Acta Bot. Croat. 36 (1977) 9—17

THE STRUCTURE AND COMPOSITION OF CRYSTALLINE INCLUSIONS OF PLANT VIRUSES

(A Survey)*

DAVOR MILIČIĆ

(Institute of Botany, Faculty of Science, University of Zagreb)

Received February 1, 1977

Introduction

Early in the development of virology as a special science the crystalline cell inclusions were discovered. Iwanovski (1903) described the hexagonal prisms in the living tobacco cells which appeared under the influence of tobacco mosaic virus (TMV). It was only half a century later that Steere and Williams (1953) established by exact methods that the hexagonal prisms were built of regularly packed virus particles. However not only TMV, but many other plant viruses can crystallize in the infected cells forming crystalline inclusions. The viral intracellular crystals will be presented in this paper.

From infected tobacco Stanley (1935) isolated a crystalline protein which had the properties of TMV and the form of paracrystalline needles or spikes. It was soon shown that the protein consisted of elongated virus particles (Bernal and Fankuchen 1937). Afterwards Bawden and Pirie succeeded in isolating the tomato bushy stunt virus, i. e. an isometric virus which crystallized in vitro in the form of dodecahedron (Bawden, 1964). Later on a large number of other viruses were obtained in crystallized form, e. g. the poliovirus and the mouse polyoma virus (Knight 1974: 17). It has also been established that two strains of the tobacco necrosis virus can crystallize regularly in two various but definite ways (Bawden 1964: 295).

Thus, plant viruses can crystallize in the infected cells and in vitro. However, in this paper only the intracellular crystalline inclusions will

* Paper delivered at the 3rd International Congress for Virology in Madrid 1975.

be presented. The inclusions can be built of virus particles but not exclusively. It has been established that some viruses can provoke the formation of protein crystals which are not built of virus particles but of another protein which is probably coded by virus.

The purpose of this paper was not to give an entire survey of all viral crystalline inclusions but only to present their most frequent types in a short form.

Crystals of the Group of Tobacco Mosaic Virus

The best studied plant virus crystals are the hexagonal prisms which appear in the plants infected with the common TMV. Wehrmeyer (1957) stated that the rod shaped virus particles were packed laterally very densely and parallelly forming layers. In the layers the ends of particles were aligned. Every prism is composed of many layers of virus particles, and all particles are oriented perpendicularly to the hexagonal surfaces and parallelly to the lateral surfaces of prisms.

The hexagonal prisms are common in plants infected with TMV and tomato mosaic virus, but they are not so frequent in plants infected by other viruses belonging to the TMV group. However, a special modification of hexagonal prisms, the so called rounded plates, are often present in plants infected with ribgrass mosaic virus, sunn hemp mosaic virus, cucumber green mottle mosaic virus and odontoglossum ringspot virus (Miličić and Juretić 1971, Miličić and Štefanac 1971). The rounded plates, similar to hexagonal prisms, are built of many layers of parallelly packed virus particles. In rounded plates the virus particles are not so densely packed laterally as in the hexagonal prisms. Owing to this property the rounded plates have not hexagonal but rounded or irregular contours. Nevertheless, all layers of virus particles are not always of the same size, the middle layers being usually larger than the marginal ones (Miličić 1968). It is important that the prisms and plates are birefringent when viewed in polarized light edge-ways but not when viewed flat (Bawden 1964: 56). The character of their birefringence is positive with regard to the direction of long axis of virus particles.

A special kind of virus aggregates are the so called *angled layer aggregates* which are formed under the influence of aucuba strain of TMV (Warmke 1968). In contrast with the hexagonal prisms and rounded plates where the particles are erect and oriented perpendicularly to the surface of the layers, in the angled layer aggregates the particles lie flat in the plane of the layer. In each layer the particles are oriented in the same direction and they are parallel to one another. The orientation of the virus particles in the next layer is rotated against the rotation in the former one at an angle of 60 degrees. The orientation of virus particles becomes, therefore, equal in every third layer (Warmke 1968). In cross sections the angled layer aggregates appear as short parallel lines interspersed with rows of dots which represent cross sections of virus particles.

