EXTRACELLULAR SYNTHESIS OF INFECTIOUS RIBONUCLEO-PROTEIN ON SUBCELLULAR STRUCTURES

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Extracellular synthesis of infectious ribonucleoprotein on [redacted] subcellular structures

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The synthesis of virus-specific RNA in cells infected by arboviruses has been studied by a number of authors (Friedman, 1968a; Yin and Lockart; Sreevalson et al., 1968; Trent et al.; V. M. Zhdanov et al., 1969a); the same has been done for proteins (Friedman, 1968b; Scheele and Pfefferkorn; Strauss et al., 1969; V. M. Zhdanov et al., 1969b). It has been shown that replication of viral RNA and polymerase activity are associated with the mitochondrial-microsome (MM) fraction of the infected cells (Martin and Sonnabend; Grimley et al.; Sreevalsan and Yin, 1969). Protein synthesis was also discovered in this fraction (V. M. Zhdanov et al., 1969a).

Since the MM fraction of cells infected by the Venezuelan equine encephalomyelitis (VEE) virus provides for the synthesis of RNA and proteins, it was of interest to study the products of the synthesis. In this article it is proved that synthesis of virus-specific ribonucleoproteids (RNP), possessing infectious properties, takes place in the MM fraction isolated from cells infected by the VEE virus.

Virus and cells. The experiments were set up with strain SPF of the VEE virus in spinner cultures of chick-embryo fibroblasts (V. M. Zhdanov et al., 1969a). Usually $0.5 \cdot 10^8$ - $1 \cdot 10^8$ cells, suspended in medium No. 199, were infected by the virus with a plurality of 50-100 CBU (conventional biological units) per cell. The procedure of labeling the virus with radioactive isotopes, purification and characterization of the RNP isolated from the virions, including its infectious properties, are described in

another of our works (L. V. Uryvayev et al.).

Fractionation of the cells. The infected and uninfected cells were rinsed in a tris-buffer (tris HCl 0.01 M, pH 7.2, NaCl 0.1 M, EDTA (ethylenediaminetetraacetate) 0.001 M), then were suspended in 5 ml of a homogenate buffer (tris HCl 0.05 M, pH 7.6, KCl 0.025 M, $MgCl_2$ 0.005 M, 2-mercaptoethanol 0.006 M, saccharose 0.25 M, macaloid 0.05%), and were left to swell for 15-20 minutes, after which they were destroyed by 15-20 tractions of a Dawes homogenizer under microscope control. The nuclei and cell fragments were removed by centrifuging at 1000 g for 10 minutes. The MM fraction was precipitated at 15,000 g for 20 minutes. It was used for subsequent experiments. All the procedures were conducted at 0-2°.

Infectious titration. Dilutions of the material were applied on the surface of single-layer cultures, and adsorption was caused to take place at 4° for 1 hour. Then the cultures were flooded by a Porterfield (1959) covering and were incubated in a thermostat with 5% carbonic acid at 37° for 48 hours. The plaques were counted, and the infectious titers were computed in terms of plaque-forming units per ml.

Determination of polymerase activity. Two systems were used for determination of the polymerase activity, namely nucleoside triphosphates and medium No. 199.

Materials and methods. The first incubation mixture contained, per ml: tris HCl, 100 micromoles, pH 7.8; KCl, 50 micromoles; $MgCl_2$, 5 micromoles; 2-mercaptoethanol, 3 micromoles; actinomycin D, 2 micrograms; adinosine triphosphate (ATP), guanosene triphosphate (GTP), and cytidine triphosphate (CTP), 1 micromole each; Phosphoenol piruvate, 10 micromoles; piruvate kinase, 50 micrograms, and H^3-Uridine triphosphate, 10 microcuries. The MM fraction in the amount of 1-2 was suspended in the incubation mixture at 35° for different periods of time; samples of 0.2 ml each were taken. The reaction was stopped by the addition of 1 ml 10% trichloracetic acid (TCA) in 0.1 mole of pyrophosphate, then 0.1% casein was added, the residue was rinsed by

5% TCA on millipore filters, was dried by alcohol, and was placed in flasks with a toluol·(PPO + POPOP) scintillator for counting the radioactivity in a Packard Tricarb liquid scintillation counter.

The second incubation medium contained medium No. 199 with 5 microcuries/ml H^3-uridine, in which 1-2 mg of the MM fraction were incubated for various periods of time. The reaction was stopped by the addition of 10% TCA, and the residue was processed in the manner described above.

For extracting the RNA, the volume of the polymerase system was increased to 3-5 ml, the reaction was stopped by quick cooling, and the RNA was immediately extracted by phenol.

Determination of the extracellular conjunction of RNA synthesis and protein synthesis. The MM fraction, isolated from $0.5 \cdot 10^8$ - $1 \cdot 10^8$ infected or uninfected cells, was suspended in 5-10 ml of medium No. 199 and was placed in an agitator at 35°. H^3-uridine (5 microcuries/ml) and C^{14} amino-acid mixture (2 microcuries/ml) were added after 20 minutes at different intervals (sic), the reaction was stopped by quick cooling. Then to a suspension of each sample of 0.2 ml of the superprecipitate liquid and the precipitate (the latter was resuspended in the initial volume of tris-buffer), 0.1% casein and 10% TCA were added, and the suspension was processed for determination of the radioactivity, in the manner described above. The remaining material was used for further study.

Centrifuging in saccharose gradients. Linear gradients of saccharose density: 5-20% for RNA analysis, 17-40 and 15-60% for RNP analysis were prepared on the basis of a homogenate buffer. Centrifuging was conducted in a 3 x 20 bucket rotor of the MSE "Superspeed-50" ultracentrifuge. The gradients were fractionated by pressing out from the test-tube bottom in an apparatus consisting of an LKB collector, a "Uvikard" (A/subscript illegible/) spectrophotometer and a registering device. The acid-

indissoluble material was precipitated by TCA and was processed for a radioactivity count.

