FSTC-HT-23-558-71

ARMY MATERIEL COMMAND

U.S. ARMY
FOREIGN SCIENCE AND TECHNOLOGY CENTER

CYTOLOGICAL STUDY OF PERITONEAL EXUDATE OF MICE IN THE COURSE OF INTERFERON PRODUCTION IN VITRO

Subject Country: USSR

This document is a rendition of the original foreign text without any analytical or editorial comment.

UNCLASSIFIED
Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)
Foreign Science and Technology Center
US Army Materiel Command
Department of the Army

2a. REPORT SECURITY CLASSIFICATION
Unclassified

2b. GROUP

3. REPORT TITLE
CYTOLOGICAL STUDY OF PERITONEAL EXUDATE OF MICE IN THE COURSE OF INTERFERON PRODUCTION IN VITRO

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)
Translation

5. AUTHOR(S) (First name, middle initial, last name)
Not Available

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
27 October 1971	12	N/A

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO.	FSTC-HT-23- 558-71
c. T702301 2301	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report) ACSI Control Number J-9747
d. Requester L. Perkins, AMXST-AB2	

10. DISTRIBUTION STATEMENT
Approved for public release; distribution unlimited.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	US Army Foreign Science and Technology Center

13. ABSTRACT

Ribonucleoprotein complexes, possessing infectious properties and capable of forming charactersitic plaques under agar, are formed in the mitochondrial fraction, isolated from cells infected with Venezuelan equine encephalomyelitis virus (VEEV) in the course of incubation in appropriate media. In the linear saccharose gradient, the sedimentation constants of these complexes varied from 80 S to 160 S, while their buoyant density in the CxCl gradient was 1.3 to 1.42 grams per cc. Virus-like particles ("pseudoviruses") with the same characteristics were also found when infectious RNA VEEV was added to a homogenate obtained from non-infected cells. They were partially resistant to ribonuclease and were not neutralized by virus-specific sera. It is suggested that on the basis of the formation of virus-like particles in systems in vivo and in vitro there is complexing between the RNA viruses and the cell proteins. Formation of informosome-type structures is possible.

DD FORM 1 NOV 68 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

UNCLASSIFIED
Security Classification

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Virology Ribonucleic Acid Protein Equine Encephalomyelitis Country Code: UR COSATI Subject Field: 06						

TECHNICAL TRANSLATION

FSTC-HT-23- 558-71

ENGLISH TITLE: CYTOLOGICAL STUDY OF PERITONEAL EXUDATE OF MICE IN THE COURSE OF INTERFERON PRODUCTION IN VITRO

FOREIGN TITLE: Not Available

AUTHOR: Not Available

SOURCE: VOPROSY VIRUSOLOGII, No. 5, 1970, pp. 537-543

Translated for FSTC by ACSI

NOTICE

BLANK PAGE

It was shown earlier than when the so-called mitochondrial fraction (MF-15) extracted from cells infected with the Venezulean equine encephalomyelitis virus (VEEV) is incubated, there is active synthesis of virus-specific nucleic acids and proteins [2, 3, 5, 6]. In addition, this fraction shows almost all of the RNA-Polymerase activity [12] and the replicative complex [6] of this virus. The final product, formed in the process of incubation of MF-15 in optimum culture media, consists of ribonucleoproteins, which are heterogeneous as to their sedimentation and density characteristics.

Following addition of virus RNA to different fractions, obtained from the homogenate of non-infected animal cells, we encountered a phenomenon which was not mentioned earlier, consisting in the formation of virus-like particles ("pseudoviruses") which have infectious activity and are capable of causing the formation of characteristic plaques under agar [1]. It follows from the materials presented below that "pseudoviruses" possess a number of the properties which distinguish them, on the one hand, from the original virus RNA, and on the other from the full-value virus. In addition, there are points of similiarity between the infectious structures formed in vivo and in vitro. The data which we have obtained make it possible to draw some conclusions regarding the nature of the ribonucleoproteins which are formed, and to assume their similarity to structures of informosome type.

Material and Methods

Virus and Cells. We used VEEV and fibroblasts from a chick embryo; the properties of this virus and the method of its culture are described in other papers by us [3, 14].

