*Acta virol.* 13 : 226—234, 1969

# SEROLOGICAL PROPERTIES OF CERTAIN ISOLATES OF RED CLOVER NECROTIC MOSAIC VIRUS

M. MUSIL

Institute of Virology, Slovak Academy of Sciences, Bratislava, Czechoslovakia

*Received January 27, 1969*

*Summary.* — Antisera against 5 isolates of red clover necrotic mosaic virus (RCNMV) were prepared. Based on the serological differences between these isolates, two antigenic groups, A and B, were established. Two of the isolates studied belonged to group A (TpM34 and MM6), another two (TpM48 and TpM49) to group B, and 1 isolate (TpM50) was found to represent a mixture of viruses belonging to groups A and B. Antisera against members of the antigenic group A contained only homologous precipitin antibody, while in antisera against virus isolates of the antigenic group B there occurred both homologous precipitin antibody and, in a lower titre, antibody to group A. The differences found between the RCNMV isolates studied were evaluated as differences between representatives of two antigenically distinct groups of a single virus species.

## *Introduction*

In studying the properties of RCNMV, considerable antigenic differences between certain virus isolates and the respective antisera were observed. Already the results of preliminary experiments suggested that the isolates of RCNMV can be divided into two distinct antigenic groups (Musil, 1969). The results of further investigations on the relationship between these two antigenic groups of RCNMV are reported in the present paper.

## *Materials and Methods*

*Virus.* Five isolates of RCNMV (TpM34, TpM48, TpM49, TpM48 and MM6 — further on designated only with the respective numbers) were used. For their origin and characteristics see Musil (1969). The isolates were propagated in bean (*Phaseolus vulgaris* L. cv. Saxa, Top crop and others) plants. In preliminary experiments, sap from infected beans, clarified by centrifugation for 15 minutes at 5000 rev/min, was employed. In the proper experiments, purified virus suspensions were used as antigens. The isolates were purified and concentrated as described previously (Musil, 1969). The suspensions used as antigens were diluted so that the dilution end points of infectious virus reached values from $10^{-6}$—$10^{-7}$ and the serological antigen titres were 8—16. At these concentrations, the purified RCNMV suspensions formed with diluted antisera sharp, distinct precipitation lines.

*Preparation of antisera.* Antisera against the individual RCNMV isolates were prepared by immunizing rabbits intravenously with purified virus suspensions (virus dilution end points $10^{-7}$—$10^{-8}$, serological titre 160). The immunization schedule was as follows:

1) The rabbits were injected with 3 doses, each of 2 ml virus suspension, at 2-day intervals. Ten days after the last injection, blood samples were taken to determine the titre of homologous precipitin antibody.

2) From 2 to 3 weeks after onset of immunization, the rabbits were again given 2 injections of 2 ml virus suspension each at an interval of 2 days. Ten days after the 2nd injection, blood samples were taken and assayed as above. When the titres of precipitin antibody reached values of 256 or 512, the rabbits were not immunized further; large blood samples were taken or the animals were exsanguinated. In case of lower antibody titres, a 3rd immunization cycle, similar to the 2nd one, was added; blood was taken 10 days after the last injection. The sera were clarified by low speed centrifugation and, after adding merthiolate as preservative, distributed into tubes which were kept at —15° C.

In the period from 1965 to 1968, the following antisera to the virus isolates examined were prepared in different rabbits: 3 sera to isolate 34 (As 34-I in 1965, As 34-II in 1966 and As 34-III in 1968); 3 sera to isolate 6 (As 6-I in 1965, As 6-II and 6-III in 1966); and 1 serum each to isolate 49 and 50 (in 1966).

*Serological tests.* Precipitin antibody in the antisera prepared against the virus isolates mentioned was assayed in tube and drop precipitation reactions and by double diffusion in agar gel. The immunodiffusion test was the basic method employed; the other reactions were used in pilot or control experiments.

