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The Quality of Virus as Affected by the Ambient Temperature

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SUMMARY

Dolichos enation mosaic virus is a serotype of tobacco mosaic virus infecting leguminous plants; it produces a large proportion of particles shorter than 3000 Å, the accepted length of infective tobacco mosaic virus. A new strain of dolichos enation mosaic virus isolated from a single necrotic lesion on French bean caused local and systemic necroses in French bean plants. It produced many more defective particles than the parent strain when infected plants were kept at 20° but not at temperatures above 32°. Both strains multiplied faster at 32° than at 20°, but the effect of increasing temperature was greater with the new strain. The effect on the new strain appeared greater when virus content was assayed by infectivity tests than when it was assayed serologically, suggesting that infectivity per unit weight of virus was also greater at 32° than at 20°. No evidence was found that leaves infected with the new strain contained free infective RNA.

The ultraviolet absorption spectra of purified preparations of the new strain produced at 20° had a greater optical density at 280 mμ than at 260 mμ, whereas the parent strain had greater density at 260 mμ, showing that some of the particles of the new strain were without RNA. The type of particles produced by the two strains differed when purified preparations in 0·06 M-phosphate buffer at pH 8 were subjected to analytical ultracentrifugation in sucrose gradient columns or fractionation through agar columns. When produced in plants at 20°, the new strain consisted mainly of ring-like particles, virus protein, some free RNA and very few infective particles. By contrast, the parent strain consisted mainly of infective virus and broken particles of various lengths, of which a particle 400 Å long was plentiful enough to give a peak in the analytical centrifuge and a zone in sucrose gradient columns. The new strain produced in plants at 32 to 36° did not differ in appearance or infectivity from the parent strain. The distribution of particle lengths in sprayed droplets, measured in the electron microscope, confirmed the difference found by other means between the type of particle in the two strains.

Changing the pH value of a purified preparation of the new strain (produced at 20°) from 5·2 to 8 released some RNA in amounts suggesting that about a third of the particles in the preparation released their RNA.

INTRODUCTION

Dolichos enation mosaic virus (DEMV) affects various leguminous plants in India (Capoor & Varma, 1948) and is serologically related to tobacco mosaic virus (Badami, 1963). From an inoculum of DEMV, kindly supplied by Dr T. S. Sadasivan of the Botany Department, University of Madras, we isolated a strain (NDEMV) causing discrete necrotic local lesions in the primary leaves of French beans (*Phaseolus*

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vulgaris L.) var. Prince, and differing in many ways from the parent culture. Bean plants infected with DEMV and grown at about 20° contain many short particles, but plants infected with NDEMV contain very many more. Although sap from beans infected with the two strains contains similar amounts of virus protein, sap from plants infected with DEMV is much more infective. However, these differences in behaviour depend on the temperature at which infected plants are kept and disappear when the plants are kept at 32° or above.

This paper describes some properties of the two strains and their behaviour in plants kept at different temperatures.

METHODS

Inocula. Sap from a batch of bean plants infected with NDEMV was stored in small tubes at −15° and used as inoculum when required. The inoculum for DEMV was fresh sap from infected beans growing in the glasshouse. Virus for purification was propagated in the primary leaves of French beans var. Prince inoculated with the aid of carborundum. From plants kept at 20° the leaves were collected about 10 days after inoculation. Plants kept at 32° or above were put at these temperatures 1 day after they were inoculated and the leaves collected 4 to 5 days later.

Virus purification. The leaves were macerated in a blender with a minimal volume of 0·2 M-phosphate buffer pH 5·2; buffer of this pH was used to prevent particles of NDEMV disrupting. The pulp was kept at −15°, thawed when required, and the sap extracted through muslin and clarified by centrifugation for 10 min. at 10,000 rev./min. The clarified sap was then centrifuged for 1½ hr at 30,000 rev./min. and the pellets containing the virus taken up in water. After 2 or 3 more cycles of differential centrifugation, taking up the pellets in a smaller volume of water each time, the preparation was dialysed against water, clarified by low-speed centrifugation and the dry weight of a sample was measured.

Infectivity assay. The relative infectivities of different preparations were compared by inoculating tobacco plants (*Nicotiana tabacum* L.) var. Xanthi, which produces local lesions with both strains. The plants were trimmed to 3 or 4 leaves by removing the oldest and youngest leaves. Usually each sample was inoculated to 8 half-leaves so that the different samples appeared the same number of times in all the plants and leaf positions. When preparations of the two strains were compared the number of lesions produced by two dilutions of one strain were fitted to a dilution curve constructed from six dilutions of the other strain.

Ultraviolet absorption. The ultraviolet (u.v.) absorption spectra of purified virus preparations were measured in a Unicam SP 800 double-beam recording spectrophotometer; optical density (E) at a single wavelength (260 mμ or 280 mμ) was measured with a Unicam SP 500 single beam spectrophotometer.

Serological tests. An antiserum against NDEMV was produced by injecting a rabbit once intravenously and thrice intramuscularly during a period of 6 weeks with a total of 5 mg. of virus. Serological tests were made, either by the tube precipitation method or by the double diffusion precipitation method in gels containing 0·75% (w/v) Ionagar (Oxoid), 0·75% (w/v) sodium chloride in 0·01 M-phosphate buffer pH 8 with 0·002% (w/v) sodium azide. When testing sap, this was first clarified by heating at 50° for 10 min. and centrifuging for 10 min. at 10,000 rev./min.

Column separation. Particles of different sizes in purified preparations suspended in

0·06 M-phosphate buffer pH 8 were separated by passage through a 70 × 2 cm. column of 4% (w/v) agar (Steere & Ackers, 1962) buffered with EDTA-phosphate buffer pH 7·5 (neutral 0·01 M-EDTA, 0·01 M-phosphate buffer pH 8, 0·001 M-sodium azide). The same buffer was used to elute the particles from the column. The rate of flow was approximately 10 ml. per hr and tubes were collected at hourly intervals.

Density-gradient centrifugation. Density gradients ranging from 10 to 40% (w/v) sucrose in 0·06 M-phosphate buffer pH 8 were made mechanically in 50 ml. centrifuge tubes. One ml. of a purified preparation containing 1 to 3 mg. of virus was overlayered and the tubes spun for 3½ hr ar 21,000 rev./min. The zones were visible in transmitted light and removed either by piercing the side of the tube or by pumping the contents of the tubes out through a LKB Uvicord monitoring at 254 mμ.