Centrifuging in cesium chloride gradients. Preformed linear density gradients of cesium chloride (ρ = 1.2 - 1.6 g/ml) were prepared on the basis of a 0.01 M phosphate buffer, pH 7.2 with 0.0015 M $MgCl_2$ and 8% formaldehyde. If the gradient fraction was used for infectious titration, the formalin was replaced by 0.33 mg beef serum albumin per ml. After centrifuging at 35,000 rpm for 15 hours in a 3 x 5 bucket rotor of the "superspeed-50" centrifuge, the gradients were fractionated by piercing the test-tube bottom, the density was determined in a refractometer, and the absorption (A/subscript illegible/) was determined in a spectrophotometer. The acid-indissoluble material was precipitated by TCA for a radiation count. For infectious titration, samples of 0.05 ml were selected from various fractions of the gradient.

Centrifuging in density gradients of cesium sulfate was conducted for separating RNA with different floating densities. The RNA samples were dissolved in a tris-buffer (tris HCl 0.1 m, pH 7.2), were placed in an 87% solution of cesium sulfate, and were centrifuged at 30,000 rpm for 40 hours in a SW-50 rotor of the "Spinco-L2" ultracentrifuge. Fractionation was accomplished in the manner described for cesium chloride gradients.

RNA was extracted by phenol, in a manner described earlier (V. M. Zhdanov et al., 1969a). Its sedimentation properties were studied in terms of saccharose density gradients, and its density characteristics were studied in terms of cesium sulfate density gradients.

Virus-specific sera were obtained after intravenous immunization of rabbits by purified virus 6 times at one-week intervals. The serum titer comprised $2 \cdot 10^4$ hemagglutination-neutralizing units.

Reagents: Actinomycin D (Merck, Sharpe and Dohm, USA), puromycin (Serva, Holland), pancreatic ribonuclease (Worthington, USA), H^3-uridine triphosphate (Radio-

isotope center, Amersham, England), H^3-uridine, C^{14}-adenin, and C^{14}-hydrolizate of chlorella (Radioisotope center, Leningrad), remaining reagents and chemicals, domestically produced.

Infectious titration of extracellular products of the mm fraction. Chick-embryo fibroblasts (CEF), processed by actinomycin D (2 micrograms/ml) were infected by the VEE virus (50-100 CBU/cell) and were incubated at 37° for 2½ hours. Then the cultures were rapidly cooled, the medium was rinsed off, and the cells were fractionated. The MM fraction was isolated from cells and was incubated in medium No. 199 at 35°. Samples were taken at various intervals. The MM fraction was removed from them by centrifuging at 15,000 g for 20 minutes, and the superprecipitate liquid was titrated for infectiousness.

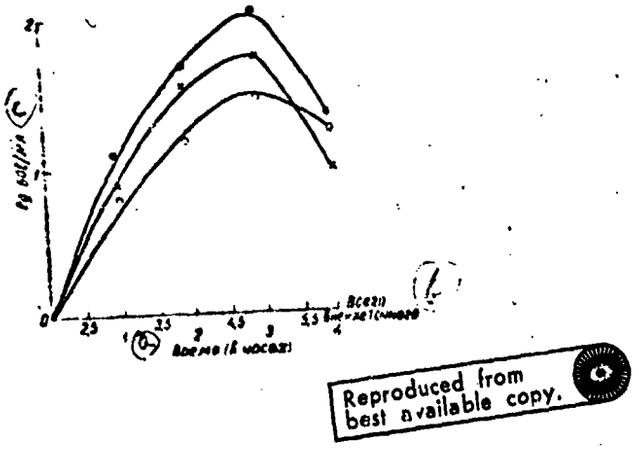

Figure 1. Kinetics of accumulation of infectious titers in the MM fraction, isolated from infected cells and extracellularly incubated in medium No. 199. Results of three experiments.

Key to Figure 1: a) time (in hours); b) total extracellular ; c) lg CBU/ml

In Figure 1 are shown the results of three experiments, from which it can be seen that infectious titers accumulate in the medium when the MM fraction was incubated at

35° for 3 hours. Maximum increase of the titer is 75-100 x (1.5-2 lg).

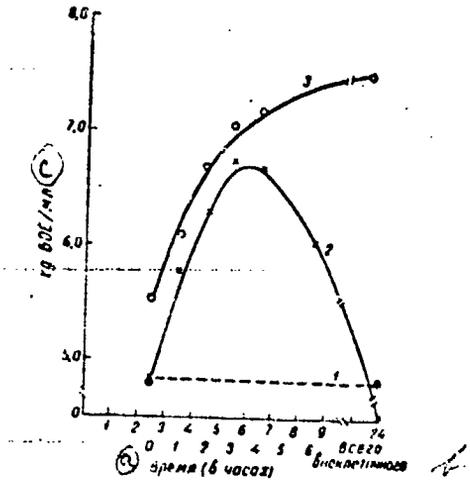

Figure 2. Kinetics of infectious titers in the MM fraction, isolated from infected cells and incubated extracellularly, and in infected cells. The MM fraction was isolated from infected cells and was incubated in medium No. 199 at 0° (1) and 37° (2), part of the cells was preserved and was incubated in medium No. 199 at 37° (3).

Key to Figure 2: a) time (in hours); b) total intracellular; (c) lg CBU/ml

In Figure 2 are shown the results of another experiment, in which the intensity of extracellular formation of the infectious material was compared with synthesis of the virus in cells. CEF, processed by actinomycin D and infected by the virus, were incubated for 2½ hours. Then the culture was cooled, washed out of the medium and divided into three equal parts. One part was incubated in medium No. 199, samples were taken at various intervals, the cells were removed by centrifuging, and the infectious titers in the medium were determined.

The other part of the cells was fractionated, the MM fraction was isolated and was incubated in medium No. 199: the samples were selected at the same intervals, the MM fraction was removed by centrifuging, and the superprecipitate liquid was

titrated for infectiousness. The third part of the cells was also fractionated, the MM fraction was isolated; it was preserved in medium No. 199 at 0°, samples for infectious titration were taken at the beginning and at the end of the observation period. From this experiment it can be concluded that infectious titers accumulate during 4 hours of incubation of the MM fraction at 37°, after which progressing inactivation of the infectious material sets in. Incubation of the MM fraction at 0° is not accompanied by accumulation of the infectious titers. During incubation of the infected cells, the infectious titers continue to accumulate during 24 hours of observation.