The so called *spikes* represent a very frequent form of bodies (Kassanis and Sheffield 1941) which show a paracrystalline structure. All virus particles are oriented along the length of the spike but their ends are not aligned (Warmke and Edwardson 1966). Therefore,

they are not arranged regularly in this direction. However, in the cross sections the virus particles are arranged in a regular way forming a crystalline lattice (Bernal and Fankuchen 1937; Fig. 1).

Kassanis and Sheffield (1941) described fibres longer than the cell so that they were often curved into figures-of-eight. The inclusions were named the *long coiled fibres* or *loops*. Afterwards, Wehrmeyer (1959) studied in detail this inclusion type and established that it was also built of virus particles. The long coiled fibres are about 1 μm thick, and retain the same thickness along their whole length.

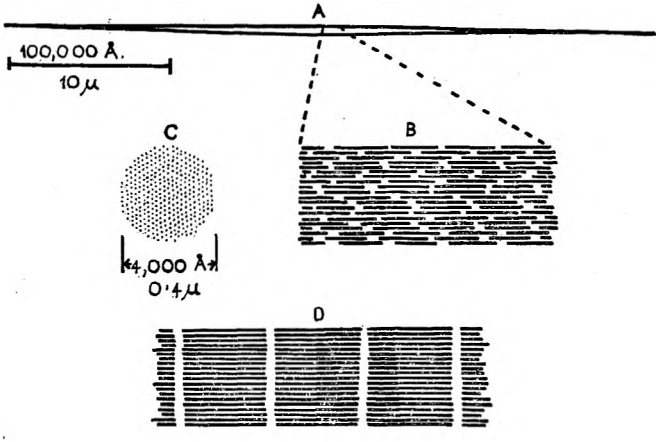

Fig. 1. A. Typical spike crystal. B. Longitudinal section. C. Transverse section. (A—C. According to Bernal and Fankuchen 1937; Nature *139*, 924). D. Longitudinal section through another form of virus crystal with particle ends aligned.

Sl. 1. A. Tipični kristal u obliku bodljike. B. Uzdužni presjek. C. Poprečni presjek. (A—C. Prema Bernalu i Fankuchenu 1937; Nature *139*, 924). D. Uzdužni presjek kroz drugi oblik virusnog kristala u kojem su svi krajevi čestica poravnati.

During electron microscopic investigations Wehrmeyer ascertained that virus particles and fibrils were oriented spirally in loops and not longitudinally as in spikes. The strength and direction of the torsion could be very different. It is characteristic of the loops that they are never positively birefringent in respect to their orientation; but in some cases a negative birefringence of some parts of loops was observed.

Virus spindles

The structure of the virus spindles is similar to the one of the spikes. The spindles are formed under the influence of some viruses belonging to the group of potato virus X, e. g. of cactus virus X and narcissus mosaic virus (Pleše and Miličić, 1966; Štefanac and Ljubešić, 1974). The flexuous particles of both viruses are about 520 nm long. The virus particles in spindles are oriented longitudinally.

Unlike the spikes the virus spindles are not homogeneous. They regularly contain enclosed long parts of cytoplasm and vacuoles. The surface of spindles is not always even and it frequently shows more or less longitudinal furrows. As the spindles have peripheral furrows and enclosed parts of cytoplasm, it often seems that they have a fibrillar structure when viewed in light microscope (Amelunxen and Thaler, 1967). The virus spindles of cactus virus X and narcissus mosaic virus can sometimes be situated in the nuclei (Milićić 1954, Amelunxen 1958, Turner 1971). The nuclear spindles of both viruses have the same form as the cytoplasmic spindles and are also built of virus particles.

The so called "zebra" structure of protein spindles sometimes appears in the spindles of cactus virus X (Amelunxen, 1956; Weber and Kenda 1952; Thaler 1966), in the spindles of an unkown virus of *Lilium tigrinum* (Thaler 1956), in the elongated virus bodies of clover yellow mosaic virus (Purcifull et al. 1966), and in the spindles of beet yellows virus (Esau et al. 1966). According to Weber and Kenda (1952) the "zebra" structure is the result of the breaks which occur repeatedly in regular intervals along the length of "zebra" spindles. In the region of the breaks virus particles are not present; this follows from the fact that the breaks do not light in a polarization microscope between crossed polarization filters. Conversely, the other parts of spindles light very strongly under the same conditions. The fact that the broken places can not be stained with organic dyes with which the spindles are stainable, leads us to the same conclusion. It is visible from electron micrographs of "zebra" spindles (Esau et al. 1966; Purcifull et al. 1966) that the virus particles are arranged in bands, very densely and parallelly, and that their ends are aligned (cf. Fig. 1 D).

