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SOME PROPERTIES OF RED CLOVER NECROTIC MOSAIC VIRUS

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Summary. — No differences in the shape and size of the virions were found between two antigenically distinct isolates of red clover necrotic mosaic virus (RCNMV). The virions of either isolate appeared isometric with a diameter of about 30 nm. On sucrose density gradient centrifugation, infectious virus was concentrated in a single zone. Infectious ribonucleic acid (RNA) was isolated from purified RCNMV; the infectivity of this RNA was less than 1% of that of the starting RCNMV suspension.

RCNMV was isolated from mosaic diseased red clover (*Trifolium pratense* L.) plants in Slovakia (Musil and Matisová, 1967); it differs in several properties from other so far known plant viruses (Musil, 1969*a*). The isolates of RCNMV obtained so far may be separated into two distinct antigenic groups (Musil, 1969*b*).

The aim of the present experiments was to obtain more information about the representatives of these two antigenic groups.

Virus. The TpM34 and TpM48 isolates of RCNMV, representatives of antigenic groups A and B, respectively (Musil, 1969*a*, *b*), were propagated in bean (*Phaseolus vulgaris* L. cv. Saxa) plants. The bean plants were infected with the given isolate by mechanical inoculation of the primary leaves and stems. After 7 days of growth in glass-wall thermostats at about 22° C, the infected plants were harvested and their leaves and stems used for virus purification.

Virus assay. Test materials were manually inoculated onto primary leaves of bean (*P. vulgaris* cv. Perlička) plants. The bean plants were used in the stage of fully expanded primary leaves and the latter were dusted with silicium carbide powder (400 mesh) before inoculation. The plants were grown in a greenhouse with fluctuating temperature. The results were read according to symptoms (necrotic lesions or vein necrosis on the inoculated leaves) usually after 2—3 days (isolate TpM34) or 3—5 days (isolate TpM48).

Virus purification. The infected plant materials were ground in a mortar with the same volume of 0.067 M phosphate buffered saline pH 7.2 (PBS). The juice was expressed through dense cloth and then shaken for 15 minutes with the same volume of chloroform. The resulting suspension was broken by centrifugation at about 4000 × g for 30 minutes and the aqueous phase centrifuged for 2 hours at 90000 × g. The pellet thus obtained was resuspended in PBS, the suspension kept overnight at 4° C, clarified by centrifuging at 4000 × g for 30 minutes and kept for a further 3 days at 4° C. The resulting precipitate was removed by low speed centrifugation and the clarified supernatant was centrifuged again for 2 hours at 90000 × g. The pellet was resuspended in a volume of PBS usually corresponding to one tenth of the original volume of plant juice used for virus purification. The dilution endpoints of either virus isolate in such purified preparations reached values of 10^{-8}, which is in accordance with values from 10^{-6}—10^{-7} determined for the starting crude juices. Thus no obvious losses of virus had occurred during the purification procedure.

Uninfected bean plants grown under the same conditions as the infected ones were subjected to the same purification procedure. A considerable amount of plant proteins was pelleted after the first high speed centrifugation cycle and dissolved after resuspension of the pellet in PBS.

To achieve better purification of virus, the partially purified samples (0.2 ml) were subjected

to sucrose density gradient centrifugation for 5 hours at 37000 rev/min and at 4° C in the SW 39 L rotor of the Spinco L-50 centrifuge. The discontinuous gradients were prepared by placing into the centrifuge tubes 1, 1.4, 1 and 1 ml of 55, 45, 35 and 25% sucrose solutions in PBS and keeping the tubes overnight at 4° C. Fractions of 3 drops each, collected by piercing the bottom of the tubes, were diluted approx. 25-fold with PBS and their absorbancy at 260 nm and infectivity determined.

Electron microscopy. The negative staining technique of Brenner and Horne (1959) was modified as follows: concentrated partially purified virus suspensions were placed onto carbon-coated perforated formvar grids. After adsorption, the preparations were washed with distilled water and stained for about 3 minutes with 2% phosphotungstic acid whose pH was adjusted to 7.2 with 1 N KOH. The solution was then sucked off and the preparations were allowed to dry. They were examined in a JEM-6C electron microscope at an instrumental magnification of $\times 50000$ and 80 kilovolts.

Viral RNA was prepared by the method of Gierer and Schramm (1956). Partially purified virus suspensions were shaken for 10 minutes with equal volumes of water-saturated phenol and 0.1% Na-bentonite. The aqueous phase separated by centrifuging for 10 minutes at $3000 \times$ g was subjected to another two such treatments with half amount of phenol. Phenol was removed from the aqueous phase by repeated shaking with peroxide-free ether, which in turn was removed by bubbling nitrogen through the solution. After adding sodium acetate to 0.1 M concentration, RNA was precipitated by adding 2 volumes of ethanol. After 20 minutes, the precipitate was pelleted by brief low speed centrifugation, dissolved in sterile PBS and the solution assayed for infectivity.

