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A Comparative Study of Red Clover Vein Mosaic Virus and Some Other Plant Viruses

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SUMMARY

Red clover vein mosaic virus (RCVMV) was compared with other viruses, in particular white clover mosaic virus (WCMV) and clover yellow vein virus (CYVV). All three have filamentous particles, those of RCVMV are 645 nm. long and have a sedimentation coefficient of 160 S, those of WCMV are 460 nm. long (119S), and those of CYVV between 700 nm. and 800 nm. long (about 140 S). RCVMV and WCMV contain about 6% ribonucleic acid with nucleotide compositions of G 31·5, A 24·1, C 22·7, U 21·7 and G 15·5, A 31·8, C 26·9 and U 25·7% respectively. RCVMV is distantly serologically related to five viruses of the potato virus S group, CYVV to eight viruses of the potato virus Y group, and WCMV to potato virus X.

RCVMV is photoreactivable; after exposure to ultraviolet radiation, preparations of the virus caused more lesions in *Chenopodium amaranticolor* when the inoculated plants were kept in the light than in darkness; this is the first virus with seemingly rigid helically constructed particles to show photoreactivation.

INTRODUCTION

Red clover vein mosaic virus [cryptogram†: R/1:⋆/5:E/E:S/Ap], first isolated from diseased red clovers in the U.S.A. by Osborn (1937) has since been found in other parts of North America and Europe. Hagedorn, Bos & van der Want (1959) and Wetter, Quantz & Brandes (1962) showed that red clover vein mosaic virus (RCVMV) has straight filamentous particles about 650 × 12·5 nm. in size, and Wetter & Paul (1961) studied its serological relationships with other viruses. The host range, symptoms, vectors and stability of RCVMV are already well known, but in this paper other properties are described and compared with those of other clover viruses, in particular clover yellow vein virus [⋆/⋆:⋆/⋆:E/E:S/Ap] and white clover mosaic virus [R/1:⋆/5:E/E:S/(Ap)], two viruses commonly found with RCVMV in naturally infected white clover in Great Britain (Gibbs, Varma & Woods, 1966). Clover yellow vein virus (CYVV), like RCVMV, is transmitted by aphids in the non-persistent manner and has so far been reported only from Great Britain (Hollings & Nariani, 1965; Gibbs *et al.* 1966). In contrast, white clover mosaic virus (WCMV) has been isolated from clovers in many parts of the world and several workers have studied its properties in detail (Bancroft, Tuite & Hissong, 1960; Bercks & Brandes, 1961; Iizuka & Iida, 1965).

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† Cryptograms are from Martyn (1968) and include information reported in this paper.

METHODS

Viruses. The isolates of the viruses used came from white clover plants naturally infected in Great Britain. RCVMV isolates were maintained and grown in *Pisum sativum* L. (Onward), CYVV in *Nicotiana clevelandii* Gray and WCMV in *Phaseolus vulgaris* L. (The Prince). The plants were grown in insect-free glasshouses at about 20°, with supplementary artificial light during the winter.

One isolate of RCVMV was used that gave countable chlorotic local lesions in the inoculated leaves of *Chenopodium amaranticolor* Coste and Reyn. *C. amaranticolor* was also used as a local lesion host of CYVV, and preparations of WCMV were assayed in *P. vulgaris.*

Purification. RCVMV was grown in Onward pea plants and purified as follows: leaves of infected peas were harvested 15 to 22 days after inoculation, when showing clear symptoms, and were then used immediately or stored at −20°. The fresh or frozen leaves were triturated mechanically with twice their weight of neutral phosphate + ascorbate buffer (equal volumes of 0·1 M-disodium hydrogen phosphate and 0·05 M-ascorbic acid). Chloroform was added and the mixture was shaken to form an emulsion which was centrifuged at 8000 ***g*** for 10 min. The aqueous phase was collected and centrifuged for 90 min. at 76,000 ***g*** to give a pellet which was resuspended in neutral 0·005 M-borate buffer. After clarification at 8000 ***g*** for 10 min. this gave the partially purified preparation. The virus-containing pellet usually resuspended readily, but some pellets from heat-clarified sap (50° for 10 min.) did not.

