

Dedicated to Prof. dr. MERCEDES WRISCHER
on the occasion of her 70th birthday.

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SOME PHYSICOCHEMICAL CHARACTERISTICS OF NATIVE AND FORMALDEHYDE TREATED TOBACCO STREAK ILARVIRUS PARTICLES

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The electrophoretic heterogeneity (three electrophoretic components) of native tobacco streak ilarvirus (TSV) preparations was demonstrated by gel immunoelectrophoresis. Formaldehyde (FA) fixation provoked enhancement of anode electrophoretic mobility and the gradual formation of a homogeneous electrophoretic pattern. Following FA fixation the three main sedimenting zones (T,M,B) revealed a certain loss of virus material as compared with unfixed, freshly purified TSV; simultaneously the nucleoprotein level in additional underlying zone(s) enhanced showing of aggregate formation. A slight shift in virus sedimentation rate towards the bottom of the gradient was another effect of FA fixation. Preparations exposed to FA for a longer time revealed a sedimentation shift in the opposite direction. A drastic decline of the main sedimenting nucleoprotein components was demonstrated with unfixed TSV for several days following purification; the B zone showed the greatest tendency to virus loss. Electron microscopy and antigenicity analysis tests confirmed that TSV aggregation occurred in preparations treated by formaldehyde. The dynamics of virus aggregate formation was monitored by immunodiffusion tests.

Key words: tobacco streak ilarvirus, formaldehyde fixation, electrophoresis, sedimentation

Introduction

Hitherto, formaldehyde has been exploited very much in the fixation of unstable plant viruses. It successfully protects the structure of virus particles, preventing protein shell dissociation into subunits and the digestion of uncoated

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nucleic acid. The protecting role of formaldehyde was first demonstrated on alfalfa mosaic alfamovirus in a high salt solution of sodium chloride (VERHOYEN 1967) and in the presence of ribonuclease (BOL and VELDSTRA 1969). Similar behaviour is shown by some other viruses from the *Bromoviridae* family. Thus LOT and KAPER (1973), as well as HABILI and FRANCKI (1974) reported the resistance of formaldehyde-fixed cucumber mosaic cucumovirus (CMV) in high salt solutions. LOT and KAPER (1976) made good use of this circumstance with the isopicnic centrifugation of CMV in caesium formate and rubidium chloride density gradients. THOMAS et al. (1983) did the same dealing with *Hydrangea* mosaic ilarvirus. In electron microscopy, the stabilization of virus particles with 1–2 % formaldehyde before staining is very common. Formaldehyde fixation also preserved the antigenicity and enhanced the immunogenic capacity of CMV preparations (FRANCKI and HABILI 1972, RICHTER et al. 1978). Its qualitative and quantitative effects, concerning the specificity of antibodies produced and their amount respectively, were demonstrated on brome mosaic bromovirus (BMV) by VON VECHMAR and VAN REGENMORTEL (1968). The most complex approach in the study of formaldehyde effects on various properties of virus particles was discussed by RICHTER et al. (1972), including the influence of formaldehyde fixation on BMV particle charge, density, aggregate formation and resistance in some extremely unfavourable conditions. Following some earlier attempts (ŠTEFANAC and KRAJAČIĆ 1991), the aim of this work was to use similar approaches in comparing the properties of native and formaldehyde treated particles of multicomponent and labile tobacco streak ilarvirus (TSV).

Materials and methods

All investigations were performed with the Cle strain of TSV identified earlier in Croatia as a distinct strain of TSV from *Clematis vitalba* (RANA et al. 1987). The virus was propagated in *Chenopodium quinoa* using 0.066 M, pH 7.3 phosphate with 0.02 M 2-mercaptoethanol as inoculation buffer. A partially purified preparation was obtained by grinding infected leaves in inoculation buffer, followed by clarification with 8.5 % n-butanol and differential centrifugation (90 000 g, 90 min; 5 000 g, 15 min). The virus pellet was resuspended in 0.01 M, pH 7.0 phosphate buffer.