A series of experiments was set up to ascertain whether the accumulation of infectious titers in the medium is a result of liberation of the residual virus adsorbed on the structures of the MM fraction. Chick-embryo fibroblasts, processed by actinomycin, were divided into three equal parts. One part was infected by the virus and was incubated for 2½ hours, then the MM fraction was isolated and was incubated in medium No. 199. Samples were taken at various intervals, the MM fraction was precipitated, and the infectious titers were determined in the supernatants and in the precipitates. The second part of the cells was not infected; from these cells the MM fraction was isolated, was mixed with the virus and was incubated in medium No. 199. Samples were selected at the same intervals, and the infectious titers were also determined in the supernatants and precipitates of the MM fraction. The third part of the uninfected cells was processed in the same manner, but RNA, extracted from the virus, was added instead of the virus..

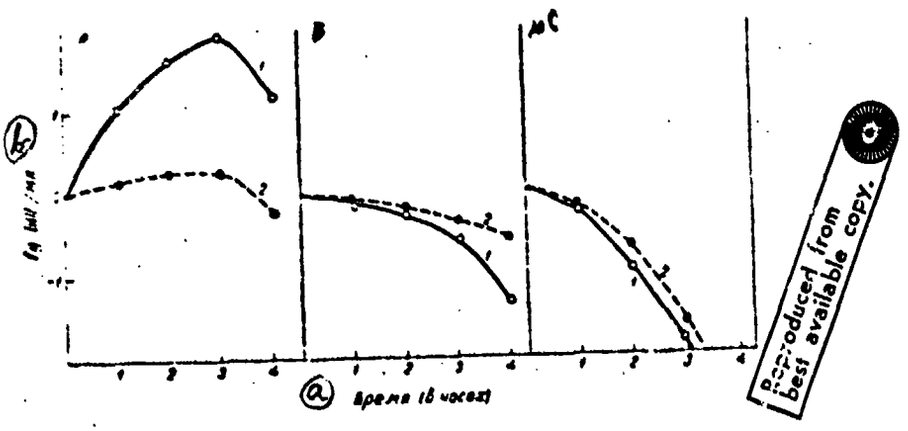

Figure 3. Kinetics of increase or decrease of the infectious titers in supernatants (1) and precipitates (2) of the MM fraction incubated extracellularly in medium No. 199 at 37°.

A - MM fraction isolated from infected cells; B - same, from uninfected cells and mixed with the virus; C - same, from uninfected cells and mixed with viral RNA.

Key to Figure 3; a) time (in hours); b) lg CBU/ml

The results of this experiment are shown in Figure 3. It can be seen that during the incubation of the MM fraction isolated from infected cells, extracellularly in medium No. 199, accumulation of infectious titers takes place in the supernatant for the duration of 3 hours, whereas the titers in the precipitates remain constant all this time; then the titers in the supernatants and precipitates of the MM fraction begin to decrease progressively. In contrast to this, addition of the virus or of viral RNA to the MM fraction, isolated from unfected cells, is accompanied by a gradual decrease of the titers in the medium and in the precipitates in comparison to the initial ones.

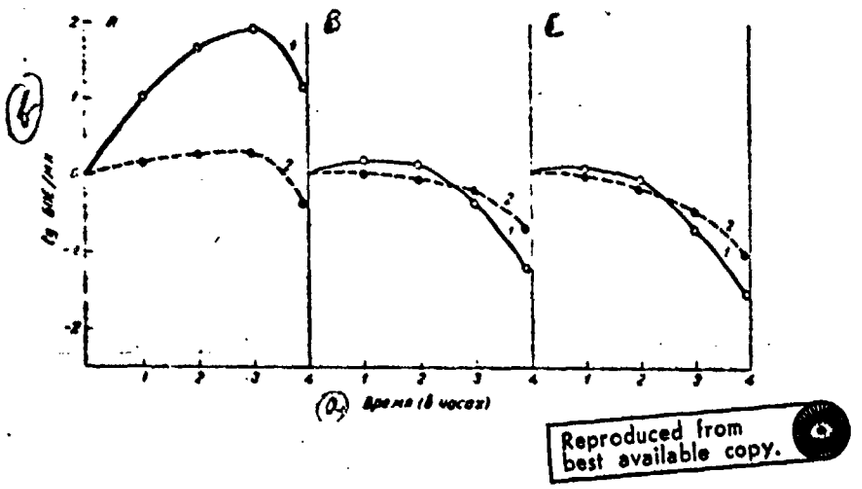

Figure 4. Kinetics of increase or decrease of the infectious titers in supernatants (1) and precipitates (2) of the MM fraction, isolated from infected cells and incubated in medium No. 199 (a), tris-buffer (B), and medium No. 199 with puromycin (C).

Key to Figure 4: a) time (in hours); b) lg CBU/ml

Figure 4 shows the results of another experiment. In this experiment chick-embryo fibroblasts, treated by actinomycin D, were infected by the virus and were incubated for 2½ hours. The MM fraction was isolated and was divided into three equal parts. One part was incubated in medium No. 199, a second part was incubated in medium No. 199 with puromycin (200 mg/ml), and the third part was incubated in a tris-buffer. Samples were taken at various intervals, and the infectious titers were determined in supernatants and precipitates of the MM fraction. From these data, it can be seen that incubation of the MM fraction in a buffer or in medium No. 199 with puromycin was not accompanied by an increase of the infectious titers, whereas, just as in the preceding experiments, accumulation of the infectious titers took place during incubation of the MM fraction in medium No. 199.

Characterization of RNA, synthesized in the MM fraction. Cells treated by actinomycin D were infected, were incubated for 3 hours, and then were fractionated. The MM fraction was isolated and was resuspended in a nucleoside-triphosphate mixture with H^3-uridine triphosphate (10 microcuries/ml), and the mixture was incubated during various intervals. In parallel experiments, the MM fraction was resuspended in medium No. 199 with H^3-uridine (5 microcuries/ml). Samples were taken at different intervals, and a radioactivity count was made of the material precipitated by TCA. The results of one of the experiments are shown in Figure 5. It can be seen that in the nucleoside-triphosphate mixture, RNA synthesis obtains a plateau after 30-45 minutes, whereas in medium No. 199 it continues for 3 hours.

