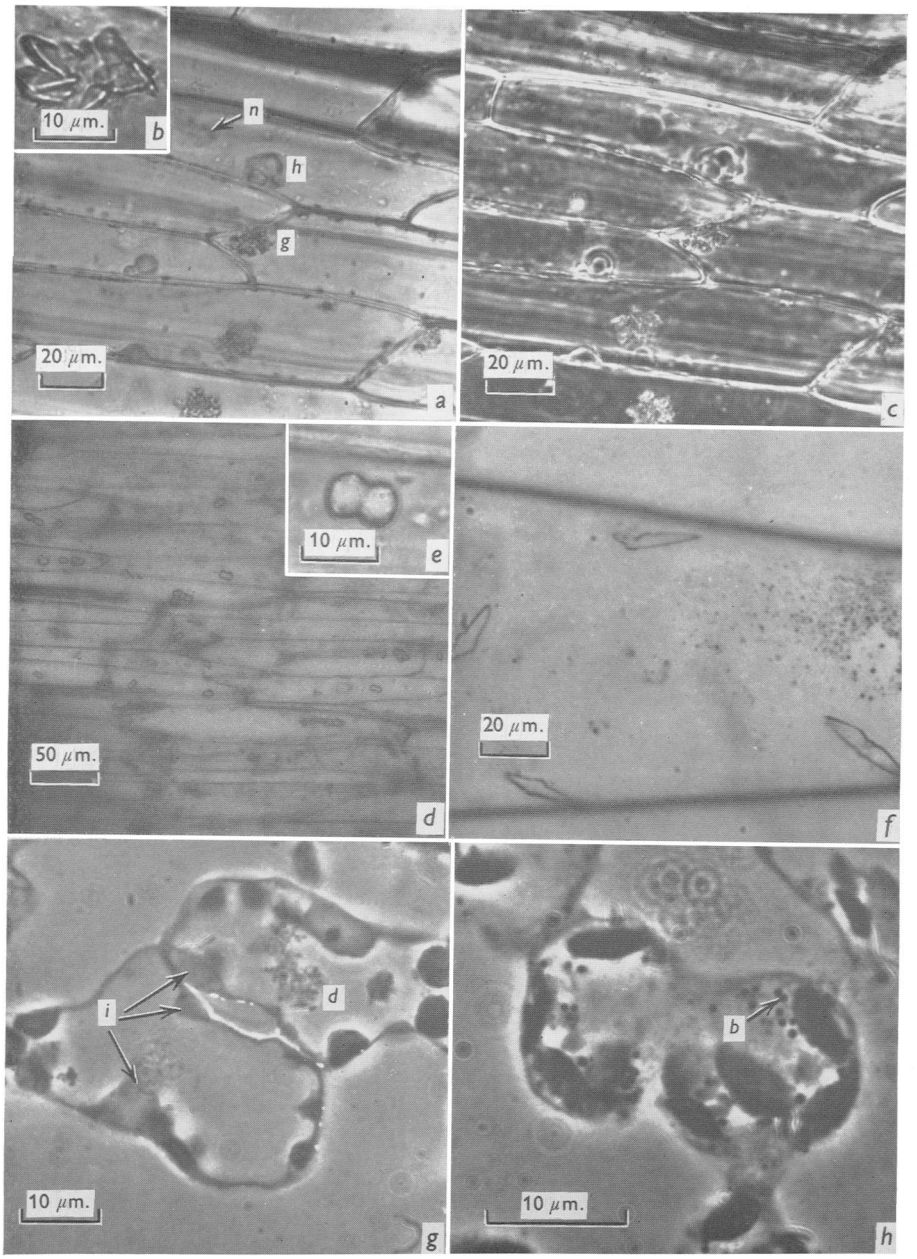

Fig. 1 (*a*) Epidermal cells infected with tobacco necrosis virus showing hyaline (*h*) and granular (*g*) inclusions, about the same size as the nucleus (*n*); (*b*) a granular inclusion magnified; (*c*) as (*a*) but in phase contrast; (*d*) epidermal cells infected with tobacco necrosis virus showing numerous hexagonal crystals; (*e*) a pair of hexagonal crystals magnified; (*f*) part of a hair from the midrib of a leaf infected with tobacco necrosis virus and showing rhombic crystals fused in pairs; (*g*), (*h*) 1 to 2 μm. sections of parenchymatous cells infected with tobacco necrosis virus and satellite virus, fixed, stained and embedded, showing inclusions (*i*), dense material (*d*) and heavily stained bodies (*b*).

