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SOME PROPERTIES OF PEA GREEN MOTTLE VIRUS, A MEMBER OF THE COWPEA MOSAIC GROUP, ISOLATED IN CZECHOSLOVAKIA

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Summary. — The properties of a virus found in peas (*Pisum sativum* L.) experimentally infected with a virus culture from mosaic diseased bean plants were studied. The virus, provisionally named pea green mottle virus (PGMV), was readily transmissible by sap inoculation. Twelve plant species of 3 families (*Fabaceae*, *Chenopodiaceae* and *Solanaceae*) proved to be susceptible to PGMV, the reactions varying from symptomless infection to local necrosis or systemic mottle (necrosis). PGMV was inactivated by heating for 10 minutes at about 65° C. It could be easily purified by several techniques. According to sucrose density gradient centrifugation, PGMV populations consisted of 2 major components. The virions appeared isometric and about 25 nm in diameter. Infectious ribonucleic acid (RNA) was obtained by phenol treatment of partially purified PGMV. Based on these properties and serological cross-reactions it was possible to include PGMV into the cowpea mosaic group. PGMV proved to be very closely related to broad bean stain virus (BBSV) and MF virus.

Introduction

In 1961, investigations into viruses affecting leguminous crops in Czechoslovakia were started at our laboratory. Among the first ones encountered was a small isometric virus, which we found difficult to identify (see Kvíčala *et al.*, 1964). This "garden bean isolate" or F1 virus did not fit any description of viruses known to occur in Central Europe at that time. It showed some resemblance to true broad bean mosaic virus (TBBMV) (Quantz, 1953) and indeed weak serological reactions between these two viruses were established (Bercks, personal communication 1963; Valenta and Gressnerová, 1966). Later we found that F1 virus was related to, but distinct from, red clover mottle virus (RCMV) meanwhile isolated also in Czechoslovakia (Musil, 1966*a*). Moreover, the F1 virus resembles quite closely another two recently described members of the so-called cowpea mosaic group, namely BBSV (Gibbs *et al.*, 1968) and MF virus (Devergne and Cousin, 1966).

In the present report we will describe some characteristic properties of F1 virus with the aim to define more precisely its position within the cowpea mosaic group. Preliminary reports on its serological relationships have been presented (Valenta and Gressnerová, 1966, 1967).

Although the designation "F1 virus" has already been used on several occasions in the literature, it was originally intended as a working label. Because, as will be shown below, F1 virus cannot be definitely identified with any known member of the cowpea mosaic group, we propose to call it "pea green mottle virus" until a general agreement will be reached about the nomenclature of the virus group under consideration.

Materials and Methods

Viruses. In addition to PGMV, the history of which is given below, the following viruses were included in some experiments: TBBMV (echtes Ackerbohnen-Mosaikvirus — Quantz, 1953), obtained from Prof. Dr. R. Bercks, Biologische Bundesanstalt f. Land- und Forstwirtschaft, Braunschweig; RCMV, strain TpM26 isolated in Czechoslovakia (Musil, 1966*a*); and BBSV (Gibbs *et al.*, 1968), obtained from Dr. A. J. Gibbs, then at Rothamsted Experimental Station, Harpenden.

Exper imental plants were grown from seeds in sterilized soil in an insect-proof greenhouse. In somenspecial experiments, peas were grown in hydroponic culture, details of which will be described elsewhere.

Diluent. Unless otherwise stated,viral inocula or sera were diluted with phosphate buffered saline (PBS; 0.9% NaCl + 0.067 M phosphate, pH 7.2). In preparing viral inocula, one part (fresh weight) infected plant material was homogenized with one part (volume) of diluent and the resulting homogenate was taken for 1 : 2 dilution of virus in further calculations.

Assay of PGMV infectivity. As stated in the Results, none of the established host plants produced such symptoms as to warrant their use for a direct assay of PGMV infectivity. The appearance of necrotic local lesions on the inoculated primary bean leaves seemed to be promising in spite of the fact that this reaction was not regularly reproducible. We attempted at improving it by applying heat shocks like those used by Rappaport and Wu (1963) and Wu (1963) with bean leaves and tobacco mosaic virus. Pinto bean leaves inoculated with PGMV were dipped for 1 minute in water at either 40 or 48° C, but the necrotic lesions appeared as irregularly and few as in the controls without heat shocks.