The immunoelectrophoretic experiments were performed in LKB-Gelman 6800 A-1 electrophoresis apparatus in 1 % agar (Difco-Noble) gel. As tested with a series of buffers differing in molarity and pH (KRAJAČIĆ and ŠTEFANAC 1990), the 0.016 M phosphate, pH 7.0 as a gel buffer and twofold molarity in the apparatus vessels, ensured the optimal immunoelectrophoretic patterns when a current of 4 mA and a constant voltage of 20 V/cm were applied for 4 h. Titre of antiserum used varied from 1/2 to 1/16 according to expected virus concentration.

Particle types were separated by density gradient centrifugation in 10–40 % sucrose gradient in a Beckman SW 25.1 rotor. Gradients were analysed on an ISCO Model 640 density gradient fractionator using a UA-5 absorbance monitor.

Partially purified preparations stained with 2 % (w/v) potassium phosphotungstate, pH 6.8 were examined in a Siemens Elmiskop I.

All serological tests were made in 1 % agar (Difco-Bacto) gel double or single diffusion plates in 0.01 M, pH 7.0 phosphate buffer with traces of sodium azide. Single radial immunodiffusion gels contained 0.01% of homologous anti-serum (titre 1/32).

The virus preparations were fixed by adding formaldehyde dropwise to final concentration of 1 % or 9 %. Some preparations were dialysed against 0.01 M, pH 7.0 phosphate buffer. All virus preparations (native, fixed and those exposed to fixation and dialysis) were kept at 4 °C.

Results

Immuno-electrophoresis. Under the conditions described, the electrophoretic heterogeneity of the native (unfixed) virus particles was demonstrated. The immuno-electrophoretic pattern (Fig. 1a) exhibited a complex precipitin arc that consisted of two components of different mobility towards the cathode and one of anode orientation.

A day after being exposed to fixation with 1 % formaldehyde, the virus preparation still showed electrophoretic heterogeneity (Fig. 1b). The precipitin pattern indicated two well expressed components, one of electrophoretic inertia or weak mobility (a few mm) towards the cathode and the other rather apart from the starting well in the direction of anode. The preparations of relatively low concentration (1–5 mg/mL) resulted in no or only slightly visible reaction. During the next month the behaviour of the virus exposed to formaldehyde was characterised by progressive enhancement of the anode electrophoretic mobility and gradual formation of the homogeneous electrophoretic pattern (Figs. 1 c, d). This phenomenon was accompanied by enlarged intensity of the precipitin arcs, due to successive focussing of virus material, and finally resulted in a single precipi-

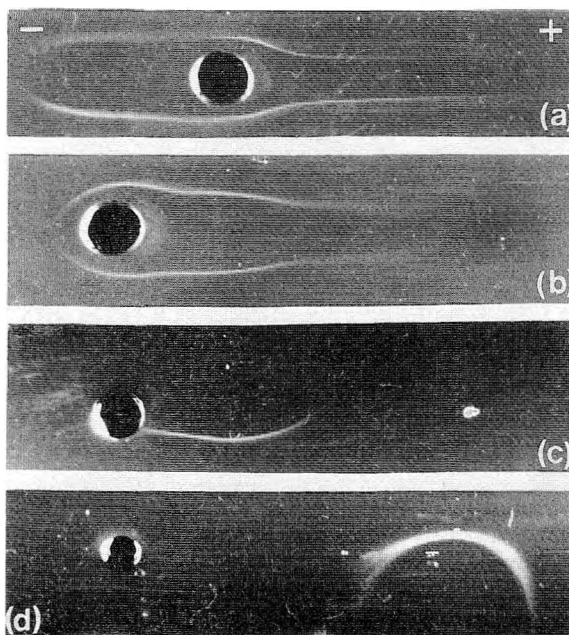


Fig. 1. Precipitin patterns obtained by agar gel immuno-electrophoresis using tobacco streak ilarvirus (TSV) anti-serum to Cle strain. Presented are the charge characteristics of: (a) fresh (1 day old) unfixed partially purified TSV preparation of Cle strain and preparations fixed in 1 % formaldehyde and stored at 4 °C for (b) 1 day, (c) 8 days and (d) 1 month (Virus concentration 10 mg/mL; serum titre 1/8 in (a), (b), (c) and 1/16 in (d)).