Electron microscopy. Preparations from leaf cells for electron microscopy were made by touching the cut edge of a leaf against a drop of water on a carbon-coated platinum grid; excess water was removed and the material mixed with an equal volume of negative stain. Before examining material from sucrose density-gradient tubes, the sucrose was removed by dialysis against 0·06 M-phosphate buffer pH 8. Preparations examined for particle length distribution were sprayed through a cascade impactor on to collodion-coated copper grids (Nixon & Fisher, 1958) or directly on to carbon-coated platinum grids. A solution of 2% sodium phosphotungstate was used as a negative stain and grids were examined in a Siemens Elmiskop I at 80 kv.

Analytical ultracentrifugation. Sedimentation analyses were made in a Spinco model E analytical centrifuge equipped with a modified u.v. optical system (Markham, 1963). U.v. absorption photographs were scanned with a Joyce-Loebl automatic micro-densitometer and sedimentation coefficients determined by the graphic method of Markham (1960).

RESULTS

Serological relationships with viruses of the TMV group

Antisera to NDEMV and to the type strain of tobacco mosaic virus (TMV) contain only a few common antibodies. When titrated against their homologous viruses at 20 μg./ml., the titre of NDEMV antiserum was 1/640, and of TMV antiserum 1/1280, but against the heterologous virus each had a titre of only 1/40. Removing the common antibodies by absorption with the heterologous virus left the titres of both antisera with the homologous virus unchanged. In diffusion tests in agar gel with either of the two antisera and the two viruses in adjacent wells, the heterologous virus produced only a faint line and the homologous virus formed a pronounced spur. Also, when the two viruses were tested against a mixture of the two antisera, the lines crossed each other, forming double spurs which were not deflected. Thus NDEMV and TMV have very few antigenic determinants in common and should be considered as serotypes rather than strains (Kassanis, 1961). In addition to the line formed by the virus near the antigen well, preparations of DEMV and NDEMV always formed a line about half-way between antigen and antibody wells. This is caused by small pieces of virus protein. Only old preparations of TMV produced this line and when such preparations were placed in wells adjacent to the Indian serotypes, spurs formed in both the virus and virus protein lines against the homologus antisera.

DEMV is closely related to the cowpea virus (Lister & Thresh, 1955) and the Indian southern sunn-hemp mosaic virus (Capoor, 1950), which were shown by Bawden

(1958) to be related to TMV. These two viruses, like DEMV, infect leguminous plants and in French beans var. Prince produce very similar symptoms. The plants are severely stunted, the trifoliate leaves show severe yellow mottle and blistering, and become very malformed (Bawden, 1958). The symptoms are influenced considerably by the environment. NDEMV differs from the others by causing necrotic local lesions in beans var. Prince and systemic necrotic lesions often extensive enough to kill the plant. However, sometimes during summer, bean plants recover from the initial severe reaction and the trifoliate leaves then show a bright yellow mottle.

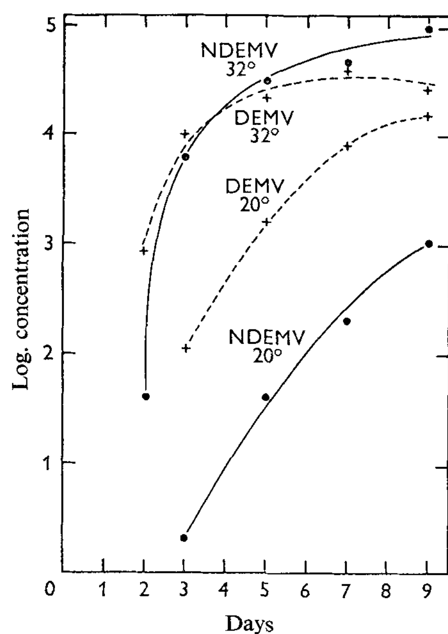
Comparison of infectivity and serological activity in sap

Sap from leaves infected 10 days previously was 10 times more infective from plants (grown at glasshouse temperatures) infected with DEMV than with NDEMV, although its antigen content was only twice as much. Electron microscopy by the cut-leaf method showed that leaves infected with DEMV contained mainly normal length rods (3000 Å), although there were many more shorter particles than is usual in plants infected with TMV. By contrast, the specific contents of leaves infected with NDEMV consisted of rings and very short particles, with only an occasional particle of normal length. Many of the larger particles also showed signs of erosion or they seemed to have broken into smaller pieces during preparation for electron microscopy (McCarthy & Woods, 1967). The appearance of cut-leaf preparations of NDEMV varied with the season, probably depending on the mean glasshouse temperature; sometimes there were few or no rings and the preparations consisted mainly of short rods, presumably broken particles.

The two strains differed more when the infected plants were grown at glasshouse temperatures averaging 20°, and behaved much alike in plants kept at 36°. For instance, in one comparison with infected plants kept for 4 days at 20° or 36°, sap from those infected with DEMV had precipitation titres of 1/40 and 1/160, and those infected with NDEMV 1/5 and 1/160 respectively. Sap from plants kept at 36° was equally infective whether they were infected with DEMV or NDEMV, but with plants at 20° sap from those with DEMV was more than 10 times as infective as from those infected with NDEMV (at equal sap dilutions, 374 lesions were obtained with DEMV and 27 with NDEMV). Similarly, although electron microscopy showed great differences between particles in plants infected with the two strains and kept at 20°, it showed no obvious differences when plants were kept at 36°, when the particles all resembled those of DEMV from plants kept at 20°.