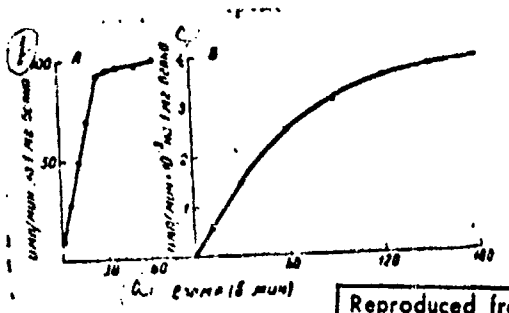

Figure 5. Kinetics of RNA synthesis in a MM fraction, isolated from infected cells and incubated in a nucleoside-triphosphate mixture with H^3-uridine triphosphate (A) and in medium No. 199 with H^3-uridine (B). Conditions of the experiment are given in the text.

Key to Figure 5: a) time (in minutes); b) pulse/min per 1 mg protein; c) pulse/min·10^{-3} per 1 mg protein

The sedimentation and density properties of RNA synthesized in the MM fraction were studied in a series of experiments. Uninfected and infected cells, treated by actinomycin D, were incubated for 3 hours and were then fractionated. The MM fraction was resuspended in medium No. 199 with H^3-uridine (5 microcuries/ml), and was incubated for various intervals of time, the RNA was extracted and was studied in terms of the density gradients of saccharose and cesium sulfate.

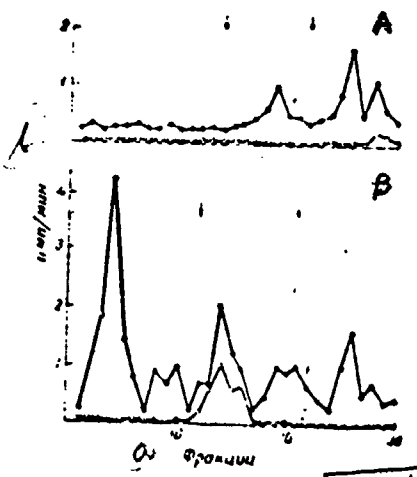

Figure 6. Distribution of the H^3-radioactivity of RNA isolated from uninfected cells (A) and from infected cells (B) and incubated in medium No. 199, in fractions of the density gradient of saccharose 5-20% after centrifuging at 20,000 rpm for 16 hours.

H^3-uridine was added (5 microcuries/ml) for 1 hour. 1/5 of each fraction was selected for a count of the total radioactivity (1) and 4/5 were treated by 2 micrograms of ribonuclease per 1 ml (2). The position of the ribosome markers RNA 28 and

18S/poorly legible/ is shown by arrows, the bottom of the gradients is here and further left.

Key to Figure 6: a) fractions; b) pulses/min

In Figure 6 are presented the results of an experiment in which RNA, isolated from uninfected and infected structures, was investigated in terms of the density gradients of saccharose. It can be seen that the RNA synthesized in the MM fraction isolated from infected cells is heterogeneous, with sedimentation constants from 9 to 40 S, whereas RNA synthesized in the MM fraction from uninfected cells has sedimentation constants from 9 to 22 S. Treatment of the gradient fractions by ribonuclease (2 micrograms/ml) reveals an RNA that is partially enzyme-resistant, with a sedimentation constant of 26 S in the infected structures; this is absent in uninfected structures.

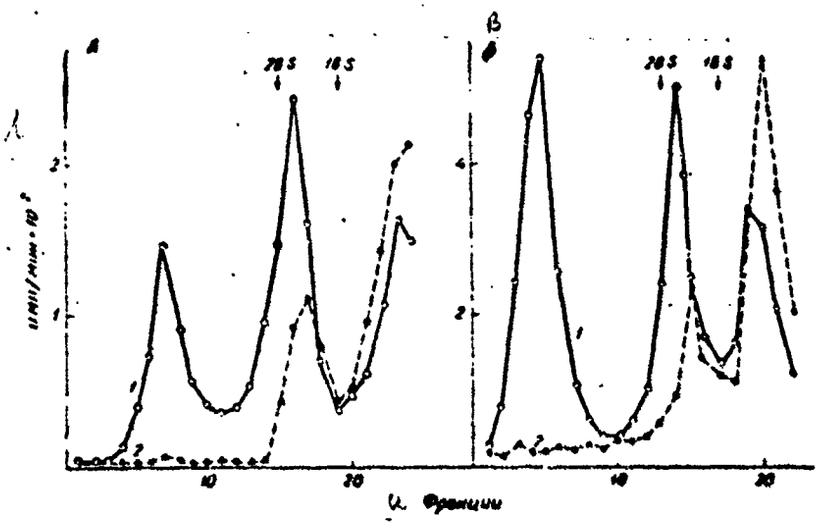

Figure 7. Distribution of H^3-radioactivity of RNA synthesized in the MM fraction isolated from infected cells and incubated in a nucleoside-triphosphate mixture (A) and in medium No. 199 (B), in fractions of the density gradient of saccharose 5-20% after centrifuging at 20,000 rpm for 16 hours.

1/5 of each fraction was selected for a count of the total radioactivity (1) and 4/5 was treated by ribonuclease (2).

Key to Figure 7: a) fractions; b) pulses/min·10^{-2}(?)

Shown in Figure 7 are the results of an experiment in which RNA synthesized in a nucleoside-triphosphate mixture was compared with RNA synthesized in medium No. 199 . From the presented data, it can be seen that in both systems, two kinds of RNA are synthesized: one of them is precipitated at 26 S and is partially resistent to ribonuclease, the other is precipitated at 40 S and is sensitive to the enzyme, the quantity of it being relatively greater in the second system.

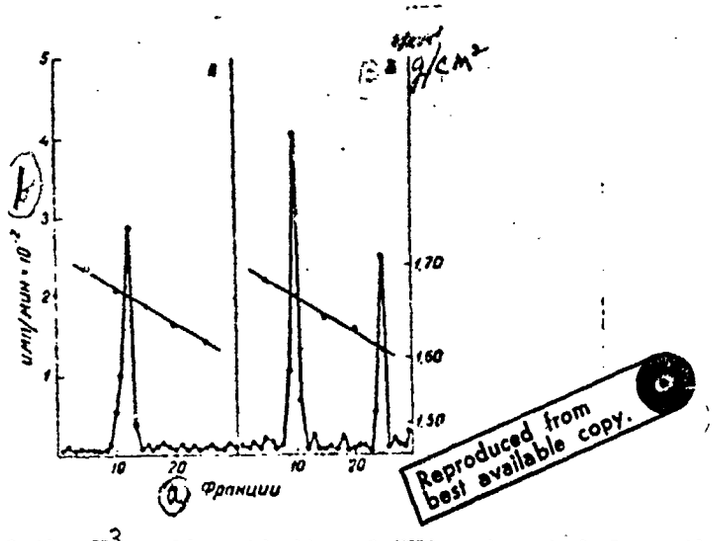

Figure 8. Distribution of the H^3-radioactivity of RNA extracted from the MM fraction, isolated from uninfected cells (A) and from infected cells (B) and incubated in medium No. 199 with H^3-uridine (5 microcuries/ml) for one hour, in fractions of the density gradients of cesium sulfate after centrifuging at 30,000 rpm for 40 hours.