pitin arc, which became visible even with preparations of relatively low virus concentration. The virus preparations exposed to fixation in 9 % formaldehyde exhibited very similar electrophoretic behaviour as those treated by 1 % formaldehyde. However, all changes listed above occurred in a few hours. As expected, dialysis of the preparations resulted in a suspension characterised by progressive changes. Moreover, in the case of insufficiently fixed preparations, the dialysis was followed by reverse direction of the changes described. The experiments were not performed in such a manner as to determine the exact value (in mm) of the plateau in electrophoretic mobility enhancement. However, there were no big differences between the final mobility of mildly and strongly fixed preparations, which differed only in the duration of the changes described.

Density gradient centrifugation. In rate zonal density gradient centrifugation the freshly purified, unfixed virus separated into three main sedimentation zones which were resolved as characteristic peaks in the UV-absorption profiles (Fig. 2a). Some preparations provoked the UV-absorption profile, which suggested that the slowest sedimented zone consisted of two particle types slightly dislocated in the gradient. The additional underlying zone probably consisted of small virus aggregates. Following formaldehyde fixation, the three main zones, and especially the two upper ones, revealed a decline of virus material; simultaneously the nucleoprotein level in the additional underlying zone(s) was enhanced (Fig. 2b). This all indicated that particle polymerization had occurred. Differently fixed virus preparations (in 1 % or 9 % formaldehyde) revealed nearly identical sedimentation patterns. Also, insufficiently fixed preparations (in 1 % formaldehyde, immediately followed by dialysis) displayed the presence of great deal of higher sedimentation rate material.

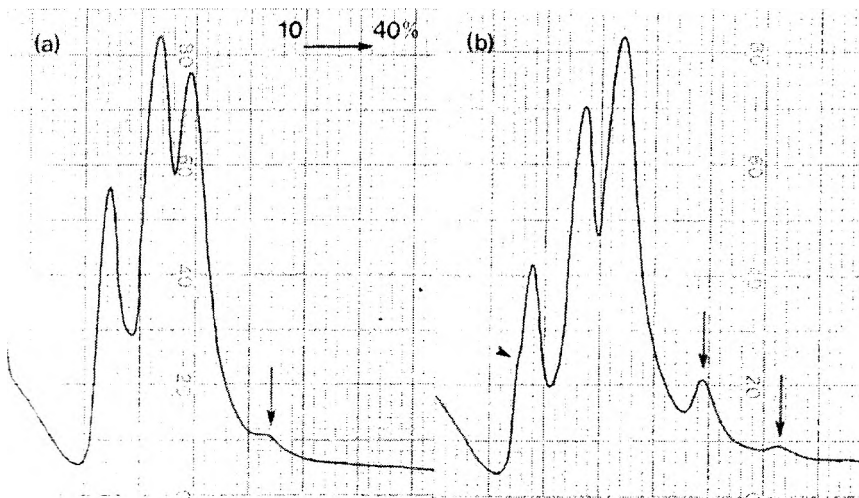


Fig. 2. Sedimentation profiles in sucrose density gradient centrifugation of: (a) fresh partially purified TSV preparation without fixation and (b) after strong fixation in 1 % formaldehyde, as analysed on an ISCO density gradient fractionator using an absorbance monitor. The slightly expressed subcomponent (arrowhead), zones of virus aggregates (arrows) more expressed after formaldehyde fixation.