Separation of Infectious RNA. The virus was initially purified using the Cheng method [10]; the titre of the original virus-containing suspensions was at least 10^9 plaque-forming units (PFU) in one milliliter. The RNA was extracted by the phenol-detergent method (phenol plus 0.5% SDS) at 65°. The RNA obtained was precipitated with 70° ethanol and stored at -20°, and was precipitated prior to titration by centrifuging for 15 minutes at 5000 g, then diluted to 1 M NaCl.

Titration of Infectiousness. The RNA cultures were placed in a single-layer FEK (expansion not available - Tr.) which was initially treated for 15 minutes with a solution of 1 M NaCl. Following 2 to 4 minutes of contact of the cells with the RNA, the single layer was covered with an agar coating according to the Porterfield recipe [13] and incubated for 2 days at 37°. Following incubation, the cells were stained with neutral red and the number of plaques formed was determined (Method No. 1). In contrast to the determination of infectiousness of RNA, processing with 1 M NaCl was not used in the titration of the mature virus; the adsorption time was 1 hour and the stain was introduced into the agar coating immediately before rinsing (Method No. 2). It is important to emphasize that during the titration of fractions obtained following centrifuging in a saccharose or CsCl density gradient, Method Number 2 was used in all cases.

Fractioning of Cells. Cells in a concentration of 10^8 were rinsed twice in a cool buffer solution (HCl 0.01 M, pH 7.2, KCl 0.1 M, ethylenediaminetetraacetate 0.002 M), five volumes of homogenized buffer solution were added (HCl 0.05 M, pH 7.6, $MgCl_2$ 0.005 M, KCl 0.025 M, EtSH 0.006 M, saccharose 0.025 M, macaloid 0.05%) and allowed to swell for ten minutes, after which they were broken up in a Downs homogenizer (10 to 15 tractions). The homogenate was centrifuged for 10 minutes at 800 g to get rid of the unbroken cells, nuclei and debris, then centrifuged for 20 minutes at 15,000 g to obtain the MF-15. The supernatant was divided into two parts, one of which was centrifuged for four hours at 150,000 g, in order to obtain a cell liquor free of ribosomes and ribosomal subunits. All of the procedures for breaking down and extracting fractions were carried out at 4°.

Sedimentation Analysis. The linear saccharose density gradients were prepared in a buffer solution of the composition indicated above. The material investigated was centrifuged for 1-1/2 to 2-1/2 hours at 38,000 rpm in a SW-39 bucket rotor. As labels we used ribosomal RNA (18S, 28S), ribosomes or subunits of ribosomes. The fractions were mixed with water and their absorption was measured in the ultraviolet with SF-4 at 260 and 280 millimicrons, after which 0.1% casein and 50% trichloracetic acid were added, the mixture was strained through millipore filters (No. 4), rinsed three times with 5% trichloracetic acid, alcohol and ether and dried. The filters were placed in flasks with a toluol scintillator (PPO+POPOP). The radioactivity was measured in a liquid scintillation counter (Packard Tricarb).

Centrifuging In The CsCl Density Gradient. The performed CsCl gradients were prepared with a density from 1.2 to 1.6 grans/cc. To study the distribution of radioactivity, the CsCl gradients were prepared from a 0.01 M phosphate buffer (pH 7.2) with 0.015 M $MgCl_2$ and 8% formalin. For titration of infectiousness, we added 0.33 micrograms per milliliter of bovine serum albumin to the gradients instead of the formalin. Following centrifuging (rotor 3 × 5 of the superspeed--50 centrifuge) for 15 hours at 35,000 rpm, the fractions were collected, the bottoms of the tubes were punctured, the index of refraction (n) was measured in a refractometer for calculating the density (ρ), optical density (A_{258}) was determined in a spectrophotometer and the fractions were precipitated on millipore filters as mentioned above. For infectious titration, we selected 0.05 milliliters of each of the corresponding fractions.

Reagents. Actinomycin D (Lyovac, Cosmegen) from Merck, Sharp and Dohme (USA), pancreatic ribonuclease from Worthington (USA), H^3-uridine (0.3 curies per millimole) and C^{14}-amino acids (0.08 curies per millimole) were obtained at the Radioisotope Center (Leningrad).