Preparations were further purified most easily by rate zonal centrifugation at 45,000 ***g*** for 75 min. in gradients (Brakke, 1960) of 10 to 40 % sucrose in neutral ascorbate + phosphate buffer. An alternative but longer method was that of restricted diffusion chromatography in agar gel (Steere & Ackers, 1962) using a 70 × 2 cm. column of 4 % agar chips (20 to 40 μm. diameter) and a buffer flow rate of 10 ml./hr.

CYVV was purified by the method of Hollings & Nariani (1965), and WCMV by a method similar to that of Bancroft *et al.* (1960) except that neutral ascorbate–phosphate buffer replaced water.

Electron microscopy. Preparations of the viruses were examined for morphology and particle length in a Siemens Elmiskop I electron microscope (Gibbs *et al.* 1966; Nixon & Harrison, 1959). As the numbers of particles of different lengths near the 'most common length' often showed a skew distribution, the modal lengths (Moroney, 1951) rather than the mean lengths ('Normallängen', Brandes & Paul, 1957) were calculated.

Sedimentation analyses were made in a Spinco Model E analytical centrifuge using schlieren and absorption optical systems. A graphical method (Markham, 1960) was used to estimate sedimentation coefficients.

Nucleotide compositions of the particles of RCVMV and WCMV were estimated by a method similar to that described by Markham (1955); the nucleic acid was released from the virus and hydrolysed in 1 N-HCl, the liberated pyrimidine nucleotides and purines separated chromatographically, using a tertiary butanol:HCl:water (70:7:23) solvent.

Antisera. Partially purified preparations of RCVMV, CYVV and WCMV were used to immunize rabbits. Each rabbit was injected intravenously four times at weekly intervals, followed by two intramuscular injections of virus in Freund's complete adjuvant. Twenty days after the last injection each rabbit was killed, its blood collected and centrifuged and the serum stored at 4° with 0·5 % phenol. Serological precipitation tests were made either by an Ouchterlony diffusion-in-gel method (Mansi, 1958) or by incubation of mixtures in tubes in a water bath at 37° (Bawden, 1950). Titres were estimated as described by Gibbs *et al.* (1966).

Some virus preparations were treated with ultrasound to disrupt the particles before use in Ouchterlony tests (Tomlinson *et al.* 1965). Five ml. of the preparation was put in a thick glass tube (75 × 14 mm.) and surrounded by ice. Into the preparation was put the terminal 0·5 to 1·0 mm. of the probe of a 60 W ultrasonic drill (MEL Equipment Co., Type E7680/3, 20 kHz); the 'step-down' titanium probe had top and bottom diameters of 18 mm. and 6 mm., respectively. Each preparation was treated for up to 17·5 min.; samples of 0·5 ml. were removed after 30 sec. (WCMV), 2·5 min. and 7.5 min. When these preparations were tested serologically the clearest bands of precipitate were obtained using 0·7 % agar gel buffered with neutral 0·05 M-diamino-ethane-tetracetate/borate buffer.

Ultraviolet irradiation. Sap from *C. amaranticolor* leaves infected with the lesion-producing isolate of RCVMV was used as inoculum in tests for photoreactivation. The sap was extracted from the leaves in distilled water, centrifuged at 8000 *g* for 15 min. and stored at −20°. The extract was thawed and centrifuged at 8000 *g* for 10 min. before irradiation with ultraviolet light for different periods of time. Each inoculum (2 ml.) was put into a glass dish and rocked continuously during irradiation with a low pressure mercury vapour lamp (Hanovia); the radiation was mainly of wavelength 253·7 nm. and was filtered to remove radiation of wavelength less than 240 nm. The inoculum was placed 20 cm. from the lamp, where the intensity of radiation was about 650 μW/cm.^{2}. In each experiment the infectivity of sap was estimated, and compared with that of various dilutions of unirradiated sap by inoculation to leaves of two similar groups of *C. amaranticolor* plants: each inoculum was tested on eight half leaves. Plants of one group were then kept in the dark for 24 hr and plants of the other in daylight with supplementary artificial light (100 foot candles) at night. After 24 hr both groups of plants were kept in the glasshouse until lesions developed.