Key to Figure 8. a) fractions; b) pulses/min·10^{-2}

Presented in Figure 8 are the results of an experiment in which RNA, extracted from uninfected and infected structures, was centrifuged in density gradients of cesium sulfate. From Figure 8 it can be seen that the RNA synthesized in infected structures is divided into two kinds, with densities of 1.66 and 1.60 g/cm^3, whereas RNA from uninfected cells forms only one peak with a density of 1.66 g/cm^3.

A series of experiments was conducted in order to study the contribution of the

cellular stage of RNA synthesis to extracellular synthesis in the MM fraction. It was shown in preliminary experiments that virus-induced RNA are synthesized in the presence of 0.5-1 mg 5-bromuridine (BUR) per 1 ml. Cells treated by actinomycin D were infected and were divided into 4 equal parts; C^{14}-adenin (2 microcuries/ml) and various concentrations of BUR were added to the cultures, the cultures were incubated for 3 hours. The results of this experiment are presented in Table 1.

Table 1.

Action of various concentrations of BUR on the synthesis of RNA of the VEE virus

BUR concentration (in mg/ml)	Radioactivity of newly synthesized RNA (in pulses/min per 10^7 cells)	% inhibition
Without BUR	14,192	--
0.5	13,549	5
0.75	12,708	11
1.0	11,345	20

Table 2.

Neutralization of products of the MM fraction by a virus-specific serum

Material	lg plaque-forming units per ml		lg of neutralization
	without serum	with serum	
Precipitate of MM fraction...	7.6	5.7	1.9
Supernatant of MM fraction...	7.0	5.6	1.4

As can be seen from Table 1, BUR in the tested concentrations only partially inhibits the synthesis of viral RNA.

In the following series of experiments, cells treated by actinomycin D were infected by the virus and were incubated in the presence of C^{14}-adenin (2 microcuries/ml) for 2 hours, then BUR (1 mg/ml) was added for 2 hours more. The RNA was extracted and was centrifuged in the density gradient of cesium sulfate. The results of this experiment are presented in Figure 9a. It can be seen that RNA extracted from cells forms two peaks with densities of 1.66 and 1.60 g/cm^3, as well as 2 supplementary peaks with densities of 1.68 and 1.62 g/cm^3. These peaks can be explained as "heavy"

chains due to the inclusion of BUR in the virus-specific RNA.

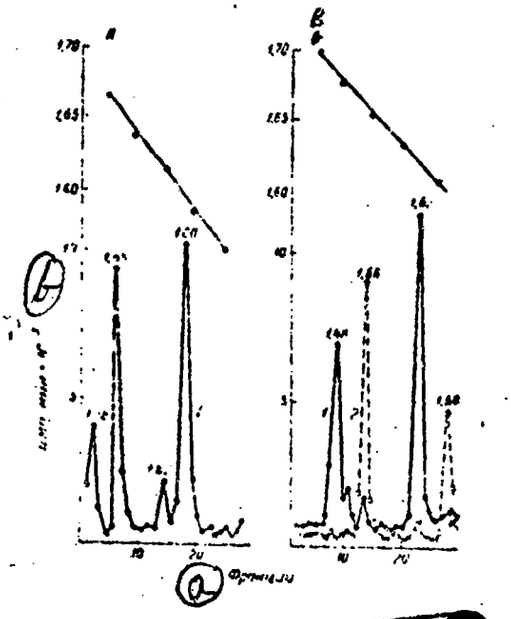

Figure 9. Distribution of C^{14} (1)- and H^3 (2)- radioactivity of RNA extracted from infected cells (A) and the MM fraction (B), in fractions of the density of cesium sulfate after centrifuging at 30,000 rpm for 40 hours. Conditions of the experiment are given in the text.

Key to Figure 9: a) fraction; b) pulses/min·10^3

Similar experiments were conducted with the MM fraction. Cells, treated by actinomycin D and infected, were incubated in medium No. 199 with H^3-uridine (5 microcuries/ml) for 3 hours, then the MM fraction was isolated and was incubated in medium 199 with BUR (1 mg/ml) and C^{14}-adenin (2 microcuries/ml) for 2 hours. The RNA was extracted and was centrifuged in the density gradient of cesium sulfate. The results of this experiment are presented in Figure 9b. It can be seen from the presented data that the RNA synthesized under these conditions contains both "light" chains (density 1.66 and 1.60 g/cm^3) and "heavy" chains (density 1.68 and 1.62 g/cm^3). It can also be seen

that the contribution of the cellular phase of RNA synthesis to extracellular synthesis is minimal or is absent, since only one of the two radioactive isotopes is included in each of the 4 kinds of RNA.

Characterization of products synthesized in the MM fraction. Chick-embryo fibroblasts, treated by actinomycin D and infected by the VEE virus, were incubated for 2½-3 hours, then the MM fraction was isolated and it was incubated in medium No. 199; H^3-uridine (5 microcuries/ml) and C^{14}-amino acid (2 microcuries/ml) were added for 20 minutes after various intervals (sic). The reaction was stopped by cooling, the MM fraction was precipitated, and the supernatants were investigated in terms of the density gradients of saccharose and cesium sulfate.