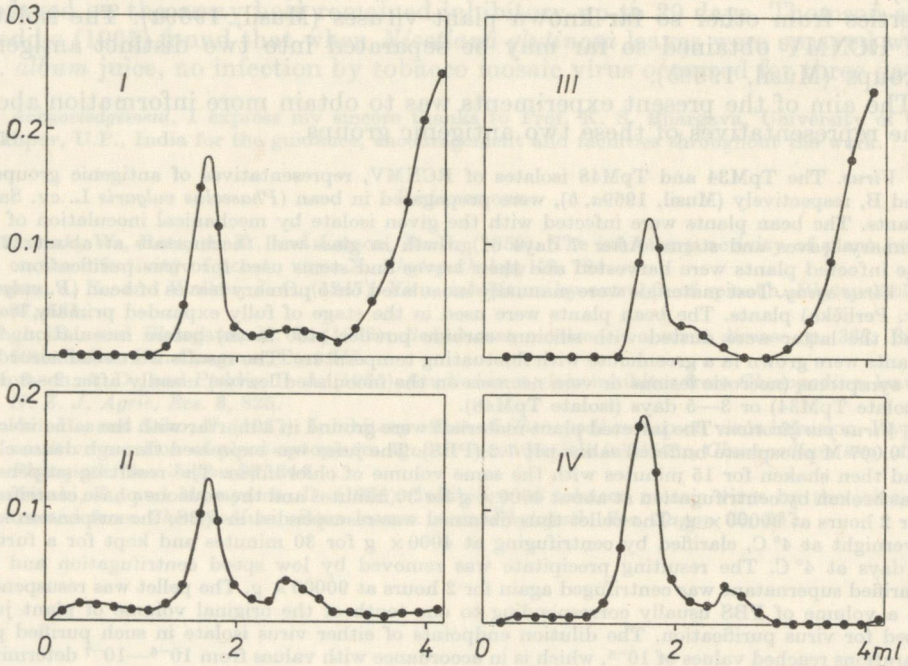

Fig. 1.

Distribution pattern of RCNMV after sucrose density gradient centrifugation
Ordinates: absorbancy at 260 nm; sedimentation is from right to left.
I and II — Isolate TpM34 after the first (I) and second (II) purification cycle
III and IV — Isolate TpM48 after the first (III) and second (IV) purification cycle

After sucrose density gradient centrifugation of either RCNMV isolate, three peaks were observed with preparations subjected to one or two purification cycles (Fig. 1). Infectivity was associated with the most rapidly sedimenting component (bottom peak). The other two (middle and top) peaks were devoid of biological activity and, on comparison with the pattern resulting after density gradient centrifugation of helathy bean proteins (Fig. 2), they may be ascribed to normal plant constituents.

The great amount of these normal plant proteins in virus preparations subjected to one purification cycle (Fig. 1 — I, III) was much reduced in preparations subjected to two purification cycles (Fig. 1 — II, IV). Most of the proteins

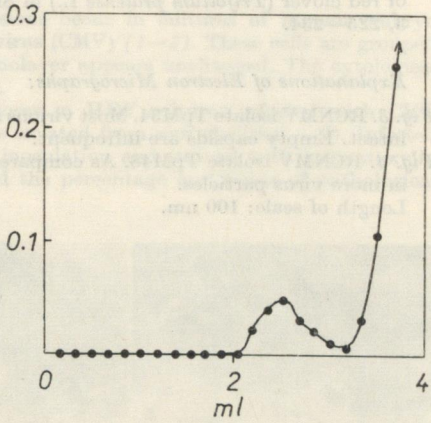

Fig. 2.

Distribution pattern of normal bean proteins after density gradient centrifugation
Normal bean plants were subjected to a treatment corresponding to the first purification cycle in the case of virus.
Ordinate: absorbancy at 260 nm; sedimentation is from right to left.

had precipitated while the virus suspensions were kept for some days at 4° C between the two purification cycles.

Based on the results of density gradient centrifugation and infectivity assay of the fractions, RCNMV may be considered as a one-component virus. The ultraviolet absorption spectrum of either isolate examined had a maximum at 260 nm.

Electron microscopy revealed no evaluable differences between the two isolates examined. The virions of both TpM34 and TpM48 appeared isometric with a diameter of about 30 nm (Figs 3 and 4).

The infectivity of RNA isolated from either isolate was less than 1% of the infectivity of the starting virus suspension. Local lesion formation was not observed when a solution, containing 100 times more RNA than its lowest detectable amount, was treated with 5 μg/ml pancreatic ribonuclease.

The present results revealed no evaluable differences between the two serotypes (A and B) of RCNMV. Based on these and previous investigations (Musil, 1969*a*, *b*), RCNMV cannot be identified with any of the known plant viruses. It is characterized by distinct host range, symptomatology, dilution end point ($10^{-6}-10^{-7}$), thermal inactivation point (about 90° C) and stability in plant sap. Moreover, no serological cross reactions between RCNMV and a number of isometric plant viruses were established.

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Explanations of Electron Micrographs:

Fig. 3. RCNMV isolate TpM34. Most virions show hexagonal projection and appear comparatively intact. Empty capsids are infrequent.

Fig. 4. RCNMV isolate TpM48. As compared with Fig. 3, the fine structure is better discernible in more virus particles.

Length of scale: 100 nm.