It seems that the "zebra" structure appears only in those spindles the virus particles of which have the ends aligned. In this case the spindle places, where two particle layers (or bands) join together (Fig. 1 D), are mechanically weak and therefore the spindles break easily. As the breaks occur in regular intervals, it seems that the tension forces, which provoke the breaks and which are probably caused by cell growth or cytoplasmic movements, are uniformly disposed along the length of spindles.

A similar process occurs in the virus spindles of beet plants infected with beet yellows virus (Esau 1966). This virus has much longer particles than the potexviruses. The virus spindles in infected beets are very frequent and they often show the "zebra" structure.

Spiral aggregates

Virus particles packed in the form of "spiral aggregates" are found in plants infected with clover yellow mosaic virus and papaya mosaic virus both of which belong to the potexviruses. In the spiral aggregates the peripheral virus particles lie tangentially in the plane of the section but the inner particles change their orientation gradually so that the centrally placed particles are cut across because they lie perpendicularly to the section (Purcifull et al. 1966; Zettler et al. 1968). Spiral aggregates were revealed not only by potexviruses but also in plants infected with pea streak virus which belongs to the group of carlaviruses (Bos and Rubio-Huertos 1972, Fig. 5 bv).

Tubular crystals

All crystals and paracrystals described so far are built of elongated virus particles. The polyhedral viruses, as for instance broad bean wilt virus, can also build elongated crystalline bodies which are called tubular crystals.

Tubular crystals of an unusual structure were found in cells infected with petunia ringspot strain of broad bean wilt virus by Rubio-Huertos (1962, 1968). The wall of every tubule is built of a layer of polyhedral virus particles while the centre of the tubule is without particles. The cross sections of tubules show that their wall is built of 9 or 10 virus particles. The tubules are not situated separately but lie in large numbers closely to one another; they are often in contact and are all oriented in the same direction, i. e. they lie parallelly. A great number of parallelly oriented tubules form aggregates which are similar to the virus spindles when viewed in light microscope. The tubule aggregates are weakly birefringent in polarised light and show a positive character of birefringence with regard to their length (Miličić et al. 1974). The tubules are fairly characteristic of broad bean wilt virus (Sahambi et al. 1973).

Several mycoviruses are also able to form tubular crystals. These crystals were found in the mushroom *Agaricus bisporus* and in the fungi *Penicillium chrysogenum* (Yamashita et al. 1974) and *P. brevi-compactum* (Hooper et al. 1972). Both *Penicillium* viruses are serologically related and belong to the group of diplornaviruses. The tubular crystals of these mycoviruses also have a wall built of one layer of virus particles.

Other true virus crystals

The broad bean wilt virus forms true crystals in the form of tetrahedra, octahedra etc. (Juretić et al. 1970) and of elongated bodies. This virus belongs to the small polyhedral viruses with a diameter of 30 nm. Many plant viruses of this group can also build crystals which are aggregates of virus particles.

According to Rubio-Huertos (1962) the true crystals of the *Petunia* strain of broad bean wilt virus belong to the cubic crystallographic system. The particles of the artichoke mottle crinkled virus, which is a member of tomato bushy stunt virus group, can also be arranged in a square or hexagonal pattern which indicates that the virus crystals have a cubic close-packed structure (Wyckoff 1948). These crystals are placed in the cytoplasm but they can sometimes be extruded into the vacuole in vesicles (Russo et al. 1968) Microcrystals in plants infected with bean pod mottle virus were established by Kim and Fulton (1972) and with radish mosaic virus by Štefanac and Ljubešić (1971).

However, not only the small one-stranded RNA viruses, but also the larger two-stranded RNA viruses can build microcrystals in infected tissues. Gerola et al. (1966) described microcrystals in plants experimentally infected with maize rough dwarf virus. Virus microcrystals were also found by Shikata and Maramorosch (1965) in the insect *Agallia constricta* which was infected with the wound tumour virus.