Since pea plants appeared to be sufficiently susceptible and could be easily grown in large numbers, we finally adopted the following procedure for titrating PGMV infectivity. Serial tenfold dilutions of the virus-containing materials were rubbed onto 2—3 leaves of young pea (*Pisum sativum* cv. Raman; other cultivars were used only exceptionally) plants, using usually 5—15 plants for each dilution. At appropriate intervals after inoculation sap was expressed from each pea plant separately and tested for the presence of virus in precipitin tests wth PGMV antiserum. At the onset of our investigations, we used drop or tube precipitin tests, for which the pea sap had to be clarified by low speed centrifugation. Later we replaced these tests by the agar double diffusion precipitin test in which the expressed pea sap was placed without any treatment directly into the appropriate wells. Based on the number of pea plants giving a positive precipitation reaction with PGMV antiserum, we calculated the percentage of infected plants for each dilution and expressed the titres in terms of ID_{50} values, using the formula proposed by Lim (1954)

Serological tests. In tube precipitin tests, equal volumes of virus suspension and serum were mixed and kept at room temperature; the results were read after about 2 hours; in some cases, the tubes were then kept overnight at 4° C and the final results read thereafter. Appropriate controls (normal serum and healthy plant extracts) were included as required. Ring precipitin tests were carried out in small glass test tubes by overlaying serum (appropriately diluted with PBS containing 10% glycerol) with the test suspension; the results were read after about 30 minutes at room temperature. Double diffusion precipitin tests in 1% aqueous agar (washed Bacto-agar Difco) were done in 5 cm Petri dishes which were incubated for several days at room temperature. The titres (virus, antibody) were expressed as reciprocals of the highest dilution still giving a positive reaction. In case of tube precipitin tests, the final dilutions of serum (virus) were taken into account.

Thermal inactivation. Virus samples distributed in thin-walled glass test-tubes were heated for 10 minutes at the given temperature in a water bath operated by a Wobser type ultrathermostat. After heating the tubes were immediately cooled under running tap water.

Electron microscopy. Virus suspensions were negatively stained with either 2% phosphotungstate (pH 6.8) or 3% ammonium molybdate (pH 5.8) and the specimens were examined under a JEM-6c electron microscope at an instrumental magnification of ×40,000. The diameter of the particles was measured from the negatives with the aid of an eye-piece micrometer at a magnification of ×14.4.

Further methods used will be described along with the results.

Results

Isolation of PGMV

PGMV was first found in experimental pea plants infected by a virus culture the history of which was as follows. In the summer of 1961, bean (*Phaseolus vulgaris*) plants grown in the garden of our Institute at Bratislava were affected by a conspicuous bright mottle. Steinerová (Kvíčala *et al.*, 1964) obtained from these beans a virus culture, designated F1. In the early passages, pea plants infected with this virus culture displayed distinct mosaic symptoms and, as revealed by electron microscopy, contained long flexuous rods. Subsequently, the mosaic symptoms disappeared and purified virus preparations obtained from infected peas consisted exclusively of isometric particles (see Mrena and Dostál, 1964). It may be concluded, therefore, that the virus possessing the flexuous rods was lost in the course of passaging and that it was probably responsible for the clear-cut symptoms observed both in the original-beans and in pea plants during the early passages. Since the isometric virus itself was found to infect beans only with difficulty, and because its presence was definitely demonstrated only in later pea passages, it may be questioned whether it was really present in the original bean plants. An unequivocal answer to this question is no more possible. In this connection it is important that several viruses of the cowpea mosaic group have been shown to be seed-borne (see, e.g., Devergne and Cousin, 1966; Gibbs *et al.*, 1968, etc.). Transmission of PGMV by seed has not yet been tested. Several attempts to demonstrate the natural occurrence of PGMV in peas grown in the same garden as the original bean plants, failed.

After the culture of the isometric virus was freed of the rod-shaped virus, it has been passed in pea plants. The lot of pea seed used in 1962 was later found to be rather heavily contaminated by the seed-borne pea leafrolling mosaic virus (Musil, 1966*b*). The results concerning PGMV (the "garden bean virus") as reported by Kvíčala *et al.* (1964) might have been, therefore, obscured by this seed-borne virus and they will not be taken into consideration in the present report.

To free the isometric virus from any contaminants, we subjected it early in 1963 to passages in peas by the limiting dilution method in combination with heat treatment (55° C for 10 minutes) of the virus suspensions used as inoculum. Repeated serological tests by the agar double diffusion method and electron microscopy of purified preparations suggested a homogeneity of the virus culture thus obtained. Since then, this virus culture has been kept under the designation F1 by successive passages in pea plants. Passaging was occasionally interrupted for short periods, during which the virus was maintained in infected pea plants kept at −20° C.