As the amounts of virus produced by the two strains in plants kept at 20° differed more at 4 days after inoculation than at 10 days, an experiment was made to measure virus increase at four different temperatures. Virus concentration was estimated both by serological and infectivity tests. The inoculum was concentrated virus plus carborundum, and the inoculated bean plants were kept for a day at glasshouse temperatures and then placed in constant-temperature glass cubicles. Samples of 6 leaves were taken at 2, 3, 5, 7 and 9 days after they were inoculated. The extracted sap was divided into 3 parts and kept at −15°. One of these was used for infectivity tests at dilutions based on the serological titre, but sometimes a second test was needed because the number of lesions did not fit the dilution curve. At first, each infectivity test compared four samples from the same temperature with four dilutions of a purified virus preparation. However, as the dilution curves from the purified preparation were similar in successive

tests, a master dilution curve was plotted to which the lesion numbers from the test samples were fitted and translated into virus concentration. The comparisons were made at 20°, 24°, 28° and 32°, but for simplicity only the results at 20° and 32° are given (Fig. 1, 2). Both strains increased faster at 32° than 20°, but increasing the temperature had more effect with NDEMV than with DEMV, and, more interestingly, increasing temperatures increased NDEMV as measured by infectivity tests much more than when measured serologically. Hence not only was more NDEMV produced at 32° than at 20°, but weight for weight it was more infective. The results at 24° and 28° were intermediate between those at 20° and 32°, so the infectivity per unit weight



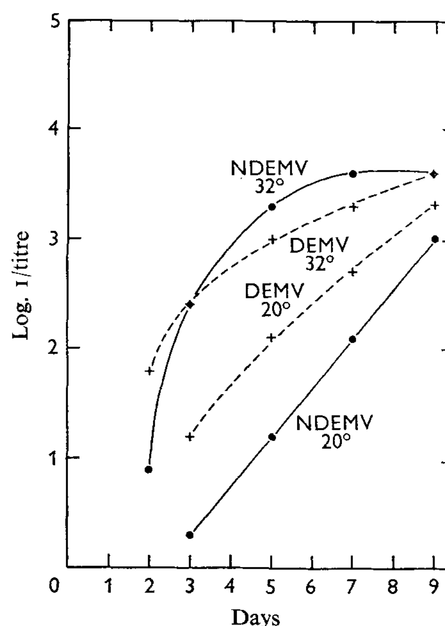


Fig. 1. Increase in infectivity of crude sap of French bean plants infected with NDEMV and DEMV and kept at 20° or 32°.

Fig. 2. Logarithm of the reciprocal of the serological titre of heat-clarified sap from plants infected with NDEMV and DEMV and kept for various times at 20° or 32°.

of NDEMV was increased by raising the temperature; as will be shown later, at temperatures above 32° the two strains produced virus of nearly the same type and of equal infectivity.

The discrepancy between estimates of the concentration of NDEMV at 20° by infectivity assays and serological tests could be caused by failure of the virus protein to combine with virus RNA, so the infectivity of extracts of infected leaves made in water and phenol was compared. Phenol extracts had less than 1 % of the infectivity of water extracts, which is about the proportion usually obtained when the nucleic acid is extracted from purified virus preparations; this suggests the leaves contained little uncoated infective RNA.

Genetic stability of the strains

Attempts were made to find whether NDEMV contained strains differing in their temperature optima for multiplication or whether it produced variants at high temperatures that are favoured by heat. No evidence for either idea was found. Plants infected with NDEMV were kept for 6 days at 36° and sap from them inoculated to healthy plants, which were kept at 20°. These plants contained a virus population as defective as the original NDEMV, i.e. it was poorly infective, and consisted mainly of ring-like particles and short rods. Also, inoculum from more than 20 single necrotic lesions produced on bean leaves by NDEMV contained only typical NDEMV. Similarly, its behaviour did not change in six successive transmissions through bean plants. By contrast, DEMV continuously produced the defective type, which could readily be isolated by repeated single lesion transfers from the occasional necrotic lesion produced in bean leaves inoculated with DEMV.

Purified preparations

The differences in appearance and infectivity between NDEMV and DEMV particles and their close similarity when infected plants were kept at higher temperatures were even more obvious when purified virus preparations were compared. The preparations were in distilled water until used, when they were diluted with 0·2 M-phosphate buffer pH 8 or 5·2 to give a final salt concentration of 0·06 M. Unless otherwise mentioned the preparations were from plants grown at 20°.

Ultraviolet absorption spectra

NDEMV preparations always absorbed more at 280 mμ than at 260 mμ, whereas DEMV absorbed more at 260 mμ than at 280 mμ (Fig. 3). As the preparations were not contaminated with plant material, this indicated that the ratio of protein to RNA was greater in preparations of NDEMV than of DEMV. NDEMV prepared from plants kept at 32° to 36° gave an absorption spectrum similar to that of DEMV at 20°.

Analytical centrifugation

Preparations of the two strains in 0·06 M-phosphate buffer pH 8 when subjected to analytical ultracentrifugation differed in the kind of components they contained and in their relative amounts. The relative proportions of these components also varied slightly between preparations as did their sedimentation coefficients. In the schlieren optical system, peaks 1, 2, and 3 of DEMV had uncorrected sedimentation coefficients of approximately 190 *S*, 70 to 80 *S* and 20 to 50 *S* respectively (Pl. 1, fig. 1 A), whereas peaks 1, 3 and 4 of NDEMV had constants of 190 *S*, 20 to 30 *S* a 2 to 10 *S* respectively (Pl. 1, fig. 2 A). The absence of peak 2 from NDEMV and of peak 4 from DEMV may mean only that the components representing these two peaks were present in a quantity too small to register. The sedimentation patterns show that whereas most of the virus in preparations of DEMV consisted of complete particles, (190 *S*), most of the material in preparations of NDEMV is virus protein (2 to 10 *S*) and rings or very short particles (20 to 30 *S*). In the ultraviolet absorption optical system, DEMV showed sharp boundaries of good contrast corresponding to peaks 1 and 2, whereas the NDEMV samples showed strong absorption right up to the front edge of peak 3 (Microdensitometer trace in Pl. 1, fig. 1 B, 2 B). Later investigation

showed that the ultraviolet absorption by NDEMV preparations was probably from free nucleic acid and oligonucleotides, rather than from the occurrence of nucleic acid in ring-like particles constituting peak 3 (Pl. 1, fig. 2B). The results obtained with DEMV suggest that neither the 2 to 10 *S* nor the 20 to 30 *S* component contains nucleic acid, but that broken particles of longer lengths do. The other difference between the two strains is a pronounced shoulder to the 190 *S* peak and the 70 to 80 *S* peak seen only with DEMV. The large amounts of broken particles of different lengths is a characteristic feature of all viruses of the group to which DEMV belongs. Some of these broken particles seem to have caused the shoulder of the 190 *S* peak but there also seems to be enough of a particular length of about 400 Å to form a peak