were also found in tobacco leaves inoculated with strains A or E of tobacco necrosis virus, usually in living cells near necrotic local lesions.

Some epidermal strips from beans infected with one or both viruses had cells containing numerous small crystals, usually rectangular, hexagonal or rhombic plates, but in one cell they appeared as two rhombic plates joining end-to-end, resembling a bow tie (Fig. 1*d*, *e*,*f*). The crystals were equally common in bean leaves infected with tobacco necrosis virus alone or both viruses. By contrast, the only crystalline forms shown by electron microscopy contained satellite virus particles, and they were smaller than the crystals seen in living cells. If this is because the crystals seen in living cells did not survive fixation, they may not be virus crystals; when crystals of either virus formed *in vitro* were fixed, embedded and sectioned for electron microscopy, they survived the treatment.

To relate the observations from the two types of microscopy, fixed and embedded leaf tissues were examined in the light microscope. The cells contained one or more inclusions, usually lying against the cell wall. They all looked alike and were stained to the same extent as nuclei, but were smoother (Fig. 1*g*). The granular inclusions and crystals seen in the living cells were not found, but cells from leaves inoculated with the two viruses had areas containing heavily stained amorphous material. Also, the same tissue had some cells that contained heavily stained small bodies, 1 to 2 μm. across, the largest of which seemed angular (Fig. 1*h*). The heavily stained bodies were probably the small crystals of satellite virus seen in the electron microscope, for they were about the same size.

Electron microscopy

No attempt was made to find the sequence of cytological changes caused by infection. In negatively stained mounts tobacco necrosis virus and satellite virus have isometric particles 26 and 17 nm. across respectively, and in thin sections they are difficult to distinguish from ribosomes, which are intermediate in size, unless the particles either formed crystalline arrays or were very concentrated. Virus was first seen 3 days after inoculation but we shall describe only advanced stages in the infection, usually one week after inoculation.

Only some cells in some areas of the tissue contained recognizable virus particles. Nevertheless, many cells in which virus was not detected were abnormal. The most notable difference between uninfected and infected tissues was the general increase in density of the cytoplasm in infected tissue, accompanied by many vesicles and prominent cytoplasmic membranes, especially in cells infected with both viruses. Many cells appeared completely disorganized, and some had lost all integrity, the cytoplasmic membranes being greatly disrupted. In many badly affected cells the tonoplast was completely broken down, and in some the plasmolemma was also degenerate. However, the most notable features of doubly infected cells were the numerous vesicles, at times a labyrinth of membranes within membranes and the tram-line effect of membranes in the electron-dense material in the vacuole. Tobacco necrosis virus and satellite virus particles were twice found enclosed within vesicles, probably trapped while the membranes were folding (Fig. 2*a*).

Another interesting change in leaves infected with both viruses was the accumulation of an amorphous, electron-dense material, seemingly of cytoplasmic origin, often occurring in the vacuoles of disorganized cells. In intensity of staining the material compares with the nucleoli. The dense material may be directly associated with virus multiplication, because it invariably contained satellite virus and tobacco necrosis virus particles (Fig. 2*b*). It was difficult to be certain that satellite virus was present either in the dense material or in the cytoplasm (Fig. 2*c*), except when the virus formed crystalline arrays; these ranged in size from 0·2 μm. to a few particles. Well-formed large rhombic plates 1 to 2 μm. long were some-