In the experiments reported below, only the "pure" PGMV (F1 virus) culture was employed.

Modes of transmission of PGMV

PGMV was readily transmissible by manual inoculation. As a rule, the leaves of the test plants were dusted with 600 mesh silicium carbide powder before inoculation. The inocula were rubbed onto the leaves using small pieces of plastic sponge.

A pilot test with starved aphids *Myzus persicae* Sulz. gave a negative result. No other attempts at insect transmission of PGMV were done so far.

Host range and symptoms

PGMV was transmitted by sap inoculation to 12 plant species belonging to three families:

Chenopodiaceae: *Chenopodium album* L., *C. capitatum* (L.) Asch., *C. murale* L., and *C. quinoa* Willd.;

Fabaceae: *Faba vulgaris* Moench, *Glycine soja* (L.) Sieb. Zucc., *Phaseolus vulgaris* L., *Pisum sativum* L., ssp. *hortense* (Neilr.) A. Gr., *Trifolium hybridum* L., *T. incarnatum* L., and *Vigna sinensis* Endl.; and

Solanaceae: *Nicotiana debneyi* Domin.

We failed to infect the following plant species (after inoculation no symptoms appeared and PGMV could not be detected in the plants either serologically or by back inoculation to pea plants): *Chenopodium amaranticolor* Coste et Reyn., *C. ambrosioides* L., *Datura stramonium* L., *Nicotiana clevelandii* Gray, *N. glutinosa* L., *N. megalosiphon* Heurck et Mueller, *N. rustica* L., *N. sylvestris* Speg. et Comes, *N. tabacum* L. cv. White Burley, and *Trifolium pratense* L.

The reactions of the susceptible plant species to infection with PGMV varied. The following categories could be distinguished:

a) systemic infection accompanied by the development of more or less obvious mosaic symptoms (*Faba vulgaris*, *Pisum sativum*);

b) local lesions on the inoculated leaves followed by systemic spread of virus (*Chenopodium murale*, *C. quinoa*, *Phaseolus vulgaris*);

c) local lesions on the inoculated leaves without detectable further spread of virus (*Chenopodium album*, *Vigna sinensis*); and

d) symptomless infection (*Chenopodium capitatum*, *Glycine soja*, *Nicotiana debneyi*, *Trifolium hybridum*, *T. incarnatum*). Systemic spread of virus in these symptomless hosts was proved either serologically or by reinoculation to pea plants or by both methods.

A brief description of the symptoms observed in the individual hosts of PGMV follows.

Chenopodium album. No symptoms were produced, but 10 days after inoculation starch-iodine lesions were demonstrated in the inoculated leaves. No back tranfers were attempted and serology gave equivocal results.

Chenopodium murale. Local necrotic lesions appeared on the inoculated leaves after 10 days or later. Afterwards, the virus became systemic, producing irregular necrotic lesions which led to distortion of leaves in the apical parts of both the main and the lateral shoots. The infection was verified by serology and infectivity tests (back transfer to peas).

Chenopodium quinoa. Local chlorotic lesions developed on the inoculated leaves about 14 days after inoculation. Later, irregular chlorotic lesions leading to distortion of apical leaves were observed. In spite of these rather distinct symptoms, we were unable to demonstrate the F1 virus in the affected *C. quinoa* plants either serologically or by back inoculation to pea plants.

Faba vulgaris. In the cultivar Považský used, no evaluable symptoms appeared on the inoculated leaves; occasionally they showed some dark necrotic areas 2—3 weeks after inoculation. The leaves above the inoculated ones frequently showed a faint but distinct green mottling, but occasionally the infection of broad bean plants remained symptomless. Infection of the broad bean plants was confirmed serologically and by back transfers to pea plants.

Phaseolus vulgaris. The reaction of garden bean cultivars to PGMV was rather erratic. In some cultivars (e.g. De Banat, Pinto, Perlička) dark necrotic local lesions developed on the inoculated primary leaves from 8—14 days after inoculation, but this local lesion formation was not regularly reproducible. Very rarely, these primary symptoms were followed by those of a systemic infection, namely by indistinct spots or ringspot-like mottling on the pinnate leaves. From the inoculated leaves of certain other cultivars (e.g. Saxa, Prince) that showed no symptoms, PGMV was recovered by back inoculation to pea plants about a month after inoculation of the bean leaves (no other intervals were tested).