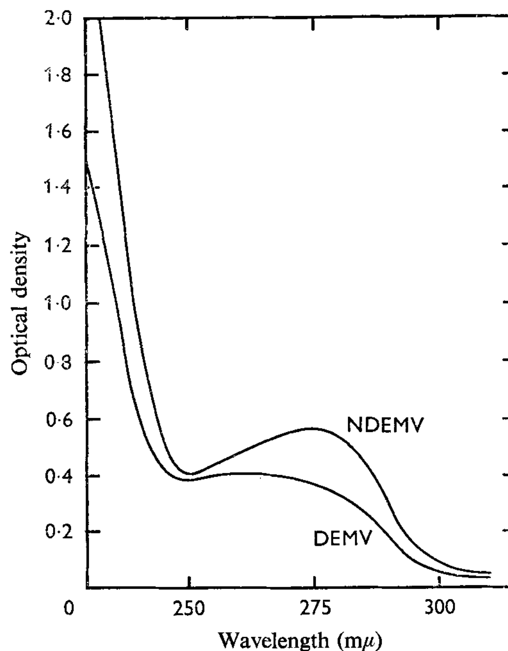


Fig. 3. Ultraviolet absorption spectra of 0·226 mg./ml. purified preparations of NDEMV and DEMV in water.

(70 to 80 *S*) on its own. The 70 to 80 *S* fraction, isolated by sucrose density gradient centrifugation, was not infective but contained RNA. The same range of broken particles is very likely present also in NDEMV, but as these amounts are probably proportional to the total amount of complete virus, which in NDEMV is very small, they do not register in the analytical sedimentation pattern. When NDEMV preparations were centrifuged at pH 5·2 the virus aggregated and the 2 to 10 *S* and 20 to 30 *S* components of NDEMV then moved as one peak with a sedimentation constant of 20 to 30 *S*. Some aggregation occurred also with DEMV but not enough to affect the sedimentation constant of the 70 to 80 *S* component or the shoulder of the 190 *S* component, though the height of the 20 to 30 *S* component was increased (Pl. 1, fig. 3).

NDEMV produced at 32° to 36° gave a sedimentation pattern similar to that of DEMV at 20° (Pl. 1, fig. 4); the patterns formed by DEMV preparations were the same regardless of the temperatures at which infected plants were kept.

Sucrose density gradient centrifugation

NDEMV formed three zones, two at about 0·6 and 1 cm. from the meniscus, which are equivalent to the two slow-moving components shown in the analytical centrifuge, and a third zone at 4 cm. from the meniscus containing the complete virus. DEMV formed two zones: one at about 2 cm. from the meniscus containing particles 400 Å long (the 70 to 80 *S* component of the analytical centrifugations), and the zone of the complete particles, which was much broader than with NDEMV but at the same position in the gradient. Also, there was a considerable extension of this zone upwards, containing broken particles graded according to their length. Samples of the zones were taken and identified in the analytical centrifuge and electron microscope. Except for isolating the 400 Å long particles of DEMV this method was of little use in separating the different fractions.

Column separations

This was by far the best method of separating the preparations into fractions of different-sized particles. When the fractions were plotted against their optical density

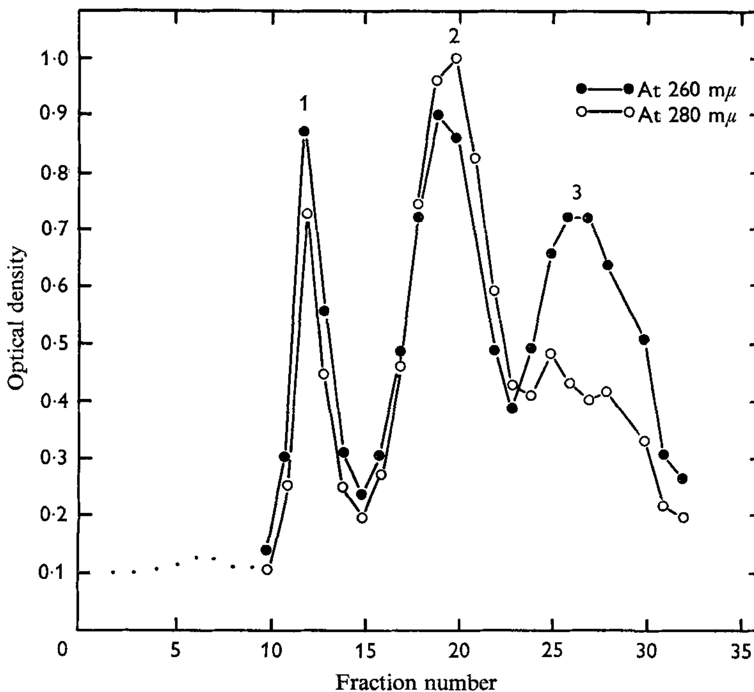


Fig. 4. Optical density at 260 mμ and 280 mμ of fractions obtained from 41 mg. of a purified preparation of NDEMV after separation on an agar column.

at 260 mμ and 280 mμ each strain yielded a characteristic pattern, Three components were identified in each separation, each having different 260/280 mμ ratios as shown by the three peaks 1, 2 and 3 in Fig. 4, 5. The relative amounts of the three components in the two strains differed and absolute amounts could be found only after the *E* values had been corrected for light scattering. However, direct comparison of the

relative amounts of the same component between the two strains could be based on the height of and area under the peaks. On this basis the amount of virus represented by peak 1 (260/280 mμ = 1·2) was much larger in DEMV than NDEMV. By contrast the amount of rings and virus protein represented by peak 2 (260/280 mμ = 0·85) and peak 3 (260/280 mμ = 1·5) were large in NDEMV and hardly present in DEMV.