Immunodiffusion tests were carried out in small Petri dishes, using an approx. 1 mm thick layer of 1% agar (Bacto-agar Difco) gel. Antisera or purified virus suspensions were placed into 5 mm wells at a distance of 6 mm; the arrangement of the wells is evident from the illustrations. The dishes were kept for 5—7 days at room temperature, thoroughly washed and the precipitation zones stained with Amido black.

The antibody titres were expressed as reciprocals of the highest serum dilution which still gave a positive precipitation reaction.

## *Results*

### *Properties of the antisera*

The homologous precipitin antibody titres of antisera prepared against the individual isolates of RCNMV varied from 128 to 512. Testing of the antisera with all 5 isolates of RCNMV studied showed that the antisera reacted not with all the isolates or, if so, the intensity of the reactions with homologous and heterologous antigens was different. Preliminary tests revealed that antisera 6 and 34 contained precipitin antibody reacting with antigens 6, 34 and 50 (Fig. 1a, b). Precipitin antibody reacting with antigens 48 and 49 was not detected in any of the 3 antisera 6 and 34, obtained from different rabbits (see Fig. 2a, b). On the other hand, antisera 48 and 49 reacted in 1 : 2 or 1 : 4 dilutions with antigens 6 and 34 by a little pronounced wide precipitation zone and with homologous antigens by a celar-cut precipitation line. With antigen 50, these antisera produced 2 precipitation lines, one of which fused with lines against antigens 6 and 34 and the other with lines against antigens 48 or 49 (Fig. 1c, e). When diluted more than 1 : 32, antisera 48 and 49 reacted only with antigens 48, 49 and 50; no precipitation lines occurred with antigens 6 and 34 (e.g., Figs 1d and 2c, d).

Antiserum 50 was found to contain precipitin antibodies reacting with both antigens 6 and 34 and antigens 48 and 49; with homologous antigen, this antiserum produced 2 mutually separated precipitation lines (Fig. 1f). One of the latter lines fused with those against antigens 6 and 34, while the second fused with the line against antigens 48 and 49.

These results suggested that antigens 6 and 34 induced in the immunized animals the formation of one group of precipitin antibody, while antigens 48, 49 and 50 induced the formation of two groups of precipitin antibody, one of which was identical with antibody occurring in antisera 6 and 34.

A comparison of the properties of 3 antisera to isolate 6, 3 antisera to isolate 34 and 3 antisera to isolate 48 (each obtained from a different rabbit) showed that, in general, the individual animals reacted to the respective antigens in a similar way. On immunization with antigens 6 or 34, the rabbits produced only one group of antibody, whereas rabbits immunized with antigen 48 (or 49 or 50) always produced two groups of specific antibody (see Fig. 2a—f).

In addition to specific antiviral antibody, all of the antisera prepared contained a low level of precipitin antibody to normal proteins of the plant host used for virus propagation.

**Table 1. Precipitin antibody titre in antisera against 5 isolates of RCNMV**

| Antigen | Antibody titre in | | | | |
|---|---|---|---|---|---|
| | As 34 | As 6 | As 48 | As 49 | As 50 |
| Healthy bean sap | 8 | 16 | 8 | 8 | 8 |
| Ag 34* | 512 | 256 | 16 | 32 | 256 |
| Ag 6 | 256 | 256 | 16 | 32 | 256 |
| Ag 48 | 0 | 0 | 512 | 512 | 128 |
| Ag 49 | 0 | 0 | 512 | 512 | 256 |
| Ag 50 | 256 | 256 | 512 | 256 | 256 |

* Purified virus suspensions were used as antigens.

Based on the results described above, the individual antisera were examined for their relative contents of each precipitin antibody group and for their relationship to the 5 isolates of RCNMV studied. In this comparative experiment, antisera prepared in 1966, namely As 6-III, As 34-II, As 48-II, As 49 and As 50, were employed (Table 1).