Results

Characteristics of the Products of Synthesis with Incubation of MF-15, Extracted from Infectious Cells. The experiments in this series followed the same system. Cells which were initially treated with actinomycin D were exposed to a massive dose of VEEV (50-100 PFU/ML) and incubated for 2-1/2 to 3 hours; the culture was then rapidly cooled, rinsed to remove the Henks solution and the MF-15 was extracted as described above. The fraction was incubated in Medium No. 199 at 37°. At various time intervals, we added H^3-uridine (20 microcuries per milliliter) and C^{14}-amino acids (2 microcuries per milliliter). The product was studied in the linear saccharose density gradient in the equilibrium density gradient of CsCl.

We showed earlier [4] that when incubating MF-15 obtained from infected cells, following the end of a latent period of growth of VEEV, there was a pronounced synthesis of virus-specific and cellular RNA and proteins and formation of ribonucleoproteins (RNP). In the sedimentation analysis, the latter were distributed in the form of two peaks, which have sedimentation constants of 80 S and 160 S (Figure 1). It should be emphasized at this point that the RNP extracted from the virions also has a sedimentation constant of 160 S [9]. In other words, at least a portion of the material synthesized by the incubation of MF-15 may be virus RNP.

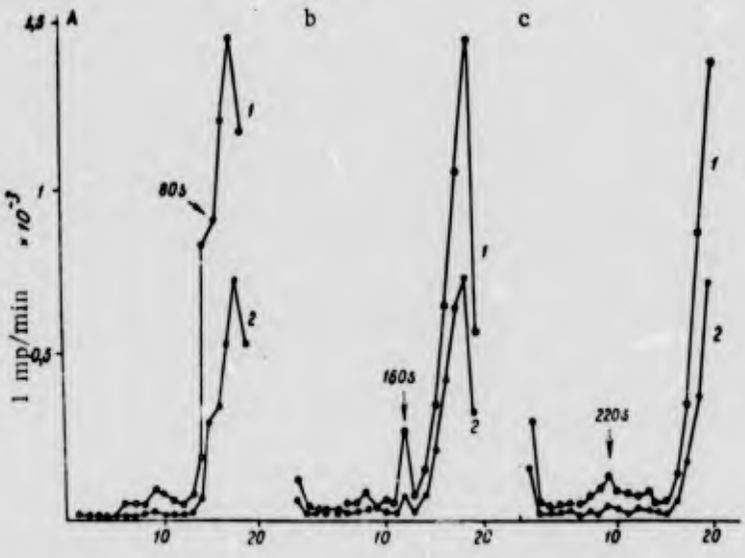

Figure 1. Sedimentation Distribution of H^3 and C^{14} Radioactivity. For the determination, we took a 1/10 volume of each fraction of the gradient. The cells were treated with actinomycin D (2 micrograms per milliliter) and infected with VEEV. H^3-uridine (20 microcuries per milliliter) and C^{14}-amino acids were added from 2 hours 40 minutes to 3 hours later. Following centrifuging at 22,000 rpm, in the saccharose gradient (15-60%) for 1-1/2 hours.

A, B, C - 3 parallel tests. Along the abscissa, we have plotted the number of fractions from the bottom of the gradient; along the ordinate, the radioactivity; 1-H^3, 2-C^{14}.

Additional information on the nature of the investigated structures was obtained following an investigation of their buoyant density and infectiousness.

From the data given in Figure 2, it follows that with an incubation of MF-15 RNP is synthesized with a density of 1.30, 1.33, 1.38 and 1.43 grams/cm^3, and only the last of these corresponds to the density characteristic of the RNP of the virions, while in the other the amount of RNP is less. A suitable calculation will show that the amount of RNP in the observed forms of RNP amount to 11, 20 and 38%. The data obtained served as a basis for the assumption that in addition to the virus-specific RNP, the incubation of MF-15 also sees the formation of "pseudovirus" RNP as a result of complexing of virus RNP with cellular proteins. In order to confirm this, it was necessary to have appropriate experiments in which we could determine the infectiousness of the RNP obtained and to check its sensitivity to the neutralizing action of antivirus serum.