Pisum sativum ssp. *hortense*. The various cultivars (Pauli, Kralický oválný, Raman, etc.) tested all behaved similarly. The growth of infected plants was usually somewhat inhibited as compared with uninfected controls. No symptoms appeared on the inoculated leaves, except that they occassionally wilted and dried earlier in infected than in uninfected plants. Symptoms developed in newly formed leaves usually about 10—14 days after inoculation They consisted of a faint clearing of the veins followed by slight, only occasionally clear-cut, green mottling. Very often, especially in late stages of disease, no clear symptoms were produced or the infection was quite symptomless, but it was possible to demonstrate the presence of virus in such symptomless plants or plant parts serologically or by infectivity tests. The virus levels varied considerably in the course of infection; a detailed report about these changes will be published later.

Vigna sinensis. In the cultivars Black systemic and Black local, small chlorotic spots appeared on the inoculated primary leaves from about 1 week after inoculation. No signs of systemic spread of PGMV were detected. Serology was negative. In other cowpea cultivars (Giant Black eye, Black eye, Lady Finger round and White acre), no symptoms were observed and serological tests with the inoculated leaves gave negative results.

Stability of PGMV

PGMV proved to be relatively stable. In sap extracted from infected pea plants with a titre of about 10^4 ID_{50}, infectious virus was demonstrated even after 9 days (the titre dropped to less than 10^2 ID_{50}), but not after 14 days at room temperature. In the same sap kept at 4° C, inactivation proceeded more slowly and some infectivity could be demonstrated even after 4 weeks (the longest period tested). In sap kept at —20° C, practically no loss in virus infectivity occurred during this period.

Table 1. Heat inactivation of PGMV in pea sap

Sap heated for 10 min at ° C	Dilution of virus (neg. $\log_{10}$)						Virus titre (log ID_{50})
	0*	1	2	3	4	5	
Unheated control	—	—	9/10	16/20	12/20	6/20	4.33
55	—	—	2/10	4/20	0/20	—	< 2
60	—	1/10	0/20	—	—	—	< 1
65	0/10	0/20	—	—	—	—	—
70	0/20	—	—	—	—	—	—

* Sap obtained after homogenizing infected pea plants in an equal part (w/v) of PBS.
Numerator: number of pea plants infected; denominator: number of pea plants inoculated.
— = Not tested.

The effect of heating on PGMV infectivity was tested several times and the results obtained with a highly infectious pea sap are illustrated in Table 1. It is evident that most infectivity was lost after heating for 10 minutes at 55° C and that infectivity was completely destroyed at a temperature below 65° C.

Antigenicity of PGMV proved to be more stable than its infectivity. In pea sap kept at room temperature, the same as mentioned above, in which infectivity was lost after about 10 days, the antigen titre determined in ring precipitin tests remained unchanged for 2 days at 128, then dropped

to 64, remaining at this level for another 6 days, and starting from the 9th day till the 15th day (the longest interval tested) the antigen titre persisted at level of 32. The effect of heating on the antigenicity of PGMV is illustrated in Table 2. It is evident that some precipitating antigen was still demonstrated after heating for 10 minutes at 65° C, under which conditions all infectivity was destroyed.

Table 2. Effect of heating on the antigenicity of PGMV

Sap heated for 10 min at ° C*	Dilution of sap (reciprocals)					
	4	8	16	32	64	128
Unheated control	++++	+++	++	+	+	±
55	+++	++	±	±	−	−
60	++	+	±	−	−	−
65	+	+	−	−	−	−
70	−	−	−	−	−	−

* The same sap as in Table 1.
±, +, ++, +++, and ++++ indicate intensity of the precipitation reaction in tubes with homologous PGMV antiserum; − means no precipitate formed.

Two cycles of freezing and thawing had no effect on the titre of PGMV antigen.

Purification of PGMV

Several modifications of different methods were tried and almost all gave acceptable results.

As starting material, above-ground parts of infected pea plants that had been kept frozen at about −20° C for various periods of time were used. In pilot experiments it was found that it was more difficult to obtain good virus preparations from fresh, unfrozen plant materials.

The frozen infected plants were weighed and homogenized in a blendor with a given volume of buffer solution. The added volume of buffer solution was varied in different experiments from one half to twice the weight of the plant material. After homogenization, the crude plant sap was expressed through a dense cloth and, in some experiments, clarified by brief low speed centrifugation.