Electron microscopy on selected fractions of NDEMV showed that those representing the front of peak 1 contained full-length particles and later fractions contained a

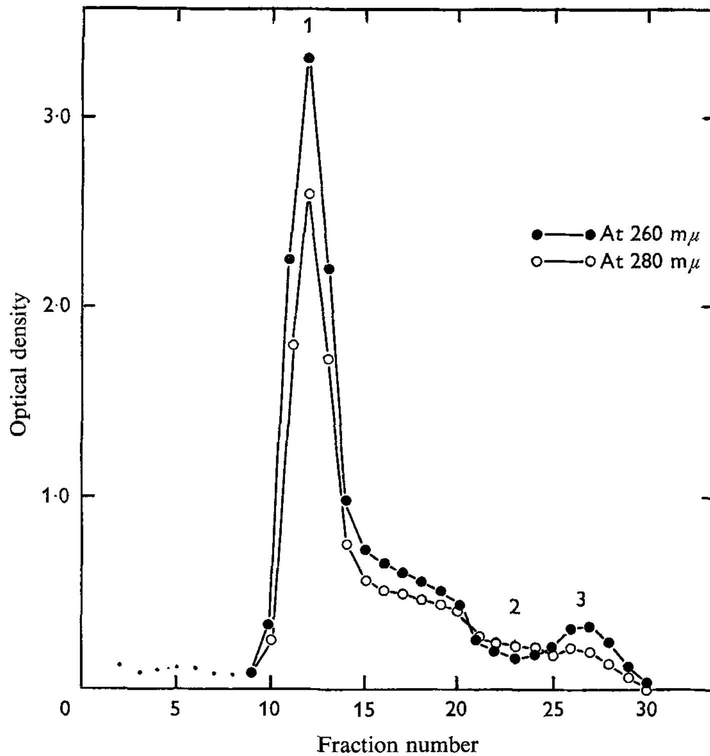


Fig. 5. Optical density at 260 mμ and 280 mμ of fractions obtained from 29 mg. of a purified preparation of DEMV after separation on an agar column.

mixture of particle sizes whose average length decreased progressively. The fractions at the apex of peak 2 (Fig. 4) contained ring-like particles or very short rods (Pl. 2, fig. 1) and those of peak 3 contained only amorphous material, However, acidifying to pH 4·5 with acetic acid caused the amorphous material to aggregate into rods of various lengths. The small 260/280 mμ ratio of peak 2 was very similar to that of 'A' protein prepared from this virus and indicated that ring-like particles were devoid of RNA. The large 260/280 mμ ratio of peak 3 (Fig. 4) was apparently caused by free RNA components being eluted at about the same position as the virus protein, because tubes from this region gave a positive result when tested with Bial's orcinol reagent and a very large 260/280 mμ ratio, after removal of protein by ammonium sulphate.

Infectivity tests with selected fractions from NDEMV preparations showed that only those of peak 1 were infective. The fractions of peaks 2 and 3 gave only an oc-

casional lesion when tasted after concentration. Recombining selected fractions from peaks 2 and 3 with the contents of a tube from peak 1 before inoculating to Xanthi plants did not enhance the infectivity of the peak 1 fractions. Further, when only particles of normal lengths of NDEMV and DEMV (tube 2, peak 1) were inoculated to beans kept at 20°, and the subsequent virus produced was purified, the whole range of particle sizes characteristic for each strain was again found.

In precipitation tube tests the fractions of peaks 1 and 2 gave a flagellar type of precipitate; fraction 20 of peak 2 (Fig. 4) produced two zones of precipitation, possibly because some small particles behaved as somatic type and larger ones as flagellar. That peak 2 of NDEMV contains a range of short ring-like particles was demonstrated by sedimenting the material in the analytical ultracentrifuge. At 29,500 rev./min. two peaks of 2 to 10 *S* and 20 to 30 *S* were formed similar to those shown in Pl. 1, fig. 2. At faster speed (59,780 rev./min.) the 20 to 30 *S* peak resolved to give three peaks of 28, 22 and 18 *S*, whereas the apex of the slowest peak still had a sedimentation constant of 6 *S*. Fraction 27 of peak 3 (Fig. 4) produced a somatic type of precipitate, which took a long time to form, and its titre was very small. In a comparison between fractions 12 (full-length particles) and 27 (unaggregated virus protein), having the same *E* at 280 mμ, the serological titres were 1/32 and 1/4 respectively. Yet when fraction 27 was brought to pH 4·5, its titre increased to 1/16 and the type of precipitate changed from somatic to flagellar.

In gel diffusion tests the virus in fractions 10 to 12 (Fig. 4), which contained only particles of normal length (3000 Å), was precipitated before leaving the antigen well. The line was formed at the very edge of the well or appeared as two whiskers on each side of the well, but the distance of the line from the antigen well increased as the particles became shorter, i.e. with fractions 12 to 14. Fractions from peak 2, in addition to giving a line some distance from the antigen well, also produced a second one much nearer to the antiserum well. This line, which was caused by the unaggregated virus protein, was the only one produced by fractions of peak 3. The results suggest that, with viruses of the TMV group, when a precipitation line is formed away from the antigen well, it indicates that the preparation contains fragmented particles.

Particle length distribution

Droplets of equal concentrations of preparations of the two strains stained with phosphotungstate were examined in the electron microscope. Lengths of about 3000 particles of each strain in ten droplets were measured. As the ratio of the number of ring-like particles to those of normal length in NDEMV preparations was 400:1, the usual histogram of length distribution would have been unwieldy. Therefore the number of particles of a given length was divided by the number of times that particular length could be accommodated in a 3000 Å particle and the results expressed as a percentage of the total population (Fig. 6, 7), giving a measure of the degree of degradation of the population, if it is assumed that all short particles arose by fragmentation from those originally 3000 Å long. Results show that only about 35 % of the populations of DEMV and 20 % of NDEMV in 0·06 M-phosphate buffer (pH 8) were 3000 Å long, whereas 30 % of NDEMV were in the form of ring-like structures and only 15 % of DEMV.

Pl. 2, fig. 2 to 4 show micrographs of purified preparations at pH 8 made from infected plants grown at 20° and 36° (DEMV produced at the two temperatures is

identical). The particle length distribution in similar preparations was compared (Fig. 8, 9). The preparations made from plants kept at 36° contained very few of the ringlike particles (shorter than 200 Å) so characteristic of NDEMV from plants grown at 20°. The general pattern of length distribution of NDEMV from plants at 36° resembled that of DEMV at 20°, although the proportion of particles with lengths between 500 to 2000 Å was slightly larger.