True crystals not built of virus particles

Under the influence of red clover vein mosaic virus (RCVMV) diagnostic true crystals appear in infected plants. These crystals are composed of protein (Rubio-Huertos and Bos 1973). They are especially frequent in the tissues which are affected by the strain BK 31 of this virus. They are easily visible in light microscope and are intensively stainable with phloxine and methylen blue. Cross sections through these threedimensional crystals show striations in electron microscope with periodicity of 11 nm. After some treatments they can disaggregate, and then it can be seen that they are composed of spherical or polyhedral particles with a diameter of 11 or 12 nm. So far the RCVMV is the only carlavirus which produces true crystals in infected plants. Some strains of RCVMV are not able to produce these crystals.

More or less similar true crystals were found in plants infected with various viruses belonging to potato Y group. These crystals were revealed in cells of host plants of bean yellow mosaic virus (McWhorter 1941). They were often placed in cytoplasm but also frequently in nucleus and nucleolus (Weintraub and Ragetli 1966). The intranuclear crystals provoked by tobacco etch virus (TEV) were especially well investigated. They were first observed by Kassanis (1939), and later were investigated in detail by Matsui and Yamaguchi (1964). The crystals are often rectangular but can sometimes be elongated showing a regular pattern with striations. The cytochemical investigations performed with light microscope (Takahashi 1962; Hooker and Summonwar 1964) showed that the crystalline inclusions of TEV were built of a protein without nucleic acid. This find was stated also by Shepard (1968) and Hayashi and Matsui (1967). Eventually Knuhtsen, Hiebert and Purcifull (1974) succeeded in purifying the intranuclear crystals of TEV. On the basis of this success they could establish by means of serological experiments that the protein of intranuclear crystals is different from the virus coat protein and from the protein of cytoplasmic inclusions, that is of pin-wheels and laminated aggregates.

Summary

The main results obtained from the research into crystalline plant virus inclusions have been presented in this report. Special attention was paid to the cell inclusions of tobacco mosaic virus.

The crystalline cell inclusions can be built of virus particles (virus crystals) or of other proteins which do not represent virus particles (protein crystals). The virus crystals can occur in the form of true crystals, paracrystalline needles, complexly formed virus spindles, and tubular crystals. The protein crystals are built of proteins probably coded by viruses but are serologically different from the virus coat protein.

References

Amelunxen, F., 1956: Beobachtungen über die Entwicklung der Eiweißspindeln bei Cactaceen. Protoplasma *45*, 164—172.

Amelunxen, F., 1958: Die Virus-Eiweißspindeln der Kakteen. Darstellung, elektronenmikroskopische und biochemische Analyse des Virus. Protoplasma *49*, 140—178.

Amelunxen, F., I. Thaler, 1967: Fine structure of cactus virus X. Z. Pflanzenphysiol. *57,* 269—279.

Bawden, F. C., 1964: Plant Viruses and Virus Diseases. 4th ed. Ronald Press, New York.

Bernal, J. D., I. Fankuchen, 1937: Structure types of protein 'crystals' from virus infected plants. Nature *139,* 923—924.

Bos, L., M. Rubio-Huertos, 1972: Light and electron microscopy of pea streak virus in crude sap and tissues of pea *(Pisum sativum).* Neth. J. Pl. Path. *78,* 247—257.

Esau, K., 1960a: Cytologic and histologic symptoms of beet yellows. Virology *70,* 73—85.

Esau, K., 1960b: The development of inclusions in sugar beet infected with the yellows virus. Virology *11,* 317—328.

Esau, K., J. Cronshaw, L. L. Hoefert, 1966: Organization of beet yellows virus inclusions in leaf cells of Beta. Proc. Nat. Acad. Sci. *55,* 486—493.

Gerola, F. M., M. Bassi, O. Lovisolo, C. Vidano, 1966: Electron microscopic observation on wheat plants experimentaly infected with maize rough dwarf virus. Caryologia *19,* 493—503.

Hayashi, T., C. Matsui, 1967: Electron microscopy of host cells infected with tobacco etch virus. IV. Virology *33,* 47—54.

Hooker, W., A. S. Summanwar, 1964: Intracellular acridine orange fluorescence in plant virus infections. Exp. Cell Res. *33, 609—612.*

Hooper, G. R., H. A. Wood, R. Myers, R. F. Bozarth, 1972: Viruslike particles in *Penicillium brevi-compactum* and *P. stoloniferum* hyphae and spores. Phytopathology *62,* 823—824.