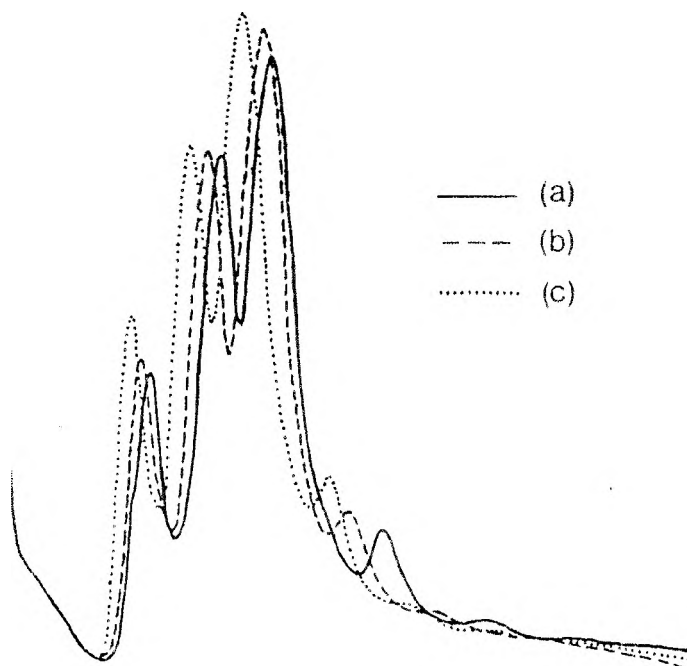


Fig. 3. Sedimentation profiles obtained by sucrose density gradient centrifugation of TSV preparation analysed: (a) 1 day, (b) 3 days and (c) 1 week after the addition of 9 % formaldehyde and storage at 4 °C. The shift in sedimentation rates towards the top of gradient are presented.

A slight shift in the sedimentation rate towards the bottom of the gradient reflected the particle condensation as another effect of the fixation procedure. This was a reversible phenomenon, because virus preparations exposed to fixation for a longer time revealed a sedimentation shift of the opposite orientation (Fig. 3). In both cases, the alteration of sedimentation value was minimally expressed at the top zone and maximally at the bottom one. It has to be mentioned that the return sedimentation shift towards the top of gradient was somewhat greater than the original one towards the bottom.

A disproportional decline of the main sedimenting nucleoprotein components characterized by adequate diminution of UV-absorption peaks was demonstrated with unfixed virus preparations several days after purification (Fig. 4). In some cases it was accompanied with an increase of material on the top of gradient suggesting the accumulation of disrupted virus particles constituents. Another time, the decrease in intact virus peaks was not followed by any increase of light material, indicating the aggregation of virus particles. Anyway, the bottom sedimenting zone exhibited the greatest tendency to lose the virus material.

Electron microscopy. Free virus particles as well as virus aggregates of different size were visible on electron micrographs immediately or several days after fixation in 1 % formaldehyde (Fig. 5). The aggregates consisted of virus particles losing their sharp outline and incorporated in a more or less amorphous mass.

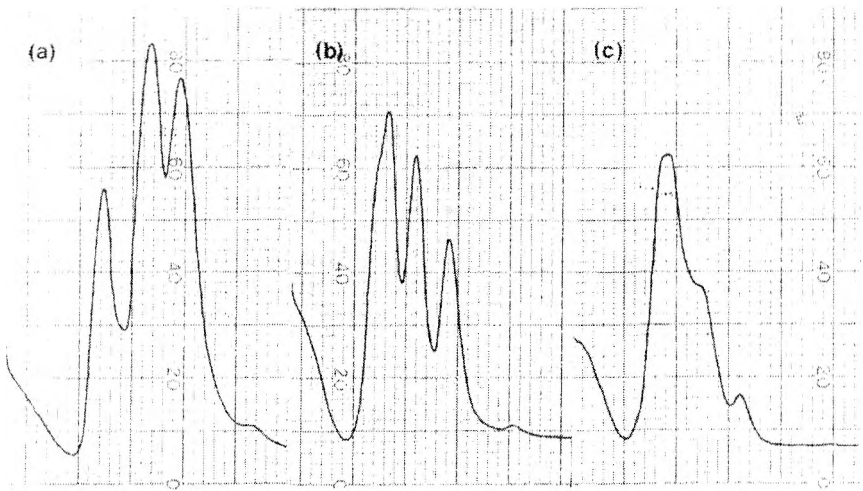


Fig. 4. Disproportional decline of the main nucleoprotein components of unfixed TSV as detected after sucrose density gradient centrifugation on a density gradient fractionator using an absorbance monitor. Partially purified virus preparations stored at 4 °C were analysed after: (a) 1 day, (b) 5 days and (c) 8 days.

