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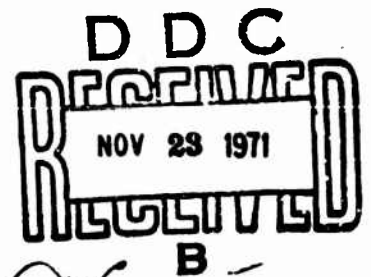
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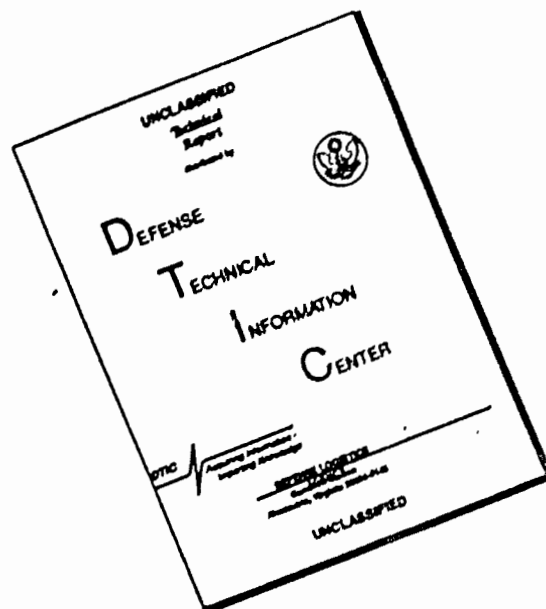
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SEDIMENTATION CHARACTERISTICS OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS

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The VEE virus was propagated in chick embryo cells, concentrated and purified. The optimal method for obtaining biologically active virus components consisted in degradation of the virus with ether-Tween. The purified VEE virus sedimented at about 380 S in sucrose gradients, the nucleoid at about 160 S. Centrifugation in CsCl gradients showed the VEE infectious material to band in two main positions: most of the three viruses banded at 1.25 g/ml., and a smaller amount at 1.42 g/ml. The main peak of hemagglutinins was detected at a boyant density of 1.25 g/ml. The site of virus and its components was determined by radiological and biological tests.

The sedimentation properties of virions and their components became in the arboviruses a subject of protracted research only recently, and detailed study was done on only a few members of the group of A. Constant, of the sedimentation of the Semlik forest virus, isolated from cells of chicken fibroblasts; it was defined at 350 S (11). At the same time in the earlier work of Cheng (4,6), the virions had a sedimentation constant of about 400 S. The density of the last equaled 1,24 g/ml CsCl (10). Grown in chicken fibroblasts, purified in columns with  $AlPO_4$ -gel, the VEE virus had a sedimentation constant of 260 S (3). Centrifuging of this virus in a CsCl gradient gave three peaks with a density of 1,20, 1,22 to 1,23 (both were infectious) and 1,18 (non-infectious material). The virus specific material of the Sindbis virus dispersed in CsCl gradient or in rubidium chloride gradient, also in three basic fractions: 1,23 - 1,24; 1,21 and 1,191 (7,13,14, 16), and the biological properties of each of them was characterized.

In 4 to 6 hours after infection of the culture by the Semlik forest virus, it was possible to isolate from the cells 140 S particles, which had the properties of ribonucleoproteid (7,10,11).

The virus of Venezuelan equine encephalomyelitis in this connection was not studied. One of the reasons is this: the difficulty of receiving highly purified virus material. We introduce the results of research on the sedimentary properties of virions and the components in a density gradient of sucrose and cesium chloride, received during work with purified material.

MATERIALS AND METHODS

The VEE virus was passivated in previously trypsinized chicken fibroblasts (monolayer or spinner culture), in No. 199 medium with hydrolysate lactalbumin and bovine serum and the crop gathered in 18 - 20 hours. The

infection titer and hemagglutinin (1'A) was defined using generally accepted methods (4,16). The average titers of the virus were  $7 \cdot 10^8$  -  $2 \cdot 10^9$  PFU/ml and 640 - 1280 HU (hemagglutinating units) per 1 ml.