In antiserum 34, the homologous antibody titre reached a value of 512. The same serum reacted with both antigen 6 and antigen 50 up to a dilution of 1 : 256. No precipitation occurred with antigens 48 and 49.

Antiserum 6 contained antibody to homologous antigen and to antigens 34 and 50 in a titre of 256. This antiserum gave no reaction with antigen 48 and 49.

In antisera 48 and 49, the homologous antibody titres reached the value of 512; the same titre was obtained with antigens 49 and 48, respectively. Antisera 48 and 49 reacted with antigen 50 up to serum dilutions of 1 : 512 and 1 : 256, respectively. With antigens 6 and 34, positive reactions were obtained with antiserum 48 diluted 1:8—1:16 and with antiserum 49 diluted 1 : 32.

Antiserum 50 reacted with the antigens tested up to serum dilutions of 1:128—1:256.

The occurrence in the antisera of groups of antibody against the different isolates of RCNMV suggested a peculiar position of the isolates 6 and 34 and their respective antisera. The results of qualitative and quantitative evaluation of the antisera 6 and 34 and their relationship to the 5 isolates of RCNMV tested make it possible to conclude that isolates 6 and 34 represent

one distinct antigenic group of RCNMV; this group will be designated as antigenic group A-RCNMV (serotype A).

In isolates 48 and 49, the presence of type A antigen was not demonstrated (Figs 1a, b; 2a, b; 3a); only another antigenic group, namely the antigenic group B-RCNMV (serotype B), was found. Since, however, in antisera 48 and 49 the presence of type A antibody, although in a lower titre, was repeatedly demonstrated in addition to type B antibody, it may be assumed that type A antigen is present in isolates 48 and 49 in serologically undetectable amounts.

In isolate 50, both antigenic groups were present, as were both groups of antibody in antiserum 50 (Fig. 3b) in about the same titre.

*Antibody absorption experiments*

Antisera were absorbed with the individual RCNMV isolates to elucidate the mutual relationships of the two antigenic or antibody groups in the given isolates of RCNMV and the respective antisera. To 0.4 ml of antisera 34-II, 6-III, 48-II, 49 and 50 were added equal volumes of sap from bean plants infected with the respective isolate of RCNMV. The mixtures were kept overnight at 4° C; the resulting precipitates were then removed by centrifugation and 0.4 ml of the respective purified RCNMV suspension (antigen titre approx. 64) was added to the clarified mixtures. After standing overnight at 4° C and removal of the precipitates by low speed centrifugation, the absorbed clarified antisera were assayed for antibody to the individual isolates

**Table 2. Antibody titres in antisera 34, 50 and 48 absorbed with healthy bean sap or individual isolates of RCNMV**

| Antiserum + antigen* | Titre of antibody against isolate | | | | |
|---|---|---|---|---|---|
| | 34 | 6 | 50 | 49 | 48 |
| As 34 + bean sap | 512 | 256 | 256 | 0 | 0 |
| + Ag 34 | 0 | 0 | 0 | 0 | 0 |
| + Ag 6 | 0 | 0 | 0 | 0 | 0 |
| + Ag 50 | 0 | 0 | 0 | 0 | 0 |
| + Ag 49 | 256 | 256 | 256 | 0 | 0 |
| + Ag 48 | 256 | 256 | 256 | 0 | 0 |
| As 50 + bean sap | 256 | 256 | 256 | 128 | 128 |
| + Ag 34 | 0 | 0 | 128 | 128 | 128 |
| + Ag 6 | 0 | 0 | 128 | 128 | 128 |
| + Ag 50 | 0 | 0 | 0 | 0 | 0 |
| + Ag 49 | 256 | 256 | 128 | 0 | 0 |
| + Ag 48 | 256 | 256 | 256 | 0 | 0 |
| As 48 + bean sap | 16 | 16 | 512 | 512 | 512 |
| + Ag 34 | 0 | 0 | 256 | 512 | 512 |
| + Ag 6 | 0 | 0 | 256 | 512 | 256 |
| + Ag 50 | 0 | 0 | 0 | 0 | 0 |
| + Ag 49 | 8—16 | 8—16 | 8—16 | 0 | 0 |
| + Ag 48 | 8—16 | 8—16 | 8—16 | 0 | 0 |

* Purified virus suspensions were used for absorption.
0 means that no antibody against the given isolate of RCNMV was detected in the absorbed serum.

of RCNMV or for excess of the added virus. To reach complete absorption of homologous antibody, 2—3 times greater volumes of purified virus suspensions had to be added to the antisera.