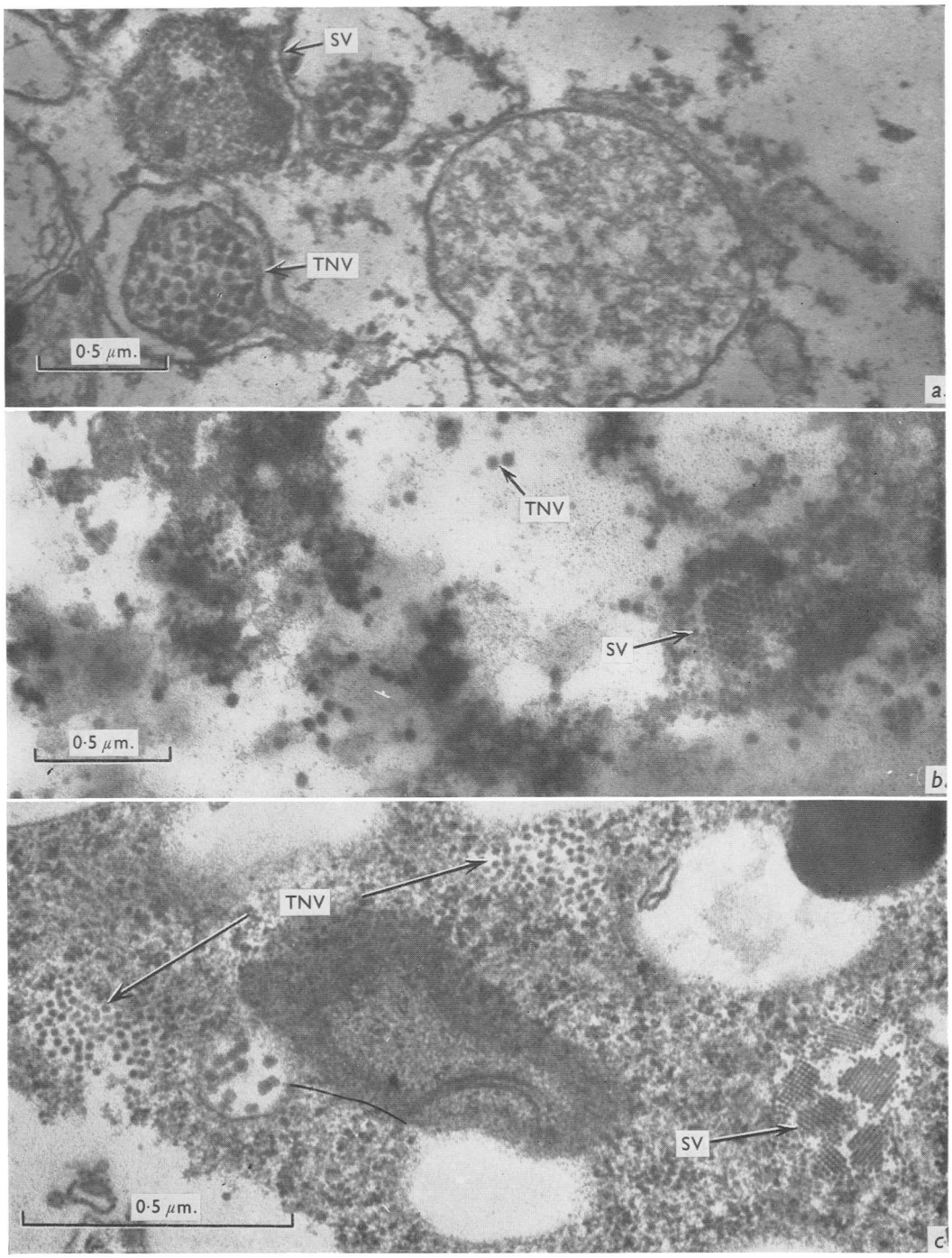

Fig. 2(*a*) Particles of satellite virus and tobacco necrosis virus enclosed in vesicles; (*b*) electron-dense material showing particles of the two viruses; (*c*) an area of the cytoplasm showing particles of the two viruses.