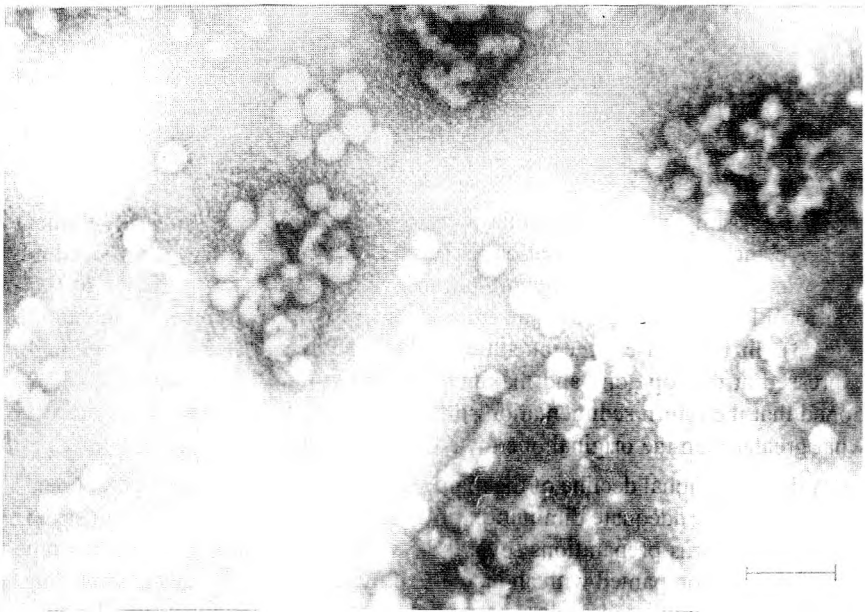


Fig. 5. Free TSV particles and virus aggregates of different sizes on electron micrograph after fixation in 1 % formaldehyde. Stained with potassium phosphotungstate. Bar represents 1 μ m.

Serology. Double radial immunodiffusion tests performed with fixed virus preparations revealed half or even quarter antigenicity of unfixed, recently puri-

fied preparations. The diffuse serological pattern and the absence of a precipitin line specific to subunits of disrupted virus particles suggested that the loss of antigenicity might be caused by virus aggregation.

The diameter of precipitin haloes produced in single radial immunodiffusion was used as a measure of the antigenic capacities of differently fixed virus preparations (Tab. 1). A day after addition of formaldehyde (9 % and 1 % FA) undialysed preparations reacted as stronger antigens than a dialysed preparation fixed in 9 % FA for 1 h. Results obtained after longer storage at 4° revealed a gradual decline in the virus antigenicity of undialysed preparations. Conversely, the dialysed preparation was characterised by increased antigenicity two weeks after fixation, with a decrease after six weeks. It has to be mentioned that a preparation diluted after formaldehyde treatment reacted as a stronger antigen than the preparation equally diluted before fixation.

Tab. 1. Diameter of precipitin haloes (used as a measure of antigenic capacity) in single radial immunodiffusion of differently fixed TSV preparations stored at 4 °C.

virus preparation (20 mg/mL) fixed in formaldehyde (FA)	diameter (in mm) of precipitin haloes obtained after:		
	1 day	2 weeks	6 weeks
1 % FA, no dialysis	18	17	16
9 % FA, no dialysis	15	12	11
9 % FA, dialyzed after 1 h	8	15	8

Purified virus preparations obtained by density gradient centrifugation gave weak results in immunodiffusion, immunoelectrophoresis and after repeated centrifugation in density gradient. In contrast to partially purified preparations, as expected, they were characterized by the loss of most of the free virus particles.

Discussion

TSV was previously described as an electrophoretically homogeneous system in free boundary electrophoresis (LISTER and BANCROFT 1970, CLARK and LISTER 1971). The second authors, however, described the electrophoretic heterogeneity in gel electrophoresis as a function of particle size, rather than charge, suggesting that virus components separated on the basis of a molecular sieving effect. In the experiment of LISTER et al. (1972) components separable by polyacrylamide gel electrophoresis, again on the basis of size, were identified as centrifugal components.