The results of experiments on absorption of antisera 34, 50 and 48 are summarized in Table 2. It was found that precipitin antibody in the sera was completely absorbed by purified preparations of homologous virus isolates. Antibody in antiserum 34 was also absorbed with both antigens 6 and 50. On the other hand, addition of antigens 48 or 49 to antiserum 34 resulted in no visible precipitation reaction and the level of antibody remained practically unchanged. In addition to antibody, also the virus added to this antiserum remained unchanged. Antiserum 6 gave results similar to those obtained with antiserum 34.

In antiserum 50, type A antibody was absorved with antigens 34 and 6, the titre of type B antibody (128—256) remaining unchanged. By contrast addition of antigens 48 and 49 to antiserum 50 resulted in absorption of typ B antibody and the titre of type A antibody remained at the level of 128—256.

The addition to antiserum 48, the same as to antiserum 49, of antigens 34 or 6 resulted in absorption of type A antibody, there being no change in type B antibody. By contrast, the addition to antisera 48 and 49 homologous antigens resulted in absorption of type B antibody; type A antibody was still present in thus treated antisera in titres of 8—16 (As 48) or 32 (As 49). Both types of antibody were absorbed from antisera 48 and 49 by antigen 50.

These results showed that antibody occurring in antiserum 34 or 6 did not react with the added purified suspensions of isolates 48 and 49. On the other hand, the addition of purified preparations of isolates 34 or 6 to antisera 48 or 49 resulted at first in absorption of type A antibody, but after adding more virus (isolates 34 or 6) both RCNMV isolates remained in the antisera along with type B antibody without undergoing a serological reaction.

The fact that by adding antigen 50 it was possible to absorb antibody from antisera 34 and 6, as well as from antisera 48 and 49, and the fact

**Table 3. Reactions of antisera 34 and 48 with purified RCNMV (isolates 34 and 48) and healthy bean sap in tube precipitin tests**

| Antiserum + antigen | Precipitation with antiserum diluted (reciprocals) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Undil. I* | 2 II | 4 III | 8 IV | 16 V | 32 VI | 64 VII | 128 VIII | 256 IX |
| As 34 + Ag 34 | +++ | +++ | +++ | +++ | +++ | +++ | ++ | + | (+) |
| + Ag 48 | + | — | — | — | — | — | — | — | — |
| + bean sap | +++ | +++ | +++ | ++ | + | — | — | — | — |
| As 48 + Ag 34 | +++ | +++ | +++ | ++ | + | — | — | — | — |
| + Ag 48 | +++ | +++ | +++ | +++ | +++ | +++ | ++ | + | + |
| + bean sap | + | — | — | — | — | — | — | — | — |

* I—IX: tube number.

The intensity of the precipitation reaction was scored from (+): indistinct precipitation till +++: heavy precipitation; — means no precipitate.

that in the thus treated antisera 34 and 6 there remained a residue of isolate 50 (antigenic group B), make it possible to assume that isolate 50 represents a mixture of representatives of either antigenic group.