Iwanovski, D., 1903: Über die Mosaikkrankheit der Tabakpflanze. Z. Pflanzenkrankh. *13,* 1—41.

Juretić, N., D. Miličić, K. Schmelzer, 1970: Zur Kenntnis des Ringmosaik-Virus der Kapuzinerkresse (nasturtium ringgspot virus) und seiner Zell-Einschlußkörper. Acta Bot. Croat. *29,* 17—26.

Kassanis, B., 1939: Intranuclear inclusions in virus infected plants. Ann. appl. Biol. *26,* 705—709.

Kassanis, B., F. M. L. Sheffield, 1941: Variations in the cytoplasmic inclusions induced by three strains of tobacco mosaic virus. Ann. appl. Biol. *28,* 360—367.

Kim, K. S., J. P. Fulton, 1972: Fine structure of plant cells infected with bean pod mottle virus. Virology *49,* 112—121.

Knight, C. A., 1974: Molecular Virology. McGraw-Hill Book Comp., New York etc.

Knudsen, H., E. Hiebert, D. E. Purcifull, 1974: Partial purification and some properties of tobacco etch virus induced intracellular inclusions. Virology *61,* 200—209.

McWhorter, F. P., 1941: Isometric crystals produced by *Pisum* virus 2 and *Phaseolus* virus 2. Phytopathology *31,* 760—761.

Matsui, C., S. Yamaguchi, 1964: Electron microscopy of host cells infected with tobacco etch virus. I. Virology *22,* 40—47.

Miličić, D., 1954: Viruskörper und Zellteilungsanomalien in *Opuntia brasiliensis.* Protoplasma *43,* 228—236.

Miličić, D., 1968: Zelleinschlüsse von Holmes' ribgrass virus, eines Verwandten des Tabakmosaikvirus. Naturwissenschaften *55,* 90—91.

Miličić, D., N. Juretić, 1971: Zelleinschlüsse der Viren der Tabakmosaik-Gruppe. Tag.-Ber. Dt. Akad. Landwirtsch.-Wiss. Berlin, *115,* 141—150.

Miličić, D., M. Wrischer, N. Juretić, 1974: Intracellular inclusion bodies of broad bean wilt virus. Phytopath. Z. *80,* 127—135.

Miličić, D., Z. Štefanac, 1971: Cell inclusions of the cucumber green mottle mosaic virus and the odontoglossum ringspot virus. Acta Bot. Croat. *30,* 33—40.

Pleše, N., D. Miličić, 1966: Vergleichende Untersuchungen an Isolaten des Kakteen-X-Virus mit Testpflanzen. Phytopath. Z. *55,* 197—210.

Purcifull, D. E., J. R. Edwardson, R. G. Christie, 1966: Electron microscopy of intracellular aggregates in pea infected with clover yellow mosaic virus. Virology *29,* 276—284.

Rubio-Huertos, M., 1962: Light and electron microscopy of inclusion bodies associated with *Petunia* ringspot virus. Virology *18,* 337—342.

Rubio-Huertos, M., 1968: Further studies on ultrastructure of plants infected with *Petunia* ringspot virus. Protoplasma *65,* 465—476.

Rubio-Huertos, M., L. Bos, 1973: Light and electron microscopy of red clover vein mosaic virus in pea *(Pisum sativum).* Neth. J. Pl. Path. *79,* 94—103.

Russo, M., G. P. Martelli, A. Quacquarelli, 1967: Occurrence of artichoke mottle crinkle virus in leaf vein xylem. Virology *33,* 555—558.

Russo, M., G. P. Martelli, A. Quacquarelli, 1968: Studies on the agent of artichoke mottled crinkle. IV. Intracellular localization of the virus. Virology *34,* 679—693.

Russo, M., G. P. Martelli, 1972: Ultrastructural observations on tomato bushy stunt virus in plant cells. Virology *49,* 128—129.

Sahambi, H. S., R. G. Milne, S. M. Cook, A. J. Gibbs, R. D. Woods, 1973: Broad bean wilt and nasturtium ringspot viruses are related. Phytopath. Z. *76,* 158—165.

Shepard, J. F., 1968: Electron microscopy of host cells infected with tobacco etch virus. IV. Virology *33,* 47—54.