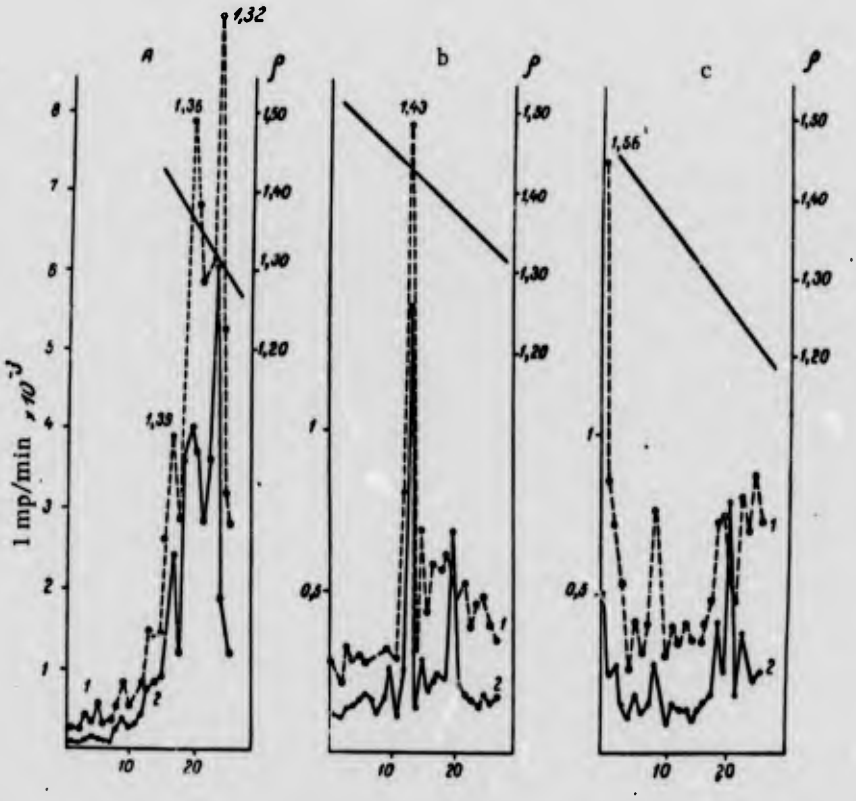

Figure 2. Density Distribution of H^3 and C^{14} Radioactivity in the CsCl Density Gradient.

For centrifuging, we used the fractions of saccharose gradient corresponding to 80 S (A), 160 S (B) and 220 S (C) (See Figure 1). Centrifuging for 15 hours at 35,000 rpm. Along the abscissa we have the number of fractions from the bottom of the gradient; along the left hand side of the ordinate the radioactivity, and along the right - the CsCl density; 1 - H^3; 2 - C^{14}.

TABLE 1. INFECTIOUS ACTIVITY AND SENSITIVITY TO THE NEUTRALIZING ACTION OF ANTISERUM OF DIFFERENT FORMS OF RNP, FORMED DURING THE INCUBATION OF MF-15[1]

Density characteristic of the material (Grams/cc)	Titre of infectiousness log PFU/milliliter		Log of the index of neutralization
	Original	Following processing	
1,30	5,6	5,3	0,3
1,33	6,3	6,1	0,2
1,38	6,1	5,7	0,4
1,43	7,1	2,3	4,8

[1]The experiment used gradient fractions corresponding to the indicated densities, obtained following centrifuging for 16 hours at 35,000 rpm.

As follows from Table 1, all the forms of RNP, synthesized in MF-15, display infectiousness. In addition, the virus-specific antiserum neutralizes practically only the RNP with a floating density of 1.43 g/cc, i.e., the RNP corresponds more than the others in its parameters to the virional. However, the other forms of RNP (1.30, 1.33 and 1.38 g/cc) apparently include cellular proteins which are not affected by the antiserum.

Virus-Like Particles Formed by the Contact of Infectious RNP With The Cytoplasmic Extracts from Non-Infected Cells. In MF-15, extracted from infected cells, together with the virus-specific RNP, there is formation of RNP which consists of virus RNA and cellular proteins, so that it is necessary to determine whether or not the formation of the virus-like particles occurs in vitro. To do this, infectious RNA VEEV was added to different fractions, obtained from a homogenate of non-infected cells; following contact at 0° for 30 minutes, we determined the infectious activity of the product.