Based on these findings an attempt was made to prepare purified suspensions of isolates 34 and 38 which would contain only either type A or type B antigen, respectively. To this end excess antiserum with heterologous antibody was added to purified preparations of isolates 34 and 48. The resulting precipitates were removed by centrifugation and thus treated virus isolates 34 and 48 were inoculated on leaves of bean (cv. Saxa) plants. Purified virus suspensions were then prepared from the infected plants. One part of these virus suspensions was used in further studies on the relationships between isolates 34 and 48 and the respective antisera. The other part was used to prepare antisera to isolates 34 and 48 in the hope that the treated isolate 48 would induce in the immunized animals only type B antibody. The antisera prepared to these "serologically purified viruses", however, did not differ from the antisera obtained previously. The new antiserum 34 again contained only one type (A) of antibody (Fig. 4a, f), while antiserum 48 (As 48-III) contained both type B and, in a lower titre, type A antibody (Fig. 4b—e).

*Relationship between isolates 34 and 48 and their antisera*

Three rows of tubes with serial twofold dilutions (1:2—1:256) of antisera 34 and 48 were prepared. To one row of tubes containing diluted antisera 34 and 48 was added an equal volume (0.3 ml) of purified isolate 34; isolate 48 was added to tubes of the 2nd row and healthy bean sap to tubes of the 3rd row. The mixtures were shaken and kept for 4 hours at about 22° C and then overnight at 4° C. Thereafter the precipitation reactions were scored (Table 3) and all tubes centrifuged for 15 minutes at about 4000 × g. The individual clarified samples were assayed for residual antibody or virus (Table 4).

In samples of antiserum 34 diluted more than 1 : 8, the absorption of antibody by the added amount of virus isolate 34 was complete. An 8-fold decrease of antibody occurred on addition of this isolate to samples of antiserum 34 diluted less than 1 : 8. In antiserum 34 diluted 1 : 64 there remained some virus, because the whole amount of virus was not bound by antibody. In samples of antiserum 34 diluted 1 : 128 and 1 : 256 there remained 2 to 4 times as much virus as in the antiserum sample diluted 1 : 64 (see Table 4).

In samples of antiserum 34 absorbed with antigen 48, the level of antibody to virus isolate 34 remained the same as in similar samples of the same antiserum absorbed with healthy bean sap. The concentration of virus (isolate 48) also remained substantially unchanged in all dilutions of antiserum 34. This suggests that no serological reaction had occurred between antiserum 34 and the purified virus isolate 48.

In samples of serially diluted antiserum 48, the addition of antigen 48 resulted in an 8-fold decrease till complete absorption of homologous type B antibody. In samples of antiserum 48 diluted more than 1 : 16 there remained an excess of virus. In sample No. V, i.e. in antiserum diluted 1 : 16, all antibody was absorbed and all virus bound by antibody (see Table 4). After adding antigen 48 to antiserum 48, the level of antibody against isolate 34 remained substantially unchanged. After adding antigen 34 to antiserum 48, the

**Table 4. Titres of residual antibody or virus in samples of diluted antisera 34 and 48 absorbed with healthy bean sap or purified RCNMV (isolates 34 and 48)**