In the course of our work different buffer solutions were tried. In the early phase, PBS was used the most often. It had no obvious adverse effects on the virus during homogenization, but when used in the later stages of the purification process, considerable losses of virus were frequently encountered. It was replaced, therefore, by phosphate buffers at pH 7.2−8, or borate buffers at pH 8−9. With pH 9 and pH 7.2−7.5 buffers there occasionally occurred some losses of virus and pH 8 buffers (phosphate or borate) were therefore employed in most of the later experiments, both for homogenizing the plant materials (0.1 M) and as diluents (0.025 M) in further stages of the purification procedure. When used for homogenization, the buffer solutions were occasionally supplemented with up to 0.4% ascorbic acid or up to 0.2% sodium sulphite (or both), and 0.01 M sodium diethyl dithiocarbamate. In some experiments, these substances were added to the crude sap immediately after homogenization. The addition of these substances did not substantially influence the virus yields, but the resulting virus preparations were less coloured than in experiments carried out in their absence.

The crude virus-containing plant saps were then treated with either ether − carbon tetrachloride by the method of Wetter (1960), or a 1 : 1 mixture of butanol and chloroform (Steere, 1956) (one volume of this mixture per 2 volumes of plant sap), or by fluorocarbon (freon; Ledon 113). The latter treatment was finally adopted as the routine method: equal volumes of

crude plant sap and freon were thoroughly homogenized in a blendor and the resulting emulsion was broken by low speed centrifugation.

The clarified saps obtained after either of the three treatments were subjected to high speed centrifugation. Pilot tests showed that centrifuging for 1—1.5 hours at approx. 75000×g did not result in complete sedimentation of the virus. Therefore, in later experiments centrifugation was carried out at approx. 100000—115000×g for 1.5—2 hours. The resulting virus pellets were resuspended in small volumes of buffer solution (see above), clarified by low speed centrifugation, and sedimented again at high speed. These cycles of high and low speed centrifugation were repeated at least twice. The final virus pellets were resuspended in a volume of buffer solution corresponding usually to 1/10 or 1/20 of the weight of starting plant material.

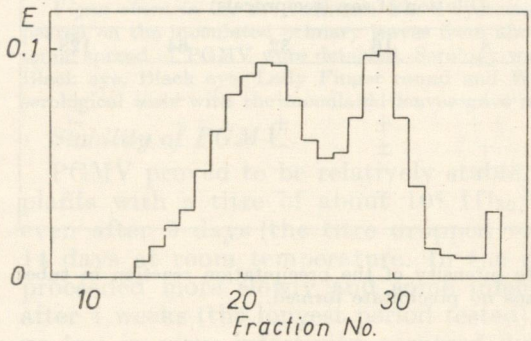

Fig. 1.

Separation of bottom and middle components of PGMV by sucrose density gradient centrifugation 45—65% sucrose gradient in 0.025 M phosphate buffer, pH 8.0; SW 39L rotor of Spinco L-50 centrifuge; 37000 rev/min for 14 hours. Fractions were taken from the bottom (from left to right).

When using PBS or buffers at about neutrality, the resuspension of the virus pellets was not complete, or the bulk of resuspended virus had irreversibly precipitated within a few hours. Alkaline buffers, mostly borate buffer pH 8, were therefore used in most of the later experiments. In this case spontaneous precipitation of virus was avoided and the virus retained its activities unaltered for sufficiently long periods of time.

In addition to the methods described above, the octanol flotation method of Čech (1966) was tried in several experiments. Equal parts (w/v) of infected plant material and 0.05 M borate buffer pH 8.5 containing 0.1% thioglycolic acid and 10% sucrose plus n-octanol (20% v/w of the plant material) were homogenized in a blendor for 5 minutes in nitrogen atmosphere. Large plant debris was removed by expressing the homogenate through dense cloth. The pulp was then centrifuged for 15 minutes at 2500×g; the resulting floating green cake was removed and the fluid phase centrifuged for 2 hours at 110000×g. The resulting virus pellet was resuspended in borate buffer, the suspension clarified (12000×g for 15 minutes) and again centrifuged for 2.5—3 hours at 110000×g. The final virus pellet was resuspended in 0.01 M borate buffer pH 8.5 containing 0.1% thioglycolic acid.

The course of the purification procedures was checked by serological assay of virus; in some experiments, infectivity titrations were included. Except of the few variants of the methods mentioned above, the yields of virus were good with all the procedures tested. However, the preparations were always more or less heavily contaminated by normal plant constituents. This was clearly shown by electron microscopy and especially by the fact that antibody to pea proteins was found in most antisera prepared by immunizing rabbits with such virus preparations. A part of the contaminating host materials could be removed by repeating the high speed centrifugation several times, but for their full removal the partially purified virus preparations had to be subjected to sucrose density gradient centrifugation.