In the majority of the experiments a virus was used marked with  $H^3$  - uridine (radioisotope center, Leningrad, specific activity 0,3 curies/ millimoles) and according to protein - with  $C^{14}$  hydrolysate chlorelli (of the same origin, specific activity 0,08 curies/ml), which was introduced into the culture medium at 5 microcuries to 1 ml immediately after infection of the cells.

The original virus suspension was given preliminary centrifuging at 5000 g. for 30 minutes, and then separated out on the centrifuge MSE (rotor 8 X 50, 28,000 rev./min, two hours). The precipitate was resuspended in 0,01 M tris-buffer pH 7,4 in which was added 0,33% crystalline bovine albumin; the 2 ml. of virus suspended matter received was layered in step-wise gradient from 60 to 15% saccharose solution (in the same buffer) and again centrifuged two hours in a bucket-rotor 3 X 20 at 2500 rev/min. The strip of material containing virus at the boundary of the two densities was gathered and diluted in buffer, layered in a 20% solution of saccharose and centrifuged another two hours. The precipitate was dissolved in the necessary buffer and used for work. In several cases the virus was preliminarily concentrated with a sulfate ammonia sediment and then purified.

For breakdown of the virus, we applied freshly distilled ethyl ether in a relation of 2:1 and the suspension of the virus and tween 80 (5 mg/ml). The mixing was done with the aid of a magnetic mixer for 20 minutes at room temperature. Then the upper layer of ether was drawn off, and the remains of the ether removed by blowing with nitrogen. In several cases for the breakdown of the virus we used desoxycholate (Michrome firm, England), at a concentration of 0,5 and 1%.

Gradient centrifuging was done at 17 - 40% and 15 - 60% density gradient of saccharose in tris-buffer in a bucket-rotor 3X20 preparatory centrifuge Superspeed MSE-50 at 25,000 rev./min for two hours. A fraction in 32 drops was gathered by means of extrusion from the bottom of the test tube in an apparatus consisting of an LKB collector, an air pump, a uvicord ( $A_{260}$ ) and registering device. The radioactivity was computed in a liquid scintillator counter Tricarb (Packard Inc.) During definition of the biological activity of the sample we diluted with 0,05 M borax buffer pH 8,6 with addition of 0,33% bovine albumin and titrated ex tempore. We defined the sedimentation constant using the method of Marin and Ames (13).

Centrifuging in a density gradient of cesium chloride (gradient with a density of from 1,15 to 1,6 g/ml was prepared in 0,01 M phosphate buffer pH 7,2 with 0,0015 M  $MgCl_2$  and 0,33% bovine albumin) was done in a bucket rotor 3X5 with indicated centrifuge for 16 to 18 hours at 35,000 rev/ min. The fractions in 16 drops were gathered by means of roasting (annealing) the bottom of the test tube; the defined refraction coefficient ( $n$ ), computed the density by empirical formula  $p^{250} = 10,8601 (n_{250}^o) - 13,4974$ . The optical density of the fractions was defined with the aid of a spectrophotometer Cf-4.

## ELECTRON MICROSCOPE

The purified virus and fractions of subviral particles from the gradient were prepared for the electron microscope as described in another of our works (3).

### RESULTS

Purification of the virus. The infectious and hemagglutinating activity and likewise the volume of the original and purified virus material is indicated in table 1.

With the aid of the described method of purification, we managed to obtain concentrated and highly purified material, and withal, a loss of virus infection which did not exceed 50%, and for viruses having hemagglutinating activity - 30%. However in the following case, it is logical to calculate the possibility of disintegration of virions in the process of centrifuging and microscope observation because of a genuine loss of hemagglutinins detached from the virions. A more detailed method of purification and the characterization of the parent and purified virus material will be presented by us in another work.

The action of detergents. Results of experiments studying the action of detergents on infectious and hemagglutinating activity of viruses is presented in table 2.