| Sample of serum* | | Titre of antibody against antigens 34 and 48 | | | | | | | | Titre of residual virus in antiserum samples absorbed with | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Before absorption | | After absorption with | | | | | | | | | |
| | | | | Bean sap | | Ag 34 | | Ag 48 | | Ag 34 | | Ag 48 | |
| | | Ag 34 | Ag 48 | Ag 34 | Ag 48 | Ag 34 | Ag 48 | Ag 34 | Ag 48 | S | I | S | I |
| As 34 | I | 256 | 0 | 256 | 0 | 32 | 0 | 256 | 0 | 0 | 0 | 8–16 | 5 |
| | II | 128 | 0 | 128 | 0 | 8–16 | 0 | 128 | 0 | 0 | 0 | 8–16 | 5 |
| | III | 64 | 0 | 64 | 0 | 4 | 0 | 64 | 0 | 0 | 0 | 16 | 5 |
| | IV | 32 | 0 | 32 | 0 | 2 | 0 | 16–32 | 0 | 0 | 0 | 16 | 5 |
| | V | 16 | 0 | 16 | 0 | 0 | 0 | 16 | 0 | 0 | 0 | 16 | 5 |
| | VI | 8 | 0 | 8 | 0 | 0 | 0 | 8 | 0 | 0 | + | 16 | 5 |
| | VII | 4 | 0 | 4 | 0 | 0 | 0 | 4 | 0 | 2 | 4 | 16 | 5 |
| | VIII | 2 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 4 | 4 | 16 | 5 |
| | IX | <2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | 5 | 16 | 5 |
| As 48 | I | 16 | 256 | 16 | 128 | 0 | 128 | 16 | 32 | 0 | 0 | 0 | 0 |
| | II | 8 | 128 | 8 | 64 | 0 | 64 | 8 | 8 | 0 | 0 | 0 | 0 |
| | III | 4 | 64 | 4 | 32 | 0 | 32 | 4 | 4 | 0 | 3 | 0 | 0 |
| | IV | 2 | 32 | 2 | 16 | 0 | 16 | 2 | 2 | 2 | 4 | 0 | 0 |
| | V | <2 | 16 | 0 | 8 | 0 | 8 | 0 | 0 | 4 | 5 | 0 | 0 |
| | VI | 0 | 8 | 0 | 4 | 0 | 4 | 0 | 0 | 8 | 5 | 0–2 | 1–3 |
| | VII | 0 | 4 | 0 | 2 | 0 | 2 | 0 | 0 | 8–16 | 5 | 4 | 4 |
| | VIII | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 8–16 | 5 | 8 | 4 |
| | IX | 0 | <2 | 0 | 0 | 0 | 0 | 0 | 0 | 8–16 | 5 | 16 | 5 |

* See Table 3.

S — Serologically determined titre of residual virus.

I — Dilution end point (neg. log) of residual infectious virus determined in bean (*Phaseolus vulgaris* cv. Perlička) leaves; + means that infectious virus was found only in undiluted sample.

0 means that no antibody or virus was detected in the respective undiluted samples.

titre of antibody against virus isolate 48 remained the same as was the case with samples of antiserum 48 absorbed with healthy bean sap. But the addition of antigen 34 resulted in absorption of antibody against this isolate (type A) which occurs in antiserum 48. In samples Nos I and II, the whole added amount of virus isolate 34 was bound by antibody, while in the other samples residual virus was demonstrated; in samples VI—IX this excess corresponded to the amount of virus originally added.

In addition to serological tests, the amount of unbound virus was also estimated by determining virus dilution end points by inoculation of bean leaves. The results of these infectivity tests were in accordance with those of serological reactions (see Table 4).

The results of these experiments showed that the addition of purified suspensions of isolates 34 and 48 (with a serological titre of approx. 16) resulted in complete absorption of homologous antibody from antisera diluted 1 : 16. At this dilution of serum an equilibrium between antigen and the respective type of antibody was reached. In the other samples there remained a residue of either antibody or virus. As far as the mutual relationship of the two virus isolates and their antisera is concerned, also these experiments supported the conclusion that isolates 34 and 48 represent antigenically distinct representatives of RCNMV.

## *Discussion*

Studies of five isolates of RCNMV revealed considerable serological differences between some of them, which were due to the existence of two antigenically distinct groups among RCNMV isolates. The finding of two antigenically distinct groups in RCNMV represents a certain analogy to the distinct groups in RCNMV represents a certain analogy to the distinct serotypes in tobacco necrosis virus (Babos and Kassanis, 1963). However, some of the differences established between the RCNMV isolates studied are of another character than those reported for tobacco necrosis virus.