We showed earlier that the methods of determining the infectious activity make it possible to differentiate the infectious RNA from the harmful virus [1]. In titrating the RNA, it is necessary to have

a preliminary processing of the cells with a hypertonic solution (Method No. 1), since the usual agar coating is used in the titration of VEEV (Method No. 2).

In all of the experiments described below, in contract to the original RNA, the virus-like particles obtained (pseudoviruses") were titrated as harmful viruses, i.e., by Method No. 2.

Table 2 shows the results of three experiments in which virus RNA was added to whole homogenate (following preliminary removal of nuclei, debris and intact cells from it), to MF-15, to the supernatant following removal of this fraction, and to cell liquor. Following the addition of infectious RNA to the indicated fraction, we observed the formation of virus-like particles ("pseudoviruses") which titrated like harmful viruses. Following contact with the cell liquor, formation of "pseudoviruses" was not observed, since the cell liquor apparently does not contain proteins that form complexes with the virus RNA.

TABLE 2. INFECTIOUSNESS OF THE PRODUCT FORMED IN FRACTIONS OF A CELL HOMOGENATE FOLLOWING INTRODUCTION OF INFECTIOUS RNA VEEV.

No.	Fraction	Log PFU/milliliter (in 3 experiments)		
		1	2	3
1	800 g -- 10 minutes	2,4	3,4	2,9
2	15,000 g -- 20 minutes (MF-15)	2,6	3,7	2,8
3	Supernatant after No. 2	2,8	4,2	3,3
4	Cell liquor	0,1	0,2	0,0
	(Original RNA (Control) [1]	2,0	3,1	2,5

[1]For titrating the RNA, we used Method No. 1 with preliminary processing of the cells with 1 M NaCl.

In subsequent experiments, we determined the sensitivity of the "pseudoviruses" to the action of RNA-ase. As a control, we used the original RNA VEEV and the intact virus. As indicated by Table 3,

the harmful virions are not sensitive to the enzyme, since the infectious activity of RNA (on the other hand) is retained completely, following processing with RNA-ase.

TABLE 3. SENSITIVITY OF VEEV, "PSEUDOVIRUSES" AND RNA TO THE ACTION OF RNA-ase.

Material	No. of fractions [1]	PFU/milliliter		Inactivation
		Before processing	After processing [2]	
VEEV	—	8,9	8,8	—
"Pseudovirus"	1	3,6	2,7	0,9
"	2	2,8	2,1	0,7
"	3	3,5	2,8	0,7
RNA VEEV	—	3,1	0	3,1

[1]For designation of fractions see Table 2.
[2]The processing was performed on the basis of a calculation of 20 micrograms of enzyme per milliliter of material for 30 minutes, at room temperature.

The "pseudoviruses" showed only partial sensitivity to enzyme digestion.

Following processing of the "pseudoviruses" with specific antiserum, there was practically no neutralization of infectious activity. In comparison to the saccharose gradient of the coefficients of sedimentation of the original virus RNA and the "pseudovirus" particles, formed following contact of virus RNA with the cytoplasmatic extracts, we observed a reliable increase in the constants of sedimentation by approximately 1-1/2 to 2 times. At the same time, the virional RNA VEEV had a sedimentation constant of 40 S, while in the "pseudovirus" particles the peak usually occurred in the 80 S region (Figure 3).