RESULTS

Properties of purified preparations

Morphology and size of particles. The particles of RCVMV are straight filaments about 12·5 nm. thick. Preparations from several sources and made in different ways were examined and the lengths of 50 to 100 particles measured in each. Particles in shadowcast preparations from *C. amaranticolor*, broad bean and pea had modal lengths of 659 nm., 650 nm. and 646 nm, respectively, with some about 1300 nm., long. Negatively stained preparations from the cut leaves of pea had particles with a modal length of 642 nm. and contained more shorter particles and fewer longer particles than the shadowcast preparations. Negatively stained particles in partially purified and purified preparations had modal lengths of 642 nm. and 644 nm., respectively. These estimates of modal length were not significantly different and the modal length for all measured particles of 600 to 700 nm. length was 645·3 nm. (mean 640·1 ± 16·6 nm.).

When RCVMV was mounted by passing the cut edge of an infected leaf through a droplet of negative stain on the microscope grid, the particles were usually randomly distributed over the grid (Fig. 1*a*). Occasionally, however, bundles of virus particles surrounded by a membrane were found. Similar aggregates were not found in comparable preparations of WCMV and CYVV, though there were some unenveloped clumps of CYVV particles.

Pellets obtained when heat-clarified sap was centrifuged resuspended less readily than pellets from sap treated in other ways. Fluid taken from around such pellets suspended in 0·05M-borate buffer contained many particles that were curved unusually (Fig. 1*d*); after 24 hr storage only straight particles were found.