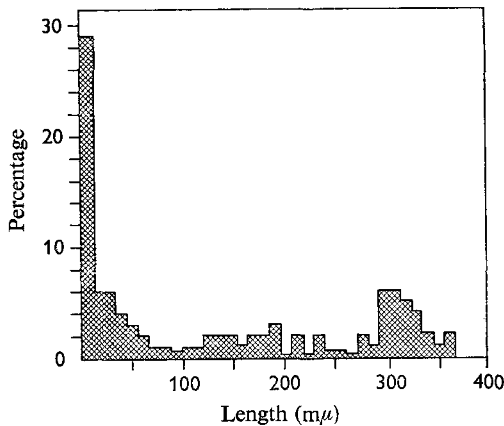


Fig. 6

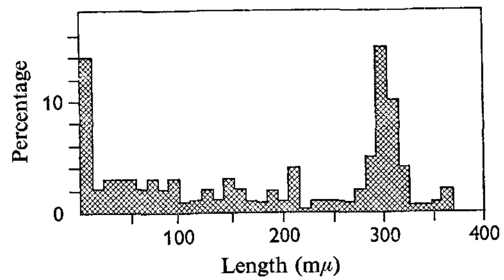


Fig. 7

Fig. 6 and 7. Histograms of particle length distribution of purified preparations of NDEMV (Fig. 6) and DEMV (Fig. 7) at pH 8. Instead of numbers of particles the vertical axis corresponds to $n_L \times$ (L Å/3000 Å), where n_L = the number of particles of a length L. The value expressed as a percentage of the population.

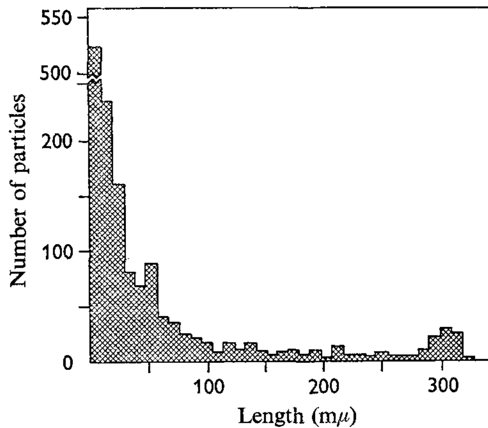


Fig. 8

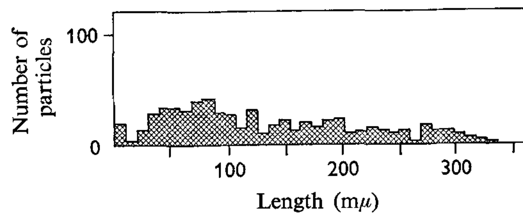


Fig. 9

Fig. 8 and 9. Histogram of particle length distribution of purified preparations of NDEMV obtained from plants grown at 20° (Fig. 8) and 36° (Fig. 9).

Changing the pH of NDEMV preparations

The extraction procedure described was used to protect the particles from premature breakage by providing conditions favourable for aggregation. Changing the pH value of purified preparations of NDEMV from 5 to 8 produced many short

particles; this may have restored them to the conditions in which they existed in the plant before extraction or it may have disrupted some particles more susceptible than others to fragmentation at pH 8. To clarify this point one sample of a purified preparation was adjusted to pH 5 and another to pH 8 in 0·06M-phosphate buffer and both centrifuged for $1\frac{1}{2}$ hr at 25,000 rev./min. Measurements of optical density at 260 mμ and 280 mμ showed that at pH 5 97% of the material was sedimented and this had an *E* 260/280 mμ ratio of 1·0, whereas the 3% in the supernatant fluid had an *E* of 1·1. At pH 8 only 5% of the material was sedimented and this had an *E* 260/280 mμ ratio of 1·2, whereas the 95% in the supernatant fluid had a ratio of 0·9. Electron microscopy showed the material sedimented at pH 5 was composed of particles with an assortment of different lengths, whereas material sedimented at pH 8 was composed mainly of particles 3000 Å long. Infectivity tests on the sedimented materials, at equivalent concentration based on their *E* 260 mμ, indicated that the material sedimented at pH 8 was about ten times as infective as that sedimented at pH 5.

Ammonium sulphate solution (adjusted to pH 8 with ammonia) was added to the supernatant fluid after centrifuging at pH 8 to produce a 30% saturated solution. The precipitate was separated by low-speed centrifugation and resuspended in 0·06M-phosphate buffer pH 8; it had an *E* 260/280 mμ ratio of 0·88 which is close to that of 'A' protein prepared from this virus. The material not precipitated by ammonium sulphate had an *E* 260/280 mμ ratio of 2 and gave a positive reaction with Bial's orcinol reagent.

The weights of material recovered in the different fractions differed with different preparations and this variability probably reflects the variations in the mean glasshouse temperature. However, to quote an average result, when 100 mg. of an NDEMV preparation were adjusted to pH 8, 5 mg. were recovered as particles 3000 Å long in the pellet from high-speed centrifugation, and the supernatant fluid contained 93·5 mg. of protein and 1·5 mg. of RNA. Had the RNA come from particles susceptible to breakage at pH 8 but containing the usual proportion of protein to RNA, this would have been accompanied by about 30 mg. of protein. Thus some 60 mg. of the original 100 mg. must have been viral protein not associated with nucleic acid.

DISCUSSION

Purified preparations of DEMV, like those of other strains of TMV infecting leguminous plants, contained unusually large numbers of particles shorter than the normal length. Their specific infectivity was only about 1% that of the type strain of TMV. Short particles, presumably caused by breakage of long particles, were more common at pH 8 than pH 5·2, but occurred at pH 5·2. Numerous short particles also occurred in sap from a cut leaf, and although this may not be evidence for the existence of such particles in unbroken cells, it showed at least that the particles were much more easily fractured than those of TMV. DEMV resembles in this respect *Odontoglossum* ringspot virus, which like DEMV is remotely related serologically to TMV, and preparations contain many seemingly broken virus particles, some of which are without RNA (Paul *et al.* 1965). Most of the short particles of DEMV seem to be of random lengths, except that there were enough particles of about 400 Å long to produce a peak 70 to 80 *S* in the analytical centrifuge. Lister (1966) showed that the

short particles of tobacco rattle virus have an important biological function because they contain the information needed to synthesize the virus coat protein. We could not demonstrate any biological activity of the 400 Å particles of DEMV; they were not infective and did not enhance the infectivity of other infective or non-infective fractions of DEMV or NDEMV.