The latter was carried out in the SW 39L rotor of a Spinco L centrifuge, using gradients prepared from either 25—55% or 45—65% sucrose solutions in 0.025 M phosphate buffer pH 8.0.

The centrifugation lasted 5 or 14 hours in the former and latter case, respectively. Fractions were collected by piercing the bottom of the tubes and their ultraviolet absorption was measured. Peak fractions were examined both in the electron microscope and in precipitin tests with PGMV antiserum.

The results were similar with both types of sucrose gradient (25—55% or 45—65% sucrose). In addition to slowly sedimenting host plant materials, there always occurred two bands (Fig. 1) which could be identified by serology and electron microscopy as corresponding to PGMV.

The ultraviolet absorption spectrum of partially purified unfractionated PGMV showed a maximum at 259 nm and a minimum at 240 nm (Fig. 2). The absorption spectrum of the components was similar but the $E_{260} : E_{280}$ ratio was 1.72 with the bottom component and 1.58 with the middle component.

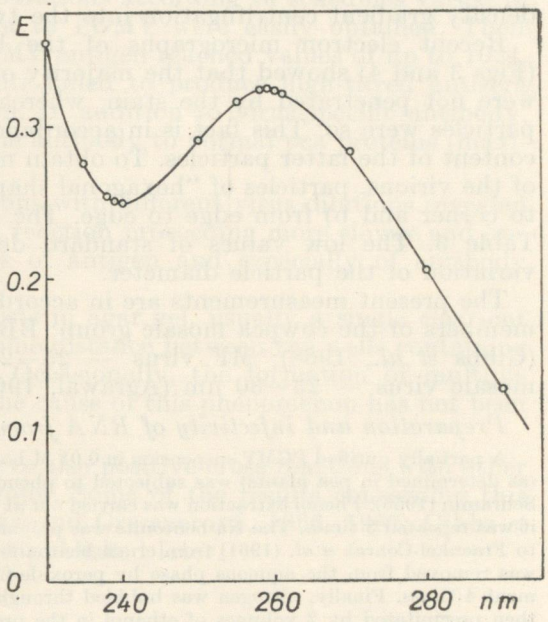

Fig. 2.
Ultraviolet absorption spectrum of purified PGMV

Electron microscopy

The first published electron micrographs of PGMV were those by Mrena and Dostál (1964). Particles with hexagonal, occasionally pentagonal outlines, suggesting icosahedral structure of the virion, were observed. Using the negative staining technique, the diameter of the virions was found to be

Table 3. Measurements of PGMV particles

Bottom component				Middle component			
a		b		a		b	
nm	Frequency	nm	Frequency	nm	Frequency	nm	Frequency
23	7	18	1	23	3	20	4
25	21	20	11	25	23	23	22
27.5	6	23	18	27.5	6	25	6
		25	4				
$\bar{x}_a = 25.0$ nm $\sigma = \pm 1.4$ nm		$\bar{x}_b = 22.0$ nm $\sigma = \pm 1.6$ nm		$\bar{x}_a = 25.5$ nm $\sigma = \pm 1.0$ nm		$\bar{x}_b = 23.0$ nm $\sigma = \pm 1.3$ nm	

a and b: measurements of "hexagonal" particles from corner to corner (a) and edge to edge (b).

approx. 30 nm. Some of the particles were penetrated by phosphotungstate, while the majority were not. These observations were made on electron micrographs of partially purified PGMV preparations, not separated by density gradient centrifugation into the two main components.

Recent electron micrographs of the bottom and middle components (Figs 3 and 4) showed that the majority of the bottom component particles were not penetrated by the stain, whereas most of the middle component particles were so. This fact is in accordance with the presumed lower RNA content of the latter particles. To obtain more exact information on the size of the virions, particles of "hexagonal shape" were measured a) from corner to corner and b) from edge to edge. The data obtained are summarized in Table 3. The low values of standard deviation indicate a low range of variation of the particle diameter.

The present measurements are in accordance with data reported for other members of the cowpea mosaic group: BBSV and TBBMV — about 25 nm (Gibbs *et al.*, 1968); MF virus — 25—29 nm (Devergne, 1964), cowpea mosaic virus — 23—30 nm (Agrawal, 1964).