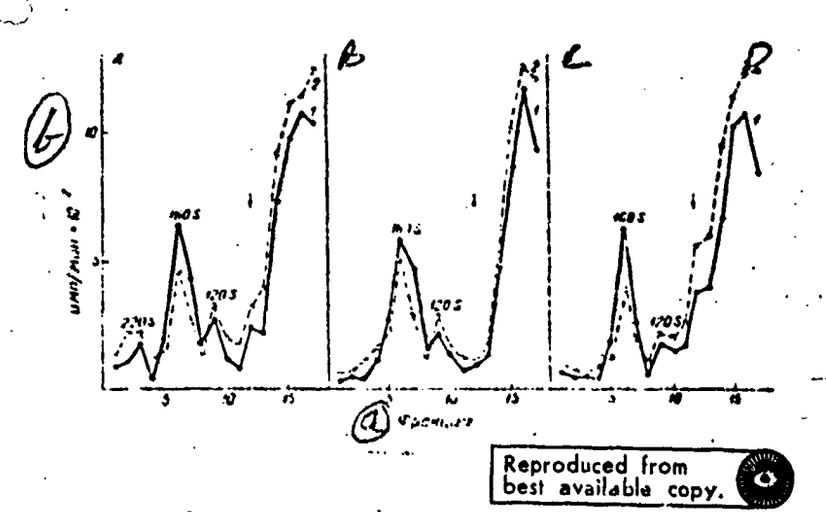

Figure 10. Distribution of H^3 (1)- and C^{14} (2)-radioactivity in fractions of the density gradient of 17-40% saccharose after centrifuging MM-fraction products, synthesized in medium No. 199, at 25,000 rpm for 2 hours.

H^3-uridine (5 microcuries/ml) and C^{14}-amino acid (2 microcuries/ml) were added from 2 hours 40 minutes to 3 hours, the MM fraction was precipitated and the supernatants were applied in a layer on the saccharose gradients. The position of the ribosomal marker-peak 80S is shown by arrows. A - without treatment; B - treatment of the material by 0.02 M EDTA; C - treatment by 20 micrograms ribonuclease per 1 ml.

Key to Figure 10: a) fractions; b) pulses/min·10^{-2}

In Figure 10 are presented the results of an experiment in which products of the MM fraction (supernatant) were centrifuged in gradients of the density of 17-40% saccharose. It can be seen from the presented data that the peaks of coinciding H^3 and C^{14} radioactivity have the sedimentation constants 160 and 120 S, and in addition, there is a peak with the sedimentation constant 220 S which is not detected in all of the experiments. A considerable quantity of radioactive material is also to be found in postribosomal fractions of the gradients. Processing of the material by EDTA (0.02 M) and ribonuclease (20 micrograms/ml) prior to centrifuging destroys the peak with the sedimentation constant 220 S and does not destroy the peaks with sedimentation constants 160 and 120 S. These data permit the conclusion to be made that the 160 and 120 S peaks are ribonucleoproteid complexes, whereas peak 220 S represents polyribosomes.

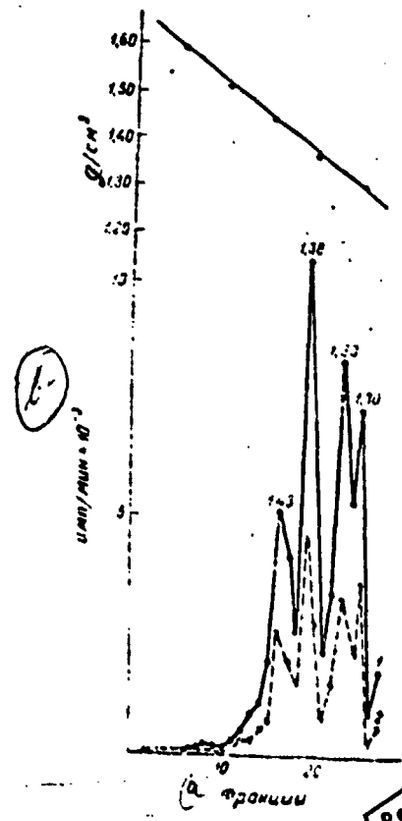

Figure 11. Distribution of H^3 (1)- and C^{14} (2)-radioactivity in fractions of the density gradient of cesium chlorate after centrifuging MM-fraction products incubated in medium No. 199, at 35,000 rpm for 20 hours.

H^3-uridine (5 microcuries/ml) and C^{14}-amino acids (2 microcuries/ml) were added from 1 hour 40 minutes to 2 hours. The MM fraction was precipitated, the supernatant was treated by 8% formaldehyde and was subjected to centrifuging in the gradient.

Key to Figure 11: a) fraction; b) pulses/min·10^{-3}

In Figure 11 are presented the results of experiment with the centrifuging of products (supernatants) of the MM fraction, isolated from infected cells and incubated in medium No. 199 with isotopes, in the density gradient of cesium chloride. Four peaks of coinciding H^3 and C^{14} radioactivity can be seen, with densities of 1.43, 1.38, 1.33, and 1.30 g/cm^3.

In a series of experiments, fractions of saccharose density gradients with different sedimentation constants of the radioactive material were collected, were fixed by 8% formaldehyde, and were centrifuged in density gradients of cesium chloride. The results of these experiments were as follows. After centrifuging in a gradient of cesium chloride, the material with a sedimentation constant of 160 S formed a peak with a density of 1.43 g/cm^3, peak 120S and postribosomal fractions of the saccharose gradients were resolved into 3 peaks with densities of 1.38, 1.35-1.34, and 1.32-1.30 g/cm^3, and the polyribosomal material (220S) was divided into several peaks with a density from 1.49 to 1.56 g/cm^3, the main part of the radioactivity having a density of 1.55-1.53 g/cm^3. These data permit the conclusion to be made that supernatants of the MM fraction, that was isolated from infected cells and was incubated in medium No. 199, contain ribonucleoproteid complexes of several kinds, as well as polyribosomes split off from MM fractions.

Investigation of the infectious properties of products of the MM fraction, and

experiments with neutralization of these products by sera. In this series, a study was made of the infectious properties of the products of an MM fraction isolated from infected cells, that were incubated in medium No. 199 after centrifuging in density gradients of saccharose and cesium chloride. Incubation took place in the presence of H^3-uridine (5 microcuries/ml) and C^{14}-amino acids (2 microcuries/ml). The MM fraction was then removed by precipitation at 15,000 g for 20 minutes, and the supernatants were investigated in the gradients. Part of the material of the gradient fractions was selected for infectious titration, and part was retained for determination of the radioactivity of the precipitated TCA.