The situation with NDEMV is much more complex, because in addition to the fact that from plants grown at 20° many more particles were broken and to much smaller sizes than with DEMV, this was not so for plants grown at temperatures of 32° or more. This increase in specific infectivity with increasing temperatures is very unusual, for with many other viruses exposing plants to high temperatures causes degradation of the viruses; they first lose their infectivity and then their serological activity (Kassanis, 1952; Lebeurier & Hirth, 1966). Indeed, heat therapy (exposing the plants for periods at 35° to 37°) is an effective method for curing many virus-infected plants (Kassanis, 1954).

NDEMV in some ways behaves like the 'thermophilic' strain isolated from the type strain of TMV by Lebeurier & Hirth (1966). The thermophilic strain multiplied better at 24° to 36° than at 16° and when produced at 16° to 20° had an E 260/280 mμ ratio of 0·8, instead of 1·1 found for virus produced at higher temperatures. When virus produced at 16° was centrifuged in CsCl density gradients, two zones were formed: the top zone contained capsids and the bottom zone complete virus particles. However, the thermophilic strain produced some capsids even at its optimal temperature of 36°. Without more information it is difficult to judge to what extent this strain resembles NDEMV. Lebeurier & Hirth (1966) suggested that the virus produced less RNA when the plants were kept cold; either the cistron of the RNA coding the synthesis of the RNA-specific replicase had been affected, or the effect depended on a defect in the synthesis of protein coded by the RNA, which in turn controlled an event permitting or preventing the specific replicase from functioning. The main reason for suggesting this explanation was that they could not demonstrate the presence of free infective RNA.

Our experience with NDEMV produced at 20° was similar, but another possible explanation for inability to find infective RNA could be that the protein was defective and any uncoated RNA was degraded. Although we cannot exclude the possibility that less RNA of NDEMV is produced at 20° than at 36°, and this leads to an excess of virus protein, there are two main ways in which NDEMV differs from the strain described by Lebeurier & Hirth. We have demonstrated that about 35% of purified virus still retained its RNA before the pH value was changed from 5 to 8 and this amount may have been even greater in the infected leaves. The second difference is that NDEMV breaks down to a much greater extent than the thermophilic strain.

Although most of the NDEMV produced at 20° was defective, a few normal-length particles were produced that remained infective for a long period (purified preparations of NDEMV without preservative, unlike TMV, may be easily degraded by growth of bacteria or fungi but retain some infectivity after many months). Moreover, as there is no evidence that NDEMV is a mixture of two genotypes, it seems that the same nucleic acid causes both good and defective protein to be produced, one bonding better than the other to form the more stable particles, and that the relative quantity of the two types of virus protein is a function of the ambient temperature. Our results suggest that there might be a certain ambiguity of the genetic code (i.e. in the transla-

tion of the genetic message related to the structure of the coat protein) and the degree of ambiguity is related to the ambient temperature. However, Wittmann (1965) found no variation in the amino acid compositions of the tryptic peptides with strains of TMV produced at different temperatures, varying from 12° to 37°.

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EXPLANATION OF PLATES

(*see overleaf*)

EXPLANATION OF PLATES

PLATE 1

Fig. 1. A, Sedimentation pattern of DEMV at a concentration of 6·8 mg./ml. in 0·06M-phosphate buffer pH 8 at 18°. The photograph was taken 12 min. after a speed of 29,500 rev./min. had been reached with a schlieren bar angle of 50°. Sedimentation is towards the right. B, Microdensitometer trace of the ultraviolet absorption of the same sample under identical conditions, but at a concentration of 0·1 mg./ml.

Fig. 2 A, Sedimentation pattern of NDEMV at a concentration of 6·8 mg./ml. in 0·06M-phosphate buffer pH 8, at 18°. The photograph was taken 12 min. after a speed of 29,500 rev./min. had been reached with a schlieren bar angle of 50°. Sedimentation is towards the right. B, Microdensitometer trace of the ultraviolet absorption of the same sample under identical conditions, but at a concentration of 0·1 mg./ml.

Fig. 3. Sedimentation patterns of NDEMV (upper) and DEMV (lower) in 0·06M-phosphate buffer (pH 5·2) at concentrations of 6·8 mg./ml. at a temperature of 16·5°. The photograph was taken 12 min. after a speed of 29,500 rev./min. had been reached with a schlieren bar angle of 40°. Sedimentation is towards the right.

Fig. 4. Sedimentation pattern of NDEMV obtained from plants grown at 36°. A purified preparation in 0·1M-potassium chloride pH 8 at a concentration of 2 mg./ml. at 19·8°. The photograph was taken 12 min. after a speed of 29,500 rev./min. had been reached with a schlieren bar angle of 50°. Sedimentation is towards the right.