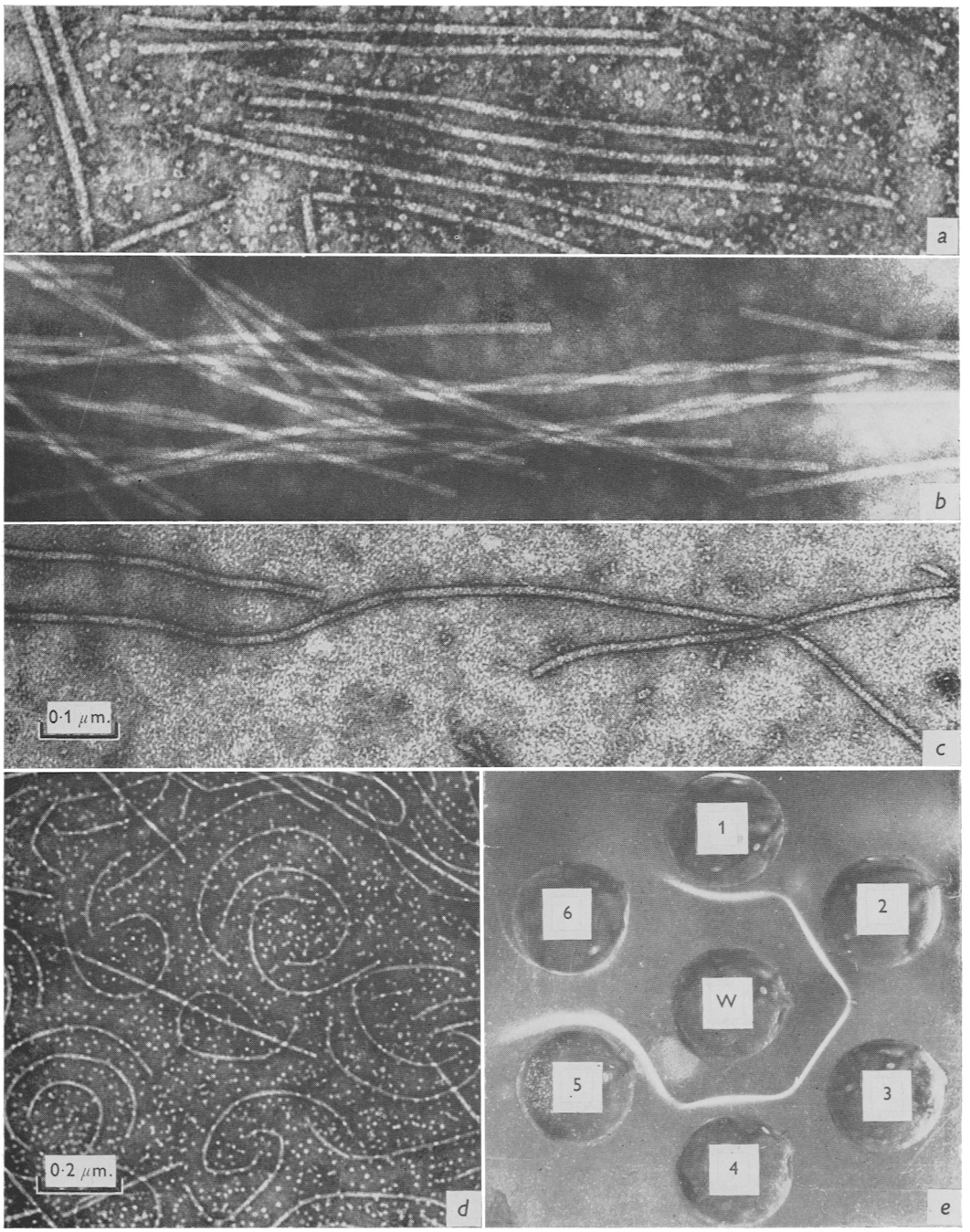

Fig. 1. (*a*) Cut leaf preparation of RCVMV mounted in sodium phosphotungstate. (*b*) Purified preparation of RCVMV stored at room temperature, showing side-to-side aggregation. Mounted in sodium phosphotungstate. (*c*) Purified preparation of WCMV stored at room temperature, showing end-to-end aggregation. Mounted in sodium phosphotungstate. (*d*) Sample taken from the top of a pellet of heat-treated RCVMV. Mounted in sodium phosphotungstate. (*e*) Precipitation pattern formed between WCMV antiserum (W) and WCMV after sonic treatment for (1) 30 sec.; (2) 2·5 min.; (3 and 4) 7·5 min.; (5) 17·5 min.; (6) untreated.

The particles of CYVV are more flexuous and vary more in length than those of RCVMV. The modal lengths of different preparations covered the range from the 670 nm. reported by Hollings & Nariani (1965) to the 767 nm. reported by Gibbs *et al.* (1966). Shadowcast and negatively stained mounts made from the same infected leaf gave particles of similar modal lengths—for example, one *N. clevelandii* leaf gave particles with modal lengths of 698 and 701 nm. respectively—and the modal lengths of particles from young and old leaves of the same plant were similar. However, virus from leaves of two batches of *N. clevelandii* gave particles of modal lengths 758 nm. and 797 nm. respectively (60 particles measured for each batch).

The particles of WCMV are flexuous filaments and in two preparations had modal lengths of 466 nm. and 453 nm.

Negative stain penetrated some RCVMV particles and revealed an axial canal, but did not penetrate particles of CYVV or WCMV. However, an axial canal could be seen in the discs and short pieces of particles in sonicated preparations of WCMV. Both RCVMV and WCMV have an axial canal of about 3·5 nm. diameter, smaller than that of the viruses of tobacco mosaic (Franklin, 1956) and tobacco rattle (Nixon & Harrison, 1959), but the same as that of potato virus X (Varma *et al.* 1968) and *Cymbidium* mosaic virus (Francki, 1966).

Purified preparations of RCVMV often aggregate and precipitate. An experiment was done to find whether the amount or rate of this aggregation depended on the buffer solution in which the virus was suspended. Twenty-three different buffer solutions were tested using various concentrations and combinations of ammonium, sodium, calcium, acetate, borate, chloride, diamino-ethane-tetracetate, phosphate and trihydroxyl-methyl-amino-methane ions and the detergent Triton X-100. The virus was most stable in neutral 0·005M-borate buffer, and this was used for all RCVMV preparations.

Preparations of RCVMV were stored at room temperature to induce aggregation and precipitation. Under these conditions RCVMV particles aggregated side-by-side, rarely end-to-end (Fig. 1*b*), whereas those of WCMV aggregated end-to-end and rarely side-by-side (Fig. 1*c*). CYVV did not aggregate, perhaps because these preparations were too dilute. The mode of aggregation seems to be specific to each virus, and unrelated to virus groupings; the particles of potato virus X, related to WCMV, also aggregate side-by-side (Kleczkowski & Nixon, 1950) like those of RCVMV.