Shikata, E., K. Maramorosch, 1965a: Plant tumour virus in arthropod host: Mycrocrystal formation. Nature *208,* 507—508.

Shikata, E., K. Maramorosch, 1965b: Electron microscopic evidence for the systemic invasion of an insect host by a plant pathogenic virus. Virology *27,* 461—475.

Stanley, W. M., 1935: Isolation of a crystalline protein possessing the properties of tobacco mosaic virus. Science *81,* 644—645.

Steere, R. L., R. C. Williams, 1953: Identification of crystaline inclusion bodies extracted intact from plant cells infected with tobacco mosaic virus. Amer. J. Bot. *40,* 81—84.

Štefanac, Z., N. Ljubešić, 1971: Inclusion bodies in cells infected with radish mosaic virus. J. gen. Virol. *13,* 51—57.

Štefanac, Z., N. Ljubešić, 1974: The spindle-shaped inclusion bodies of narcissus mosaic virus. Phytopath. Z. *80,* 148—152.

Takahashi, W. N., 1962: Effect of viral infection on the nuclei of the host. Phytopathology *52,* 20.

Thaler, I., 1956: Eiweißkristalloide von *Lilium tigrinum.* Protoplasma *45,* 486—490.

Thaler, I., 1966: Eiweißkristalle in Pflanzenzellen. In: Protoplasmatologia. Bd. II/B/2b. Springer Verlag, Wien—New York.

Turner, R. H., 1971: Electron microscopy of cells infected with radish mosaic virus. J. gen. Virol. *13,* 177—179.

Warmke, H. E., 1968: Fine structure of inclusions formed by Aucuba strain of tobacco mosaic virus. Virology *34,* 149—159.

Warmke, H. E., J. R. Edwardson, 1966: Electron microscopy of crystaline inclusions of tobacco mosaic virus in leaf tissue. Virology *30,* 45—57.

Weber, F., G. Kenda, 1952: Die Viruskörper von *Opuntia subulata.* Protoplasma *41,* 378—831.

Wehrmeyer, W., 1957: Darstellung und Strukturordnung eines Tabakmosaikvirus-Einschlußkörpers in der Zelle. Naturwissenschaften *44,* 519.

Wehrmeyer, W., 1959: Entwicklungsgeschichte, Morphologie und Struktur von Tabakmosaikvirus-Einschlußkörper unter besonderer Berücksichtigung der der fibrillären Formen. Protoplasma *51,* 165—241.

Weintraub, M., H. W. Ragetli, 1966: Fine structure of inclusions and organelles in *Vicia faba* infected with bean yellow mosaic virus. Virology *28*, 290—302.

Wyckoff, R. W. G., 1948: The electron microscopy of macromolecular crystals. Acta Cryst. *1*, 292—294.

Yamashita, S., Y. Doi, K. Yora, 1973: Intracellular appearance of *Penicillium chrysogenum* virus. Virology 55, 445—452.

Zettler, F. W., J. R. Edwardson, D. E. Purcifull, 1968: Ultramicroscopic differences in inclusions of papaya mosaic virus and papaya ringspot virus correlated with differencial aphid transmission. Phytopathology *58*, 332—335.

SADRŽAJ

GRADA I SASTAV KRISTALIČNIH UKLOPINA BILJNIH VIRUSA

Davor Miličić
(Botanički zavod Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu)

U ovom prikazu izneseni su glavni rezultati koji su dosad postignuti na području istraživanja kristaličnih uklopina biljnih virusa. Posebna pažnja posvećena je uklopinama koje u napadnutim stanicama stvara virus mozaika duhana.

Ti kristali mogu biti izgrađeni ili direktno od virusnih čestica (virusni kristali) ili od samog proteina koji ne predstavlja virusne čestice (proteinski kristali). Virusni kristali mogu zadobiti oblik pravih kristala, parakristaličnih iglica, kutnih slojevitih agregata, složeno građenih virusnih vretena i tubularnih kristala. Proteinski kristali izgrađeni su vjerojatno od proteina koje kodira virus i koji se serološki razlikuju od proteina virusne kapside.

Prof. dr Davor Miličić
Botanički zavod
Prirodoslovno-matematičkog fakulteta
Marulićev trg 20
Yu 41000 Zagreb (Jugoslavija)