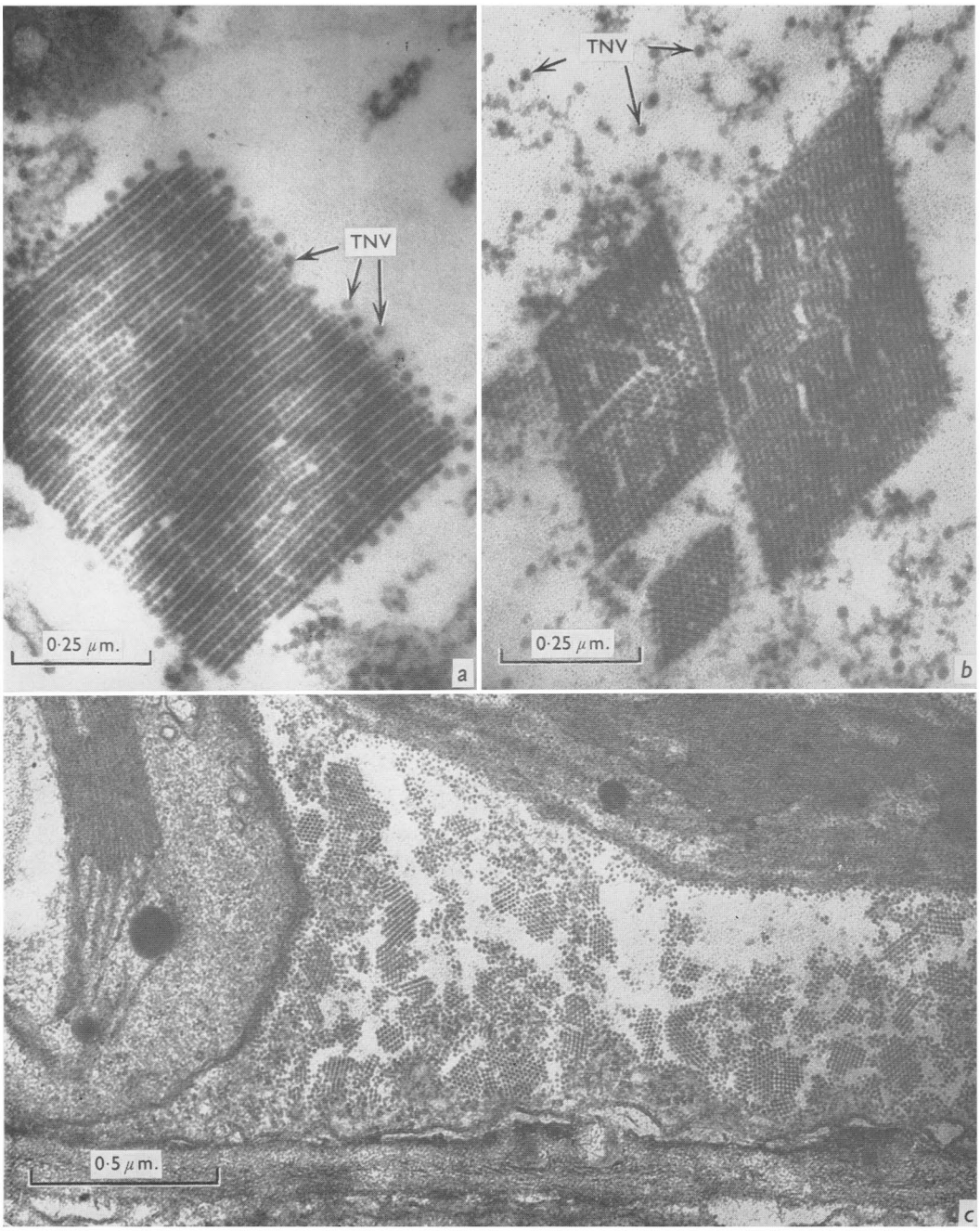

Fig. 3(*a*), (*b*), (*c*) Crystals and crystalline arrays of satellite virus, surrounded in (*a*) and (*b*) by particles of tobacco necrosis virus.