Sedimentation coefficients. Purified RCVMV preparations had a single, homogeneously sedimenting component with a sedimentation coefficient of 160·9 S when extrapolated to infinite dilution by the least squares method. This coefficient depended both on virus concentration and on amount of impurity in the preparation; in the most pure and concentrated preparations it was about 135 S and in less pure preparations as small as 75 S.

Purified WCMV preparations contained two components with sedimentation coefficients at infinite dilution of 119S and 134·5S. Electron microscopy showed that most of the particles were of about 470 nm., the modal length, although some were twice as long. The numbers of these two lengths were related to the amounts of the two components shown by sedimentation analysis. Their relative sedimentation coefficients are as anticipated for the monomer and end-to-end dimer of long thin particles (Lauffer, 1944).

Preparations of CYVV, at the small concentrations obtained, contained a single component with a sedimentation coefficient of about 140 S.

Ultraviolet absorption. Purified preparations of RCVMV and WCMV were dialysed for several days against many changes of buffer to remove ascorbic acid remaining from the extracting buffer. Each virus then had an absorption peak at 262 to 263 nm. and gave an

absorption spectrum characteristic of a nucleoprotein with a small content of nucleic acid.

The 280/260 absorption ratio for RCVMV was 0·850 (mean for five preparations) and for WCMV was 0·845 (mean for three preparations), suggesting that they contain 6·25 % and 6·5 % nucleic acid respectively (Paul, 1959).

Nucleic acid composition. The nucleic acids of RCVMV and WCMV contain guanine, adenine, cytosine and uracil, and are presumably ribose nucleic acids. Table 1 shows the analyses of the base ratios.

Table 1. *Base composition of the nucleic acids of RCVMV and WCMV*

	Molar ratios (%)	
Base	RCVMV*	WCMV†
Guanine	31·5 ± 0·7	15·5 ± 0·9
Adenine	24·1 ± 0·1	31·8 ± 1·0
Cytosine	22·7 ± 0·7	26·9 ± 0·7
Uracil	21·7 ± 0·1	25·7 ± 1·0

* Mean of four estimates. † Mean of six estimates.

Table 2. *Serological tests with RCVMV and antisera to other viruses of the potato virus S group*

	Reciprocal antiserum titre	
Antiserum prepared against	Homologous	Against RCVMV
RCVMV	1024	1024
*EKV (German isolate of RCVMV)	2048	2048
*Cactus virus 2	1024	4
†Carnation latent virus	16304	8
‡Chrysanthemum virus B	Not done	8
*Passiflora latent virus	1024	8
Potato virus M	Not done	< 1
Potato virus S	1024	4

Antisera kindly provided by *the late J. Brandes, †M. Hollings, ‡R. A. Hakkaart.

Serological properties. Precipitation tests were made to show antigenic relationships between the viruses. Diluted preparations of virus and antisera were mixed in tubes and incubated at 37°; sap from infected plants clarified by centrifuging at 8000 *g* for 10 min. was used as antigen. The RCVMV, WCMV and CYVV antisera had homologous titres of 1/1024, 1/4096, 1/256 respectively when tested with sap of infected plants, and titres of 1/8, 1/1 and 1/4 when tested with extracts of healthy plants.

Table 2 gives the results of tests with RCVMV. The German and English isolates of the virus seemed identical and distantly related to five viruses of the potato virus S group (Brandes & Wetter, 1959). This confirms the results of Wetter & Paul (1961).

Table 3 summarizes the results of tests with WCMV, which confirm those of Bercks & Brandes (1961) showing WCMV to be serologically related to potato virus X; Bos, Delevic & van der Want (1959) failed to detect this relationship, perhaps because their antisera were too specific.

CYVV did not react with antisera to viruses related to either RCVMV (potato virus S group) or WCMV (potato virus X group), but reacted with antisera to viruses of the potato virus Y group (Brandes & Wetter, 1959). Table 4 summarizes these tests, which confirm and

extend those of Hollings & Nariani (1965), showing that CYVV is distantly serologically related to seven of twelve other viruses of the potato virus Y group.