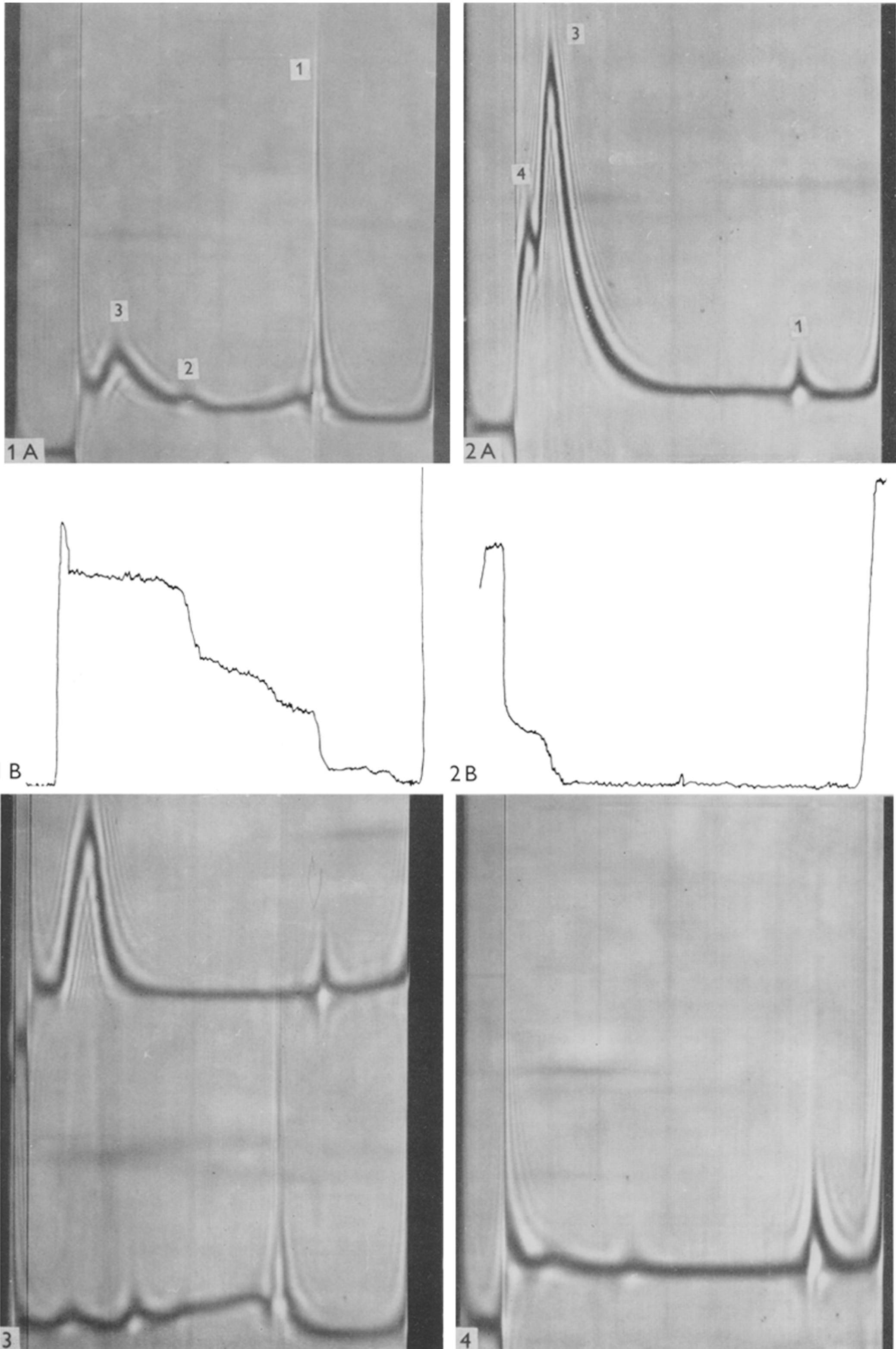
PLATE 2

Fig. 1. Electron micrograph of the contents of tube 20 after separation of NDEMV on an agar column, showing mainly ringlike or short particles.

Fig. 2. Electron micrograph of a purified preparation of NDEMV from plants grown at 20°, with many ringlike particles in side view.

Fig. 3. Electron micrograph of a purified preparation of NDEMV from plants grown at 36°.

Fig. 4. Electron micrograph of a purified preparation of DEMV from plants grown at 20°.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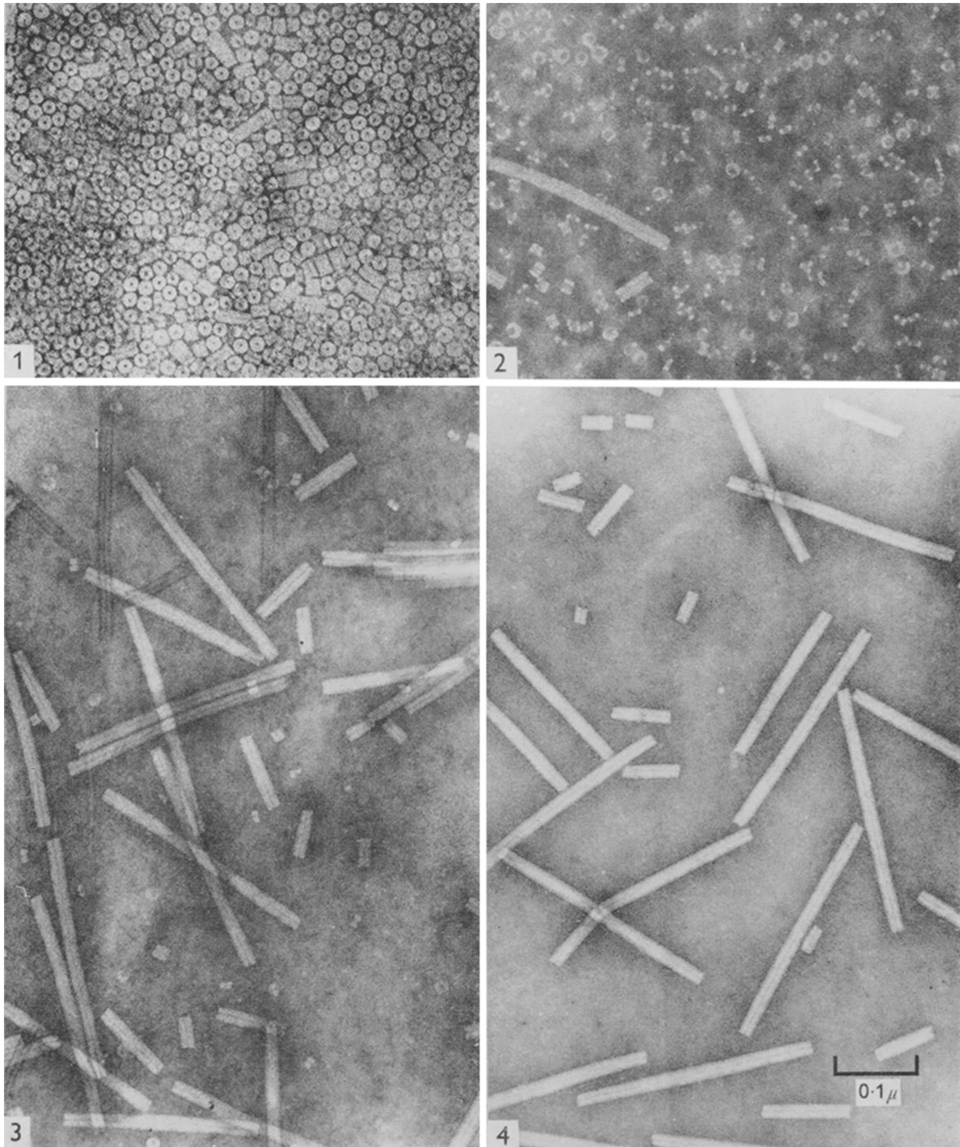

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