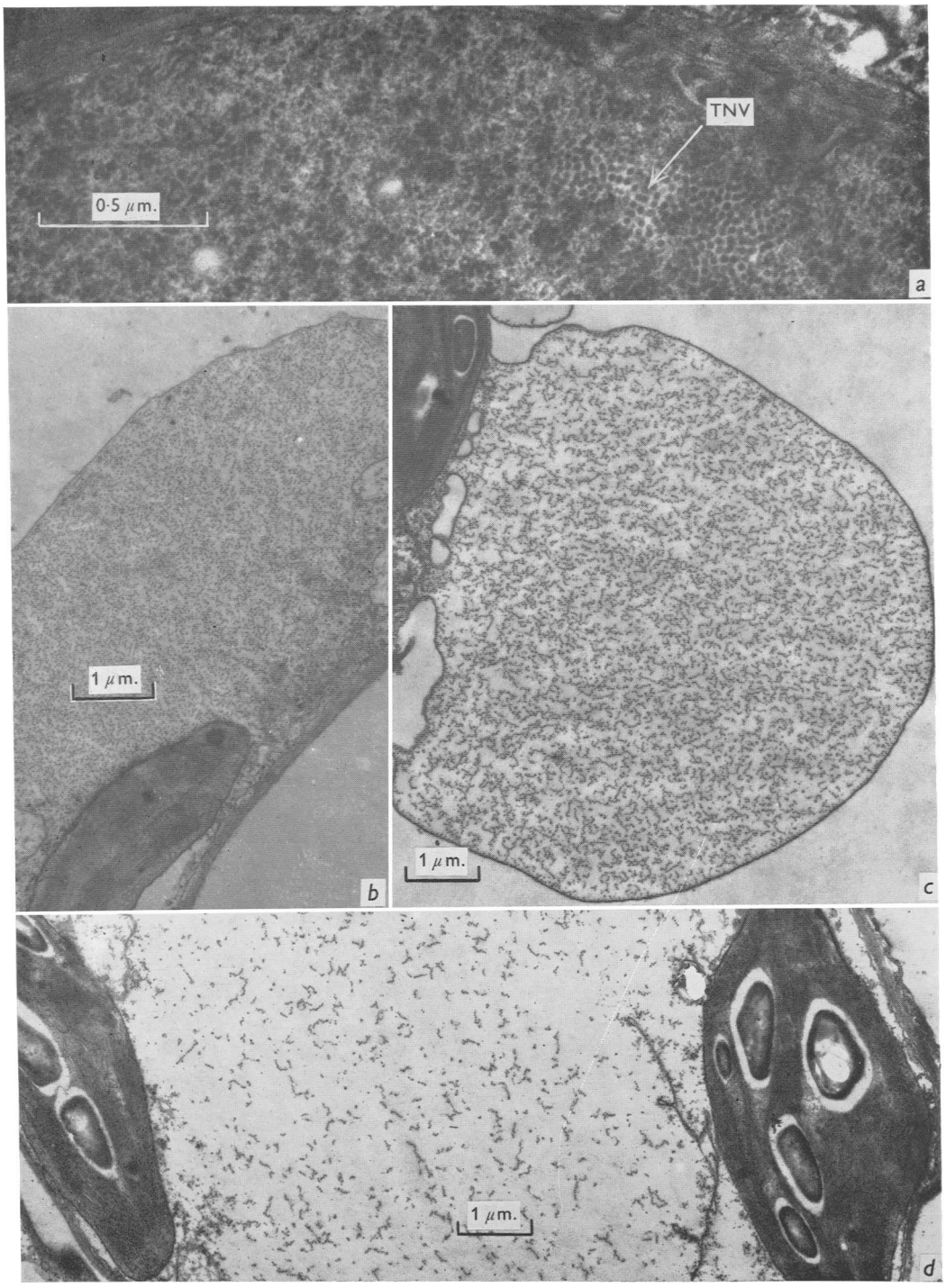

Fig. 4 (*a*) Part of the cytoplasm showing an accumulation of tobacco necrosis virus; (*b*) an accumulation of tobacco necrosis virus at high concentration; (*c*), (*d*) areas containing less tobacco necrosis virus than in (*b*) but the particles are aligned in little strings.