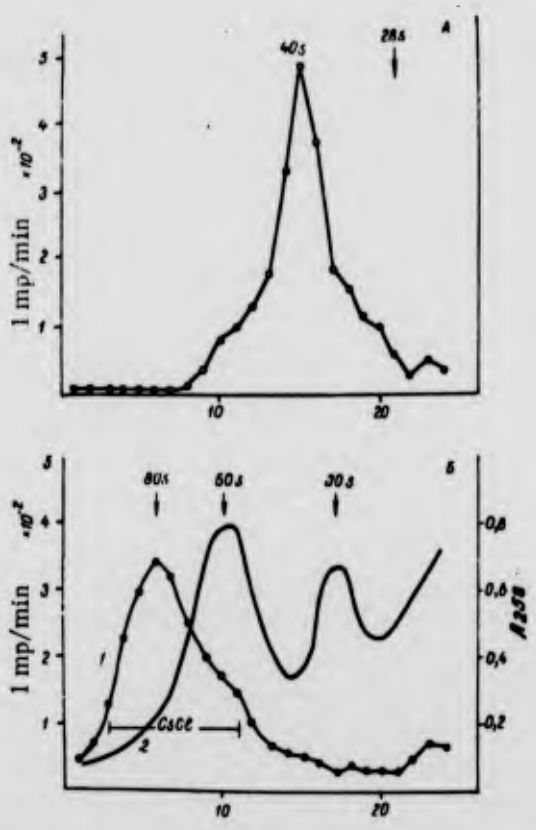

Figure 3. Sedimentation Distribution of Labeled RNA VEEV. The preparations were centrifuged at a 5 to 30% gradient for sampling of saccharose at 37,000 rpm for 2-1/2 hours. A - Distribution of RNA (the arrow indicates the position of the marker peak - 28 S). B - Distribution of virus RNA, displaced before fractioning with homogenate from cells of the FEK.

Along the axis of the abcissa, we have plotted the numbers of fractions from the bottom of the gradient; along the left hand side of the ordinate the radioactivity, and along the right hand side - the absorption at 258 millimicrons; 1 - radioactivity; 2 - absorption.

In special experiments, we studied the density distribution of "pseudoviruses" in the CsCl gradient. With this purpose in mind, we selected from the saccharose gradient those fractions which corresponded to the peaks which were formed as the result of interaction of the virus RNA with the components of the cytoplasmatic extract. As we can see from Figure 4, the "pseudoviral" particles formed 4 homogeneous peaks, whose floating density was 1.30, 1.33, 1.38 and 1.42 grams per cc. Each of these types of RNP has an infectious nature.

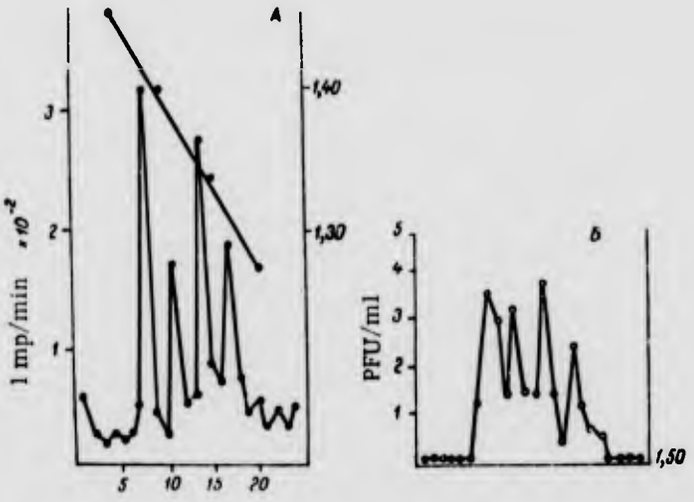

Figure 4. Density Analysis of Products Arising Following Interaction of RNA VEEV With Cell Homogenate.

The distribution of H^3 radioactivity (A) and infectiousness (B) with centrifuging of fractions from the saccharose gradient in the equilibrium CsCl gradient (selected fractions from the 3rd to the 11th were indicated in Figure 3). Titration of the infectiousness was performed using Method No. 2. Along the abcissa, we have plotted the number of fractions from the bottom of the gradient; along the left side of the ordinate: A - radioactivity; B - infectiousness; along the right side of the ordinate, we have the density (in grams per milliliter).

The material of the fractions from the 3rd to the 11th on the saccharose gradient (indicated in Figure 3, B) was centrifuged in the CsCl density gradient until equilibrium. Following centrifuging in the fractions of the gradient we determined the radioactivity (A) and infectiousness (B). Method No. 2 was used for titrating the infectiousness.

Hence, the virus-like particles formed in vitro upon the introduction of infectious RNA into the homogenate of fibroblasts of the chick embryo correspond to different forms of RNP which are formed during the incubation of MF-15, separated from the infected cells.