Table 3. *Serological tests with WCMV and potato virus X (PVX)*

Antiserum prepared against	Reciprocal antiserum titre	
	Against PVX	Against WCMV
PVX	3200	8
WCMV	32*	4096

* Granular precipitates formed in this test, in contrast to the flocculent precipitates in the other three tests.

Table 4. *Serological tests with CYVV and antisera to viruses of the potato virus Y group*

Antisera prepared against	Reciprocal antiserum titre	
	Homologous	Against CYVV
CYVV	256	256
*Bean common mosaic virus	128,000	80
*Bean yellow mosaic virus	32,000	20
Bean yellow mosaic virus	64	4
Cocksfoot streak virus	256	< 1
Henbane mosaic virus	256	< 1
†Lettuce mosaic virus	—	< 8
Pea mosaic virus	128	2
Potato virus Y	256	< 1
*Sorghum red stripe mosaic virus	2048	80
*Soybean mosaic virus	2048	20
*Turnip mosaic virus	64,000	40
*Watermelon mosaic virus	—	0

* Tests by the late J. Brandes (personal communication).
† Antiserum kindly provided by J. A. Tomlinson.

Infective sap or purified preparations of all three viruses gave no visible bands of precipitate in Ouchterlony tests, presumably because the filamentous particles of the viruses did not diffuse through the gel. The tests were repeated using purified preparations of RCVMV and WCMV (*c.* 1 mg./ml.) that had been treated with ultrasound. The samples of WCMV treated for 2·5 and 7·5 min. gave clear bands of precipitate (Fig. 1 *e*), whereas those treated for longer or shorter times did not. The RCVMV treated for 7·5 and 17·5 min. gave the clearest bands of precipitate, but these were less obvious than those of WCMV and formed in the gel close to, or in, the sides of the antigen wells. The difference between WCMV and RCVMV was not caused by differences in the concentrations of virus or the titre of the antisera, but RCVMV may be more resistant to breakage by ultrasound than WCMV (Fig. 2).

Sonicated preparations of potato virus X reacted strongly in gel diffusion tests with homologous antiserum, but not with WCMV antiserum; treated preparations of WCMV reacted with the homologous antiserum but not with PVX antiserum. The reason for the difference between the results of the tube and Ouchterlony tests with these two viruses is not known.

Photoreactivation. These experiments were made with an isolate of RCVMV that produces local lesions on *C. amaranticolor.* Brief exposure to ultraviolet light decreased the infectivity of purified RCVMV preparations considerably; the decrease from a given exposure

was greater when the inoculated plants were kept in darkness than when they were kept in light. Hence some of the damage caused to RCVMV by ultraviolet radiation is photoreactivable; the amount of photoreactivation increased with longer exposure to ultraviolet light (Table 5).