times found and they were similar to those formed *in vitro* (Kassanis, 1962). Many of the crystals were surrounded by tobacco necrosis virus particles (Fig. 3*a*, *b*, *c*). Wherever satellite virus was identifiable, tobacco necrosis virus particles were always very close to it. By contrast, tissues infected with the two viruses sometimes had cells or parts of cells that contained only tobacco necrosis virus. Tobacco necrosis virus accumulated in three different ways: (1) in narrow bands or slightly bulging regions of cytoplasm, mixed with ribosomes, membranes and cell organelles; (2) in well-defined areas in which the virus was greatly concentrated and apparently free from contaminants; (3) as in (2) but the virus was less concentrated (Fig. 4*a*, *b*, *c*, *d*). In (3) the virus particles were lined in little strings, which suggests that these areas may be the granular inclusions containing crystals seen in living cells by light microscopy. The size, shape and frequency of (2) and (3) corresponded with the smooth inclusions seen in fixed cells by light microscopy, and their identity was checked by cutting alternate sections for light and electron microscopy.

In disorganized cells the areas containing virus seemed to have been discharged with the cytoplasm into the vacuoles. This effect was seen more often in cells infected with the two viruses than with tobacco necrosis virus alone and such cells were those that contained the large rhombic satellite virus crystals.

DISCUSSION

Our main interest was to know the spatial relationship of the two viruses in the infected cells and the evidence was conclusive that, wherever satellite virus occurred, tobacco necrosis virus was very near by, as might be expected with satellite virus being dependent on tobacco necrosis virus for its multiplication. In this respect the results resemble those reported with adeno-satellite virus, but not on the ratio of the two viruses in infected cells. Atchison, Casto & Hammon (1966) found that the nuclei of some cells from an infected culture of rhesus monkey kidney cells contained many adenovirus crystals and a few scattered small particles thought to be satellite virus. Other nuclei in the same culture had numerous satellite virus particles in large and small crystalline forms and only the occasional adenovirus particle. They suggested an interference between the two viruses within cells. Our results seem to show a discrepancy between cytological observations and serological tests on the relative concentration of the two viruses, as much more tobacco necrosis virus than satellite virus was seen in infected cells than expected from serological tests. Possibly the areas of tissues containing the large satellite virus crystals cut badly and were discarded, but a more probable reason is that the electron-dense material contains much satellite virus but the particles can be identified only when they form crystalline arrays.

There is little doubt that the areas containing concentrated, seemingly uncontaminated tobacco necrosis virus correspond to the hyaline inclusions, but it is less certain that the areas containing the aligned particles of tobacco necrosis virus correspond to the granular inclusions that contain crystals. Doubt arises from the fact that when pellets of crystals of tobacco necrosis virus formed *in vitro* were fixed and sectioned they survived the treatment. However, the crystals used were formed in a salt-free solution after many months at 4° and the solubility of tobacco necrosis virus crystals depends on the conditions in which they form. Crystals formed by quick crystallization in the presence of salt dissolve more easily than those formed slowly from salt-free solutions. Even then, the crystals of tobacco necrosis virus formed *in vitro* that were sectioned tended to come apart and form rows of particles, while those of satellite virus did not, although they were from quick crystallization. Hence, even the crystals seen outside the inclusions in living cells may have contained tobacco necrosis virus but have come apart during fixation, especially as Edwardson, Purcifull &

Christie (1966) reported crystalline forms in cells infected with tobacco necrosis virus alone. They used Dalton's chrome-osmium fixation, which probably has less effect on the tobacco necrosis virus crystals.

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(*Received* 18 *December* 1969)