Discussion

The data obtained make it possible to conclude that the products of the synthesis of MF-15, separated from infected cells, are RNP,

distinguished by different sedimentation and density characterisitcs; one of them corresponds to the RNP of the virions in its properties. Each of these forms of RNP have infectiousness, but they differ in sensitivity to the action of the antiviral serum. The latter fact indicates that the composition of the formant RNP may involve both virus-specific as well as cellular proteins. In fact, when virus RNA is added to the homogenate from normal cells, there is also a non-specific complexing of RNA with the cell proteins. The structures which are formed, which we have called "pseudoviruses", have infectious properties, are not sensitive to the neutralizing action of immunizing sera and are stable to a significant degree with respect to RNA-ase.

The study of the sedimentational properties of the "pseudovirus" has shows that the complexing of virus RNA with the components of the cell extracts leads to an increase in the rate of sedimentation by approximately 2 times. A similar phenomenon of "loading" of RNA was observed in the study of different types of cell and virus RNA [13].

The density of the "pseudovirus" and the infectious RNP was always less than the density of the ribosomal particles (1.5 to 1.6 grams/cc). This makes it possible to assume that the subunits of the ribosomes do not enter into the composition of the virus-like particles formed in vivo and in vitro. Apparently, in the systems investigated, the "loading" is caused by the formation of complexes of virus RNA with soluble cell proteins. The latter, as we have shown, are removed from extracts following centrifuging at 150,000 g for 2 to 4 hours. The different density distribution of these complexes may have an effect either on the variability of the ratio of RNA and protein in them, or on the fact that the different proteins may play the role of a "loading" factor.

Apparently, the complexing of RNA with the proteins of the homogenate has the nature of a general relationship. It is possible to have formation of structures of the informosomal type [7, 8]. Similarity of the "pseudovirus" particles to the informosomes is indicated by the general phenomenon of "loading" and the coincident density characteristics in the CsCl gradient. In our case, the buoyant density of one of the constant peaks (1.42 g/cc) forming the "pseudovirus" corresponds with the density of the informosomes. It should be emphasized that the values of the buoyant density for the virus RNA tested is much higher (1.66 g/cc). Hence, following contact with the cytoplasmatic extracts, the rate of sedimentation increases or the buoyant density decreases. This leads us to assume that the "pseudoviruses" may be viewed as specific stoichiometric complexes of RNA with less dense components of the cell homogenate, which

is also the case in the formation of informosomes. In other words, the basis of the formation of "pseudoviruses" and informosomes may be a common mechanism.

REFERENCES

1. Yershov, F. I., L. V. Uryvayev, V. M. Zhdanov, et al., *Dokl. AN SSSR*, Vol. 190, No. 1, p. 212, 1970.

2. Yershov, F. I., L. V. Uryvayev, V. M. Zhdanov, *Dokl. AN SSSR*, No. 2, p. 458.

3. Yershov, F. I. and V. M. Zhdanov, *Acta virol.*, Vol. 12, p. 136, 1968.

4. Yershov, F. I., L. V. Uryvayev and V. M. Zhdanov, *Vopr. Virusol*, No. 3, p. 322, 1970.

5. Zhdanov, V. M., F. I. Yershov and L. V. Uryvayev, *Dokl. AN SSSR*, Vol. 187, No. 3, p. 667, 1969.

6. Zhdanov, V. M., F. I. Yershov and L. V. Uryvayev, *Dokl. AN SSSR*, Vol. 189, No. 3, p. 647.

7. Ovchinnikov, L. P., A. S. Voronina and A. S. Stepanov, et al., *Molek. Biol.*, Vol. 2, p. 752, 1968.

8. Spirin, A. S., N. V. Belitsina and M. A. Aytkhozhin, *Zh. Obshchey Biol.*, Vol. 25, p. 321, 1964.

9. Uryvayev, L. V., V. M. Zhdanov, F. I. Yershov, et al., *Vopr. Virusol.*, No. 3, p. 330, 1970.

10. Cheng P.-Y., *Virology*, Vol. 14, p. 124, 1961.

11. Girard, M. and D. Baltimore, *Proc. Nat. Acad. Sci. (Wash.)*, Vol. 56, p. 999, 1966.

12. Martin, E. M. and J. A. Sonnabend, *J. Virol.*, Vol. 1, p. 97, 1967.

13. Porterfield, J. S., *Bull. Wld. Hlth. Org.*, Vol. 22, p. 373, 1960.

14. Zhdanov, V. M., F. I. Yershov and L. V. Uryvayev, *Virology*, Vol. 38, p. 355, 1969.