Preparation and infectivity of RNA from PGMV

A partially purified PGMV suspension in 0.01 M borate buffer pH 8.5 with a titre of $10^{3.5}$ ID_{50} (as determined in pea plants) was subjected to phenol extraction by the method of Gierer and Schramm (1956). Phenol extraction was carried out at 4° C in the presence of 0.1% Na-bentonite; it was repeated 3 times. The Na-bentonite was prepared by differential centrifugation according to Fraenkel-Conrat *et al.* (1961) from crude bentonite of Czechoslovak origin (Lastovce). Phenol was removed from the aqueous phase by peroxide-free ethyl ether, repeating the ether treatment 4 times. Finally, nitrogen was bubbled through the fluid to remove ether and RNA was then precipitated by 2 volumes of ethanol in the presence of Na-acetate. The precipitate was dissolved in a volume of PBS corresponding to the volume of the starting virus suspension.

The RNA thus obtained showed an ultraviolet absorption spectrum typical of RNA with an absorption maximum at 258 nm and an $E_{260} : E_{280}$ ratio of 2.14. It was assayed for infectivity in pea (*Pisum sativum* cv. Raman) and bean (*Phaseolus vulgaris* cv. Perlička) plants. The results are shown in Table 4. It is evident that the infectivity of the RNA preparation was about 3 log units less than that of the original virus. Some of the necrotic local lesions formed on the inoculated bean leaves were cut out, homogenized in a small volume of PBS and the homogenate was inoculated onto pea plants. PGMV was later detected in these pea plants serologically.

Table 4. Infectivity of RNA prepared from PGMV

Inoculum	*Pisum sativum*	*Phaseolus vulgaris*
RNA — undiluted	7/10*	24/28**
RNA — diluted 10^{-1}	2/10	18/44
RNA — undil. + 5 μg/ml ribonuclease	0/10	0/24

* Number of plants infected over number of plants inoculated.

** Number of leaf halves with local necrotic lesions over number of leaf halves inoculated.

Serology

By injecting rabbits intravenously or intramusculary (with adjuvant) with partially purified PGMV preparations according tu schedules varied in the different experiments, antisera to PGMV were easily obtained. Their precipitin antibody titres with PGMV antigen reached values of up to 1024, occasionally 2048, but some rabbits failed to produce high-titred antisera even after prolonged immunization. In addition to virus-specific antibody, most of the antisera contained some antibody to normal pea proteins (maximally in a titre of 16).

Testing of different serum dilutions with different virus dilutions revealed clear-cut optima, the precipitation reaction proceeding more slowly and less precipitate being formed in zones of antigen and especially of antibody excess.

In double diffusion precipitin tests in agar gel, usually a single clear-cut precipitation line was formed at some distance between the wells containing antibody and virus, respectively. Occasionally, the formation of multiple precipitation lines was observed; the cause of this phenomenon has not been definitely elucidated.

PGMV antigen and antiserum gave also positive cross-reactions with other members of the cowpea mosaic group. Some of the results concerning this point have been published (Valenta and Gressnerová, 1966, 1967; Gibbs *et al.*, 1968). A detailed report will be published later.

Cross protection tests

PGMV and TBBMV. In several experiments, pea (*Pisum sativum* cv. Raman) plants were inoculated with a mixture of PGMV and TBBMV, or inoculated first with PGMV or TBBMV and after about a week challenged with TBBMV or PGMV, respectively. The pea plants invariably developed clear-cut symptoms of TBBMV infection. Either virus multiplied in the doubly infected plants to levels similar to those found in control plants infected with the respective virus alone. Thus no evidence of a cross protection between PBMV and TBBMV was obtained.

PGMV and RCMV. In experiments carried out in pea plants, no clear-cut evidence of a cross protection between PGMV and RCMV was obtained.

Broad bean (*Faba vulgaris* cv. Považský) plants that were inoculated first with PGMV and then challenged with RCMV, did not always show the systemic necrosis characteristic of RCMV infection. In one of the several experiments carried out, groups of 10 broad bean plants each were challenged with RCMV 5, 7, 9, 11 and 13 days after inoculation with PGMV, respectively. One month after inoculation, the numbers of necrotic plants were 6, 1, 0, 2 and 3 respectively. All 10 control plants inoculated with RCMV alone were necrotic and 10 controls inoculated with PGMV alone were almost symptomless. These results suggest that PGMV infection at least delayed or inhibited the development of necrosis following challenge with RCMV, but that PGMV infection did not afford an absolute protection to broad bean plants from subsequent challenge with RCMV.