The results of our investigation differ from previously reported data, indicating the complex electrophoretic behaviour of TSV particles during fixation procedure. It is reasonable to suppose that our electrophoretic components differ in charge. As virus preparations gradually change their electrophoretic mobility during formaldehyde fixation, becoming finally homogeneous, it seems that it could not be the consequence of particle size changes.

A shift in sedimentation rate reflected TSV particle condensation as another effect of the fixation procedure. In contrast, RICHTER et al. (1972) have observed by density gradient centrifugation and sepharose filtration that particle size of BMV did not alter, since virus sedimentation zone or elution peak kept its position after the formaldehyde treatment. Similarly, LOT and KAPER (1973) could not demonstrate the influence of formaldehyde on particle density in this virus. However, by isopicnic centrifugation the same authors (LOT and KAPER 1976) revealed the small density alteration of CMV caused by formaldehyde fixation. The changes recorded in our experiments with TSV were not big, but significant enough to be pointed out.

In unfixed (native) partially purified TSV preparations, a considerable decline in the nucleoprotein level of particular sedimenting zones was demonstrated. The most expressive changes were always observed in the bottom zone. In addition, this zone exhibited the most significant shift in particle sedimentation rate following formaldehyde fixation. It is thus evident from the sedimentation alteration study that the stability of TSV particles was conversely related to the particle diameter.

Electron microscopy, as well as antigenicity analysis by immunodiffusion tests, confirmed that the aggregation of the virus under the influence of formaldehyde occurred. Single radial immunodiffusion tests revealed the dynamics of virus aggregate formation during a six-week period (Tab. 1). At the beginning of the fixation procedure the evident aggregation effect resulted in the decline of virus antigenicity, which was proportional to formaldehyde concentration. That is, mildly fixed virus preparations showed better antigenicity than strongly fixed preparations. The optimal time for beginning the dialysis seemed to be correlated with obtaining the plateau of electrophoretic mobility enhancement. This could be the moment of an optimal fixation effect, which was followed by the progressive decline of preparations caused by virus aggregate formation.

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Sažetak

NEKA FIZIČKOKEMIJSKA SVOJSTVA NATIVNIH I FORMALDEHIDOM FIKSIRANIH ČESTICA ILARVIRUSA CRTIČAVOSTI DUHANA

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Imunoelektroforezom u gelu utvrđena je elektroforetska heterogenost, tj. tri elektroforetske komponente, nativnih pripravaka ilarvirusa crtičavosti duhana (TSV) (sl. 1a). Fiksiranje virusa formaldehidom (FA) uzrokovalo je povećanu elektroforetsku pokretljivost u pravcu anode i postupno oblikovanje homogenog elektroforetskog obrasca (sl. 1b, c, d). Nakon fiksiranja s pomoću

FA tri glavne sedimentacijske zone (T, M, B) pokazale su stanovito smanjenje količine virusa (sl. 2b) u usporedbi s nefiksiranim, svježe purificiranim TSV (sl. 2a); istovremeno se povećala količina nukleoproteina u dodatnim donjim zonama, što je bio dokaz oblikovanja virusnih agregata. Daljnji učinak fiksiranja s FA bio je lagan pomak u brzini sedimentacije prema dnu gradijenta; pripravci izloženi fiksiranju duže vremena pokazivali su sedimentacijski pomak u suprotnom smjeru (sl. 3). U nefiksiranih pripravaka TSV-a utvrđeno je drastično smanjenje glavnih sedimentacijskih nukleoproteinskih komponenta u toku nekoliko dana nakon purifikacije (sl. 4); pritom je najveću tendenciju gubitka virusa pokazivala zona B. Elektronska mikroskopija (sl. 5) i testovi analize antigenosti (tablica 1) potvrdili su pojavu agregiranja TSV-a u pripravcima kojima je dodan FA. Dinamika stvaranja agregata praćena je pomoću imunodifuzijskih testova.