As is logical from table 2, already in 5 minutes after the treatment of a virus with an ether titer, the hemagglutinating increases, and the infection titer steadily decreases. Sodium desoxycholate caused a fast and apparently deep breakdown of virions, and it wholly inactivated the infection. The optimum effect is received during the breakdown of virions with ether and tween: a persistent increase of hemagglutinin titers and less expression of inactivation of virus infection. Therefore in the majority of the experiments we used this treatment of virus suspensions.

The sedimentation constant of virions and their components. Experiments in defining the constants of sedimentation of a purified virus in a density gradient of saccharose of 15 - 60% showed that the peak of radioactivity ( $H^3$  - uridine and  $C^{14}$  - amino acid) corresponded with the maximal quantity of infectious material and the highest titers of hemagglutinins in these samples (sketch 1). This fact as noted allows reliable confirmation that the maximal indicators are due to viruses preserved in the course of centrifuging (whole virions), and to determine the position of the virions on a sedimentogram. A comparison of their position with the position of a known marker (ribosomes of chicken fibroblasts 74S) allows computation of the sedimentation constant of a virus which is equal to 380S.

At the same time in the sedimentogram, a second peak (fraction 13-14) of radioactivity ( $H^3$  - and  $C^{14}$  - marker) is usually clearly revealed, having above all a low infection ( $6 \cdot 10^3$  PFU/ml), complement-binding activity (32-64 CB part.) and nearly free of hemagglutinating activity (4 HA/ml). The change of position on the sedimentogram, the correspondance of peaks of protein and nucleic markers, and also the presence in these fractions of infectious material and complement-binding activity allows, with a known probability ratio, consideration of this supplementary peak as a product of desintegrated virions, namely S-antigen or ribonucleoprotein (RNP), the constant of which is equal to 160S.

Treatment of the purified virus with tween and ether for 20 min and subsequent centrifuging in a saccharose gradient of 17 - 40% led to a change in the sedimentogram (sk. 2). The greatest radioactivity was revealed in the lighter peak, infectious activity - in the light and in the heavy peak to about the same degree - although very low - not more than  $2 \cdot 10^2$  PFU/ml. Infection in fractions 4-5 and especially 12-14 was revealed inconsistently (in 2 out of 7 experiments), and only in cases where the material of these fractions was introduced into the culture fabric immediately after removal of the gradient. After storage in a frozen state for a certain amount of time, infectious activity was not revealed. The maximal titers of hemagglutinating (up to 128 HA/ml) was noted in the last samples.

The material received after treatment of the virus with desoxycholate did not give a characteristic picture on the sedimentogram. The dispersion had a "sawtooth" character without the expression of peaks. It was not possible to discover virus-specific material with biological tests.

Buoyant density of the virion and its components. The density of dispersion of the purified virus treated with tween and ether is shown in sketch 3 (a, b). The basic peak of radioactivity (corresponding with  $H^3$ - and  $C^{14}$  - markers) was situated in the fractions with a density of 1,25, corresponding with the highest titers of infection and caused hemagglutinating of material which was neutralized with specific immunization serum.

On the sedimentogram of the virus broken down by detergents, one peak of radioactivity appeared very clearly in the fraction, having a density of 1,42. The preliminary experiments did not succeed in clarifying the infectious activity in this fraction. It was possible to observe an analogous picture of dispersion of the radioactive material during use of material fixed with formaline.

#### ELECTRON MICROSCOPE

The preparations received from the various fractions of cesium chloride gradient were studied under an electron microscope Jem-7G (sk. 4). The intact virions (a) were received from fractions with a density of  $1,25 \text{ g/cm}^3$ , and the threads of ribonucleoprotein from fractions with a density of  $1,42 \text{ g/cm}^3$ . The threads of ribonucleoproteids had a diameter of about 20Å. The more

detailed analysis of the morphology of subvirus structures and virions was done in another of our works (3).