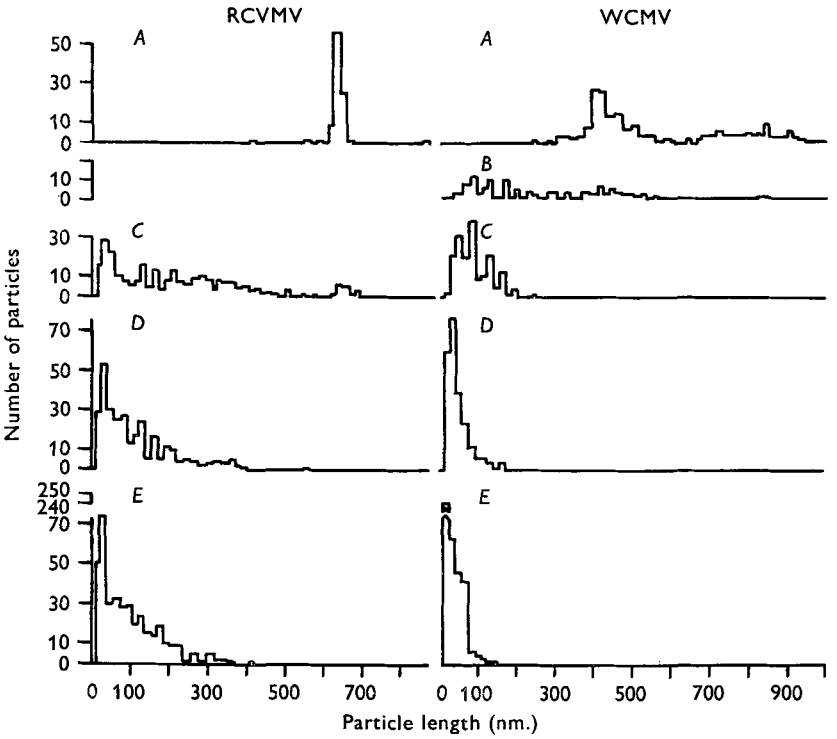

Fig. 2. Lengths in nm. of particles in purified preparations of RCVMV and WCMV after sonic treatment. *A*—untreated; *B*—treated for 0·5 min.; *C*—2·5 min.; *D*—7·5 min.; *E*—17·5 min.

Table 5. *Photoreactivation of ultraviolet-irradiated RCVMV*

Time of irradiation (sec.)	Infectivity of irradiated sample as percentage of control					
	Expt 1		Expt 2		Expt 3	
	Dark	Light	Dark	Light	Dark	Light
0	100 (495)	100 (309)	100 (49)	100 (40)	100 (135)	100 (63)
15	—	—	—	—	23·7	52·2
30	6·1	30·1	6·1	32·5	6·2	41·2
60	0·4	8·1	2·0	15·0	2·2	28·6

The numbers of lesions on eight half leaves of *Chenopodium amaranticolor* inoculated with the unirradiated control sample are given in parentheses.

DISCUSSION

Our estimate of the lengths of RCVMV particles is close to that of Wetter *et al.* (1962), but we found no evidence that the length of RCVMV particles depended on the host plant from which they were obtained (Hagedorn *et al.* 1959). Hollings & Nariani (1965) reported that the particles of CYVV had a mean length of about 670 nm., which suggested that CYVV

might be a member of the potato virus S group of viruses (Brandes & Wetter, 1959) and related to RCVMV. However, J. Brandes (personal communication) found that the particles of a CYVV isolate from Hollings were about 760 nm. long, and Gibbs *et al.* (1966) reported

Table 6. *The sedimentation coefficients of various viruses with filamentous particles*

Virus group	Virus	Sedimentation coefficient (s)	Information source
Potato S	RCVMV	161	This work
	Carnation latent	175	A. Varma and R. D. Woods, unpublished
	Pea streak	137*	Rosenkranz & Hagedorn (1967)
Potato X	WCMV	119	This work
	WCMV	112	Pratt & Reichmann (1961)
	Potato virus X	124	Lauffer & Cartwright (1952)
	Potato virus X	118	Reichmann (1959)
	Clover yellow mosaic	121	Pratt & Reichmann (1961)
Potato Y	CYVV	About 140*	This work
	Sugar cane mosaic	140–155*	Bancroft *et al.* (1966)
	Sugar cane mosaic	171	Shepherd (1965)

* Sedimentation coefficient not corrected for concentration or impurities.

that the particles of a different CYVV isolate were 767 nm. long. The modal lengths of CYVV particles reported in this paper (minimum 698 nm., maximum 797 nm.) show that the lengths of the particles of one CYVV isolate can vary; this may explain the conflicting earlier reports. Most of the preparations we measured had modal lengths between 700 nm. and 800 nm., suggesting that CYVV is a member of the potato virus Y group rather than of the potato virus S group. This was confirmed by the serological tests.

Our results and those of others (Table 6) show that the particles of viruses of the potato virus X group have sedimentation coefficients between 110 S and 125 S, and can thus be readily distinguished from viruses of the potato virus S and potato virus Y groups, whose particles sediment between 140 S and 170 S. Viruses of the potato S group are readily distinguished in electron micrographs from those of the potato X or potato Y groups; however viruses of the potato X and Y groups are less easily distinguished from one another, and accurate estimates of sedimentation coefficients may be useful for this purpose, especially with viruses such as CYVV whose particles are fragile and break when prepared for electron microscopy.