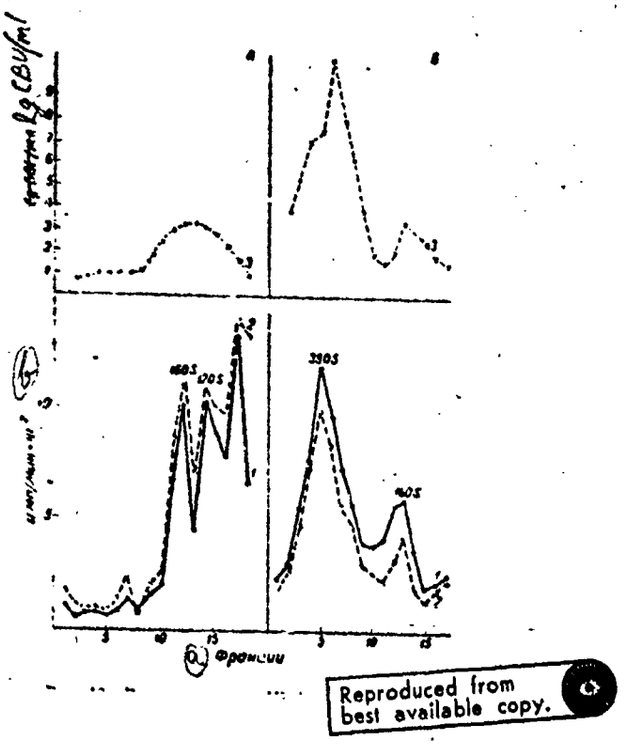

Figure 12. Distribution of H^3 (1)- and C^{14}(2)-radioactivity and infectious titers (3) in fractions of the density gradient of 50-60% saccharose after centrifuging MM-fraction products, isolated from infected cells, and incubated in medium No. 199 (A), at 22,500 rpm for 1 hour 40 minutes, and after centrifuging a virus labeled by

H^3-uridine and C^{14}-amino acids in a parallel gradient (B).

The MM fraction was isolated from cells treated by actinomycin D 2½ hours after infection, and was incubated in medium No. 199 for 3 hours. H^3-uridine (5 microcuries/ml) and C^{14}-amino acids (2 microcuries/ml) were added from 2 hours 40 minutes to 3 hours . The MM fraction was removed by precipitation, and the supernatant was investigated in the gradient.

Key to Figure 12: a) fraction; b) pulses/min·10^{-2}

Figure 12A presents the results of experiments with the centrifuging of products in sucarose gradients. It can be seen from the presented data that maximal infectiousness is associated with peak 160S, although the infectiousness peak is not sharp, and infectious material is found in all fractions of the gradient. However, the peak is not linked to contamination of the fractions by the residual virus present in the system, since the distribution of infectious titers within the fractions of the parallel gradient is a different one, and the principal part of the infectious material is associated with lower fractions of the gradient (380S), which contain the precipitating virus (Figure 12B). Here only an insignificant part of the virus is destroyed spontaneously, and releases ribonucleoproteids with a sedimentation constant of 160S.

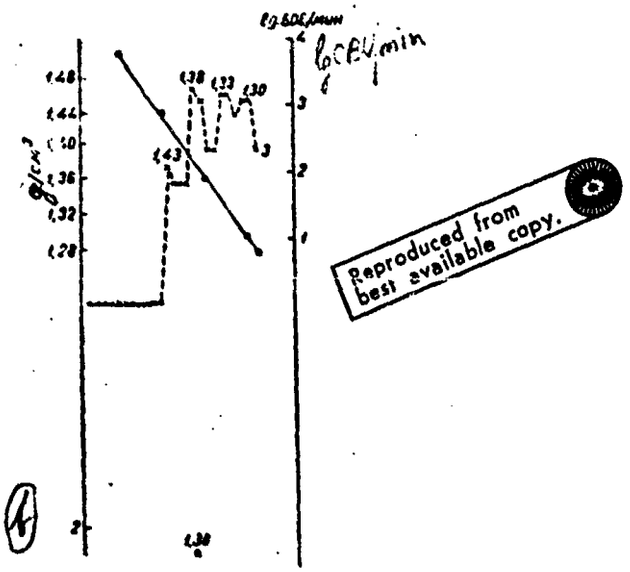

Figure 13. Distribution of H^3(1)- and C^{14}(2)-radioactivity, and infectious titers (3) in fractions of the density gradients of cesium chloride after centrifuging products of synthesis (supernatant) of the MM fraction isolated from infected cells and incubated in medium No. 199, at 35,000 rpm for 12 hours. The conditions of the experiment are the same as in Figure 11, but the formaldehyde was replaced by 0.33 mg beef-serum albumin per 1 ml.

Key to Figure 13: a) fraction; b) pulses/min·10^{-3}

The distribution of infectious titers in the density gradient of cesium chloride is shown in Figure 13, from which it can be seen that the peaks of the infectious titers of the gradient fraction coincide with the peaks of H^3- and C^{14}-radioactivity, which have a density from 0.3 to 10.130 g/cm^3.

A series of experiments was conducted to study the nature of the proteins that are associated with virus (infectious) RNA. Chick-embryo fibroblast was infected, was treated by actinomycin D, was incubated for 2½ hours; the MM fraction was isolated and was incubated in medium No. 199 for 3 hours. The the MM fraction was precipitated in a centrifuge, and the infectious titers were determined in the presence of 10^3 neutralizing units of virus-specific serum, and without the serum. The results of one experiment are presented in Table 2.

It can be seen from Table 2 that the virus-specific serum only partially neutralizes the products formed during incubation of the MM fraction from infected cells in medium No. 199.

Experiments were conducted with a virus-specific serum which neutralizes products of synthesis of the MM fraction, which have different density during centrifuging in cesium-chloride gradients. The conditions of the experiments are similar to those of the preceding ones, and after incubation in medium No. 199, supernatants of the MM fraction were centrifuged in density gradients of cesium chloride without

their fixation by formaldehyde. The gradient fractions were collected, and the infectious titers were determined in them in the absence and in the presence of the virus-specific serum. The results of one of the experiments are presented in Table 3.

Table 3.