#### DISCUSSION

With the help of the method of salting out, with subsequent gradient centrifuging, it was possible to receive highly purified and concentrated preparations of infectious virus (infection titer up to  $3 \cdot 10^{10}$  PFU/ml), having hemagglutinating activity. Such virus had a sedimentation constant of 380S near the value received for the Semlik forest virus (4,6,11). In other work, the sedimentation constant of the VEE virus was significantly lower - 260S (3). The basic peak of the virus-containing material during centrifuging in a gradient of cesium chloride was linked with the fraction, having a density of 1,25. As has already been indicated, the buoyant density of other virions studied as representatives of the arboviruses varied between 1,21 - 1,24 (8,9,14). The difference in density of 0,02 g/ml is explained by the dissimilar quantity of lipids and other host material adsorbed on the virions. In connection with this it is logical to underline the great significance of the dependence of this value on the type of virus, the type of material in which the virus is grown, and the method of purification used for the receipt of the virus material.

The optimal method for receipt of the virus S-antigen turned out to be treatment of the virus with tween and ether: the highest, and most stable for a selected period of time, titers of hemagglutinins and complement-binding antigens, likewise the decreased (in comparison with other methods) lowering of infection bear witness to the less significant inactivation of the products of virion breakdown. At the same time residual infection can be caused by the virions which are being conserved, since it is possible to discover it both during the usual titration and the hypertonic method (15, 18). The dispersion in the gradient bears witness on the existence of both types of particles. The data received by us allows the conclusion that there does exist in the VEE virus, ribonucleoprotein which has a sedimentation constant of 160S and a density of 1,42 g/ml. From 140 S particles received from the chicken fibroblasts infected by the Semlik forest virus (near in value to the 160S particles from the virions of the VEE virus), it was possible to isolate 42S-RNA which confirms the link of these particles with virions.

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Table I. Biological Activity of Virus-containing Material

| Вирус VIRUS        | Volume Объем (в мл) IN ML | TITER ТИТР       |             | % очистки по белку (по Лоури) |
|--------------------|---------------------------|------------------|-------------|-------------------------------|
|                    |                           | БОЕ/мл           | ГAE/мл      |                               |
| Исходный ORIGINAL  | 1200                      | $7.5 \cdot 10^4$ | 320-640     | 99.0                          |
| Очищенный PURIFIED | 12                        | $3.0 \cdot 10^6$ | 20000-40000 |                               |
|                    |                           | PFU/ML           | HAP/ML      |                               |

← % of purification by protein (acc. to Lowry)

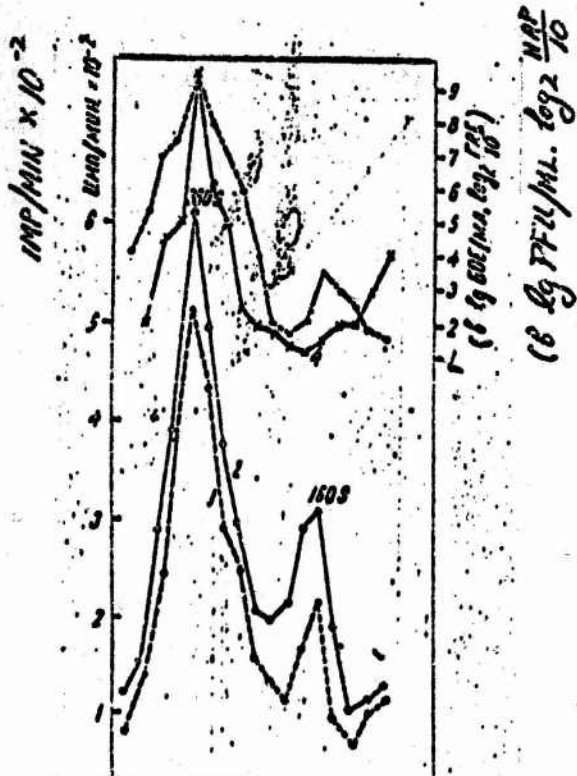
Table 2. Influence of Detergents on the Biological Activity of the VEE Virus.

Note: Original virus 320 HAP/ml and  $3 \cdot 10^5$  PFU/ml (HAP - hemagglutinating particles or units)