To compare the estimates of the nucleotide composition of the nucleic acids of RCVMV, WCMV and other similar viruses we computed a classification from the compositions. Fig. 3 shows an agglomerative hierarchical classification, computed by the 'Centclas' program of Lance & Williams (1966) and Williams, Lambert & Lance (1966), from the nucleotide composition of RCVMV, WCMV and other plant viruses with rod-shaped or filamentous particles. Fig. 4 shows the mean nucleotide composition of the major clusters defined by this classification. The nucleic acid of RCVMV is quite different from that of the other viruses in that guanylic acid is its most common nucleotide. Our estimate of the nucleotide composition of WCMV is closer to that of Fry, Grogan & Lyttleton (1960) than to that of Miki & Knight (1967).

Not all plant viruses with anisometric particles are photoreactivable. Tobacco mosaic and tobacco rattle viruses, which have straight tubular particles, are not photoreactivable, although their protein-free nucleic acids are (Bawden & Kleczkowski, 1955; Cadman & Harrison, 1959). Of the viruses with longer particles that have been tested, all have flexuous

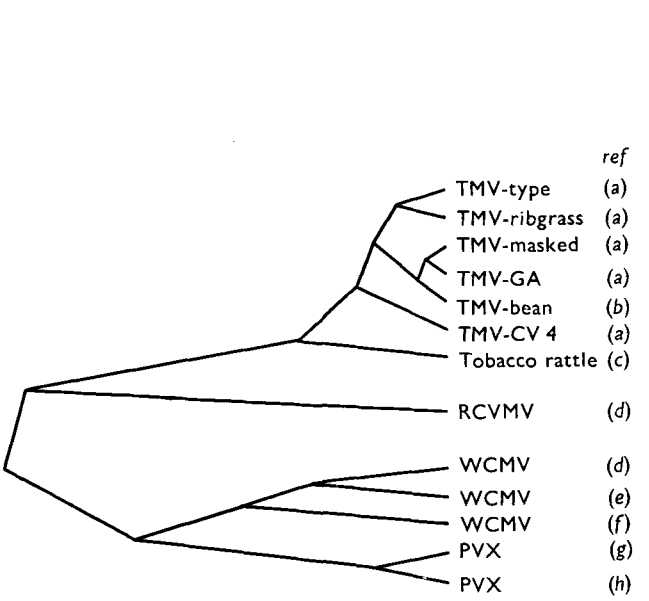

Fig. 3

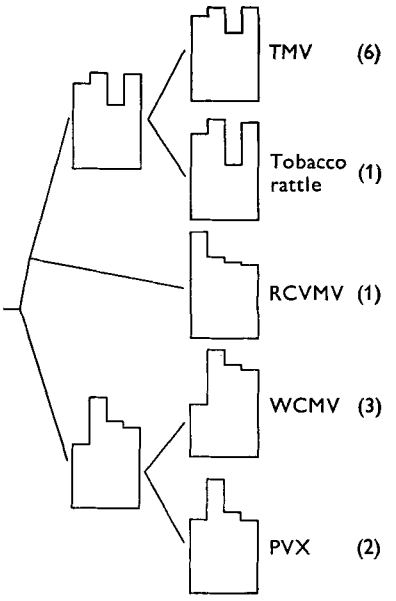

Fig. 4

Fig. 3. A dendrogram illustrating a classification of some plant viruses with rod-shaped or filamentous particles. Computed from the composition of their nucleic acids, using the 'Centclas' program of Lance & Williams (1966); agglomerative hierarchical classification, non-metric coefficient/centroid sorting. Data from: (*a*) Knight (1952); (*b*) R. H. Symons, personal communication; (*c*) Semancik & Kajiyama (1967); (*d*) this work; (*e*) Fry *et al.* (1960); (*f*) Miki & Knight (1967); (*g*) Dorner & Knight (1953); (*h*) Markham (1959). Acronyms as in text.

Fig. 4. The mean nucleotide composition of the viruses in the clusters defined by the classification in Fig. 3. Each histogram shows, left to right, the molar proportion of guanine, adenine, cytosine and uracil. In parentheses is the number of estimates for different strains or by different workers contributing to the histogram.

particles and all are photoreactivable (potato virus X and turnip mosaic virus (Bawden & Kleczkowski, 1955); clover yellow mosaic virus (Chessin, 1965)). RCVMV has particles as straight as the 'non-photoreactivable viruses' but as long as those of the 'photoreactivable viruses', showing that the flexuousness of the particles is not correlated with photoreactivability.

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