Neutralization of products of the MM fraction after their separation in the density gradient of cesium chloride

Density of the products (in g/cm^3)	lg plaque-forming units in 1 ml		lg neutralization
	without serum	with serum	
1.30	5.6	5.3	0.3
1.33	6.3	6.1	0.2
1.37	6.1	5.7	0.4
1.43	7.1	2.3	4.8

It can be seen from these data that the virus-specific serum does not completely neutralize all the 4 fractions of the infectious material, which have densities from 1.43 to 1.30 g/cm^3. Most sensitive to neutralization is the material with the density of 1.43 g/cm^3.

The aim of this work has been to study the synthetic potencies of subcellular structures isolated from cells infected with the VEE virus. It has been shown earlier that the replicative complex of arbovirus is associated with the MM fraction (the fraction of large cells) of infected cells (Friedman and Berezesky, 1967; Martin and

Sonnabend; V. M. Zhdanov et al., 1969c), and the recently described virus-specific structures -- "factories"/"fabriki"/ (Bykovsky et al.) are also associated with this cell fraction. In the present work it has been shown that the MM fraction isolated from infected cells possesses polymerase activity, and virus-specific antigens have been discovered in it by the method of immunofluorescence (F. N. Yershov et al.).

It therefore appeared expedient to study, in this system, the extracellular synthesis of RNA and proteins, and the formation of ribonucleoproteid complexes. After several attempts at combining noncellular systems of protein and RNA synthesis, we arrived at medium No. 199, which contains, in good balance, the predecessors of both systems. Although the predecessors of RNA in this system are puridine and pyramidine bases, the presence of functionally active mitochondrias in the system provides for their phosphorylization to nucleoside triphosphates.

Study of this system showed that the MM fraction, isolated from infected cells and incubated in medium No. 199, provides for intensive synthesis of RNA and proteins during 3-4 hours incubation. Furthermore, 2 kinds of RNA are synthesized in the MM fraction: virus-cpecific RNA, and cellular RNA, apparently of mitochondrial origin, since its sedimentation properties correspond to those of mitochondrial RNA (Comarosan et al., 1968). Synthesis of the latter is relatively resistant to the action of actinomycin D (Dubin and Montenecourt). The virus-specific RNA synthesized in this system is discovered in two forms: sensitive and resistent to ribonuclease. This corresponds well to newly published data (Sreevalsan and Yin, 1969) and with our results, which showed that the products of polymerase reaction differ in relation to the incubation time: with short-time incubation (10-15 minutes) the principal product of the polymerase reaction is bifilar RNA, while a considerable quantity of monofilar RNA accumulates during prolonged incubation. Both kinds of virus-specific RNA separate well in gradients of saccharose and cesium sulfide. In experiments with the weighting of RNA by BUR, it was also shown that virus-specific RNA is synthesized in the investi-

gated system extracellularly, and is not a product of the completion of incomplete chains synthesized in the cell.

We did not try to characterize in detail the protein types synthesized in this system (this is an object of subsequent research); however, experiments with a serologic neutralizer of the products of synthesis showed that at least part of them are virus-specific.

Our attention has been concentrated on study of the products formed in the system under investigation. Their analysis in the density gradients of saccharose and cesium sulfate showed that the end products of synthesis of the MM fraction are ribonucleoproteid complexes, which differ among themselves with respect to sedimentation properties and floating density. It has been shown earlier (L. V. Uryvayev et al.) that ribonucleoproteid isolated from VEE virions has a sedimentation constant of about 160S and a floating density of 1.43 g/cm^3. Similar characteristics have been obtained during study of the ribonucleoproteid of other arboviruses (Streevalsan and Allen, 1968; Dobos and Faulkner). A component with these properties was regularly being discovered in our experiments with extracellular synthesis in the MM fraction; however, ribonucleoproteid complexes with other sedimentation and density characteristics were also discovered.

During titration of products of the MM fraction, incrementation of infectious titers in the incubation medium was established, and infectious properties of various ribonucleoproteid complexes, divided in the density gradients of saccharose and cesium chloride, were discovered. The increase of infectious titers is not linked to elution in the medium of the residual virus, adsorbed on structures of the MM fraction, since, in the first place, an increase of the infectious titers does not take place during incubation of the MM fraction at 0°; in the second place, during incubation of the MM fraction with the virus or with viral RNA at 37° the infectious titers decrease in the

medium and on the structures; in the third place, decrease of the infectious titers takes place also during the incubation of the MM fraction from infected cells at 37° in a tris-buffer or in medium No. 199 with puromycin. In all the cases, the cause of the decrease is the thermal deactivation of infectious material, which during incubation of the MM fraction in medium No. 199 is compensated by a much more intensive incrementation of the newly synthesized infectious ribonucleoproteid.

Infectiousness of the gradients of saccharose and cesium chlorate is also not associated with contamination of the gradients by the residual virus, since in controlled experiments with the centrifuging of VEE virions, a different type of distribution of the infectious titers in the gradients was obtained, than the type obtained during the centrifuging of products of the MM fraction.

Therefore both the general increase of infectious titers in the medium after extracellular incubation of the MM fraction, isolated from infected cells, and the infectiousness of individual fractions of the gradient, corresponding to various kinds of RNA, should be considered as being associated with extracellular synthesis of the infection material.

The infectiousness properties of the total product, as well as those of its individual components, were not completely neutralized by virus-specific sera. This testifies to the fact that both virus-specific and cellular proteins participate in the formation of complexes with virion RNA.

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EXTRACELLULAR SYNTHESIS OF INFECTIOUS RIBONUCLEOPROTEIN ON SUBCELLULAR STRUCTURES

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Summary

The synthesis of the infectious ribonucleoprotein virus was effected in the mitochondrial-microsomal (MM) fraction isolated from the chicken fibroblast cells infected with the virus of the Venezuelan form of equine encephalomyelitis (VEEE) 2½ 3 hours from the moment of inoculation and then incubated in the N199 medium or in the system for defining the polymerase activity. Following incubation of the said system for 3—4 hours the infection titre of the product increased 75 -100-fold by comparison with the initial one. It is shown that such a rise of the infectiousness had been caused not by elution of the residual virus with the subcellular structures into the medium, but by the synthesis of the infectious material de novo. In this system both the single- and two-band RNA in synthetized, being then associated with virus-specific and cellular proteins and forming ribonucleoprotein complexes. The latter possess infectious properties and are partly neutralized with the virus-specific serum.