Virus isolates 6 and 34 are considered to represent members of one antigenic group — group A. Isolates 48 and 49 represent members of a second antigenic group — group B. A mutual relationship between members of these two groups follows from the properties of antisera prepared against members of the antigenic group B. Members of antigenic group A (i.e. isolates 34 and 6) induced in immunized animals the formation only of homologous antibody giving no cross-reaction with members of the other antigenic group (group B). From this point of view, the virus isolates 34 and 6 appear as serologically distinct from isolates 48 and 49. By contrast, isolates 48 and 49 (members of antigenic group B) induced in immunized animals the formation of homologous antibody and, in addition, of type A antibody, although in a lower titre. At first we assumed that the formation of both types of antibody was due to a different quantitative representation of both antigenic groups in isolates 48 and 49, in a way similar to that found with isolate 50. But the finding of both types of antibody in antiserum from a rabbit immunized with "serologically purified" isolate 48 did not exclude another explanation

than the originally assumed mixture of members of two antigenic groups. Experiments on isolate 50 clearly demonstrated that this isolate in fact represents a mixture of members belonging to both A and B antigenic groups.

The findings obtained on the serological properties of the five isolates of RCNMV studied make it possible to consider the distinct antigenic groups of RCNMV as either antigenically distinct strains of one virus species or representatives of two serologically related virus species. At present we incline to the former eventuality, i.e. that the question is of representatives of antigenically distinct groups of a single virus, displaying a certain unilateral serological relationship. This relationship is manifested by the formation of both types of antibody in animals immunized with representatives of the antigenic group B-RCNMV. This view is supported by certain other facts concerning the shape and size of the virions of either antigenic group (Marcinka *et al.*, 1969). It cannot be excluded, however, that further informations on the properties of representatives of individual antigenic groups of RCNMV might lead to another evaluation of the established differences with respect to classification.

*Acknowledgement.* Thanks are due to Miss M. Augustínová for technical assistance in the experiments and photodocumentation, and to Dr. V. Valenta for helpful suggestions.

## References

Babos, P., and Kassanis, B. (1963): Serological relationships and some properties of tobacco necrosis virus strains. *J. gen. Microbiol.* **32**, 135—144.

Marcinka, K., Musil, M., and Mrena, E. (1969): Some properties of red clover necrotic mosaic virus. *Acta virol.* **13**, 247—250.

Musil, M. (1969): Red clover necrotic mosaic virus, a new virus infecting red clover (*Trifolium pratense*) in Czechoslovakia. *Biológia (Bratislava)* **24**, 33—45.

*Explanation of Figures:*

Immunodiffusion reactions with RCNMV.

*Fig. 1.* Central wells — antisera: *a* — As 6, dil. 1 : 2; *b* — As 34, dil. 1 : 2; *c* — As 48, dil. 1 : 2; *d* — As 48, dil. 1 : 8; *e* — As 49, dil. 1 : 2; *f* — As 50, dil. 1 : 2. Peripheral wells — antigens 34, 6, 50, 49 and 48.

*Fig. 2.* Reaction of antisera
*a* — As 6-I, As 6-II and As 6-III, diluted 1 : 2;
*b* As 34-I, As 34-II and As 34-III, diluted 1 : 2;
*c* — As 48-I, As 48-II and As 48-III, diluted 1 : 2;
*d* — As 48-I, As 48-II and As 48-III, diluted 1 : 32;
*e* — As 34-II (dil. 1 : 2), As 50 (dil. 1 : 2) and As 49 (dil. 1 : 2); and
*f* — As 34-II (dil. 1 : 32), As 50 (Dil. 1 : 32) and As 49 (dil. 1 : 32)
with antigens 34 and 48.

*Fig. 3.* Central wells — antisera: *a* — As 34, dil. 1 : 2, *b* — As 50, dil. 1 : 2, and *c* — As 48, dil. 1 : 32.
Peripheral wells: antigens 50, 34, 48, 50, 6 and 49.

*Fig. 4.* Reactions of
*a* — As 34, dil. 1 : 2, *b* — As 48, dil. 1 : 2, and *c* — As 48, dil. 1 : 16 with antigens 34 and 48
*d* — As 34 and As 48 diluted 1 : 2, and *e* — the same diluted 1 : 16 with antigen 34;
*f* — As 34 and As 48 diluted 1 : 2 with antigen 48.