Discussion

Based on the morphological, physical and antigenic properties, PGMV clearly belongs to the cowpea mosaic group. The virions of PGMV are isometric and about 25 nm in diameter. According to density gradient centrifugation experiments, at least two classes of viral particles were present in purified preparations. They evidently corresponded to the so-called bottom and middle components of viruses of the cowpea mosaic group (see, e.g., Bancroft, 1968), although no experiments have yet been carried out with PGMV to show as to whether their biological function is the same as with other members of the group.

The antigenic and biological properties seem to be the two most important criteria in attempting to evaluate the relationship of PGMV to the other members of the cowpea mosaic group. To simplify the matter, we shall take into consideration mainly those members of the group, which have been shown more closely antigenically related to PGMV than the rest (Valenta and Gressnerová, 1966, 1967; Gibbs *et al.*, 1968). This means that a priori we shall consider cowpea mosaic, bean pod mottle, radish mosaic, squash mosaic and TBBM viruses as distinct from PGMV.

Immunodiffusion, cross precipitin and cross absorption tests revealed a considerable degree of similarity, but not identity, of PGMV and RCMV antigens (Valenta and Gressnerová, 1966, 1967, and unpublished; Gibbs *et al.*, 1968). Moreover, the biological properties (especially host range and symptoms) of PGMV differ in several respects from those of RCMV as reported by Sinha (1960) or Musil (1966*a*). It appears justified, therefore, to consider these two viruses as distinct, though rather closely related. The latter suggestion seems to be supported also by the results of cross protection tests described above, in which PGMV afforded to broad bean plants at least some protection against challenge with RCMV.

Serological tests revealed a close relationship of PGMV to two other members of the cowpea mosaic group, namely to the so-called "broad bean mosaic" (mosaïque de la fève — MF) virus reported from France by Devergne (Devergne and Smith, 1964; Devergne and Cousin, 1966) and to BBSV occurring in England and described by Gibbs *et al.* (1968). The degree of relatedness of PGMV to MF virus cannot be definitely evaluated, because no complete set of cross reactions was yet carried out. In the experiments done so far (Valenta and Gressnerová, 1967) only MF antiserum from Dr. Devergne was examined with F1 virus antigen and double diffusion tests suggested antigenic identity. However, cross-absorption tests seem to be more sensitive in determining antigenic differences between members of the cowpea mosaic group and thus should be carried out to clarify the question definitely. Concerning the biological properties, there is hardly any substantial difference between PGMV and MF virus as judged from the description of MF virus presented by Devergne and Cousin (1966). The MF virus only seems to produce in certain hosts somewhat more severe symptoms than PGMV.

The antigenic relationship between PGMV and BBSV has been studied in some detail (Valenta and Gressnerová, 1967 and unpublished; Gibbs *et*

al., 1968) and especially our cross-absorption tests suggested a very great similarity of the two antigens. The physical properties of BBSV (Gibbs *et al.*, 1968) are very similar to those of PGMV. Moreover, the two viruses apparently do not substantially differ in their host ranges, although the information about BBSV as presented by Gibbs *et al.* (1968) is rather difficult to evaluate definitely. In our limited comparative tests, PGMV produced in peas less severe symptoms than BBSV; in *Phaseolus vulgaris* cv. Perlička local lesion formation on the inoculated primary leaves was regular with BBSV, while it was erratic with PGMV.

Based on the available informations it seems justified to consider BBSV, PGMV and MF virus as more closely related to each other than to the other members of the cowpea mosaic group. Perhaps it would be even possible to consider them as representatives of a single virus species. But there is especially one problem which should be solved before making such decision. It has been namely shown (see, e.g., Bancroft, 1968) that the phenomenon of mutual complementation between the bottom and middle components of viruses of the cowpea mosaic group is strictly specific and that it does not occur between components derived from distinct viruses. No such experiments have yet been carried out with BBSV, PGMV and MF virus.

In plant virology, cross protection tests in susceptible plants have been frequently used to establish relationships among viruses. However, this method might prove unsuitable in the case of the 3 viruses under consideration, especially in view of the rather unstable symptom expression in most hosts and difficulties in serological analysis of mixtures of the viruses involved. A pilot cross protection test with BBSV and PGMV gave ambiguous results.

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Explanation of Electron Micrographs:

Particles of PGMV negatively stained with ammonium molybdate. ×120000.

Fig. 3. Bottom component.

Fig. 4. Middle component. In some cases, central capsomeres (60—70 Å in diameter) composed of 5 or 6 subunits (~25 Å in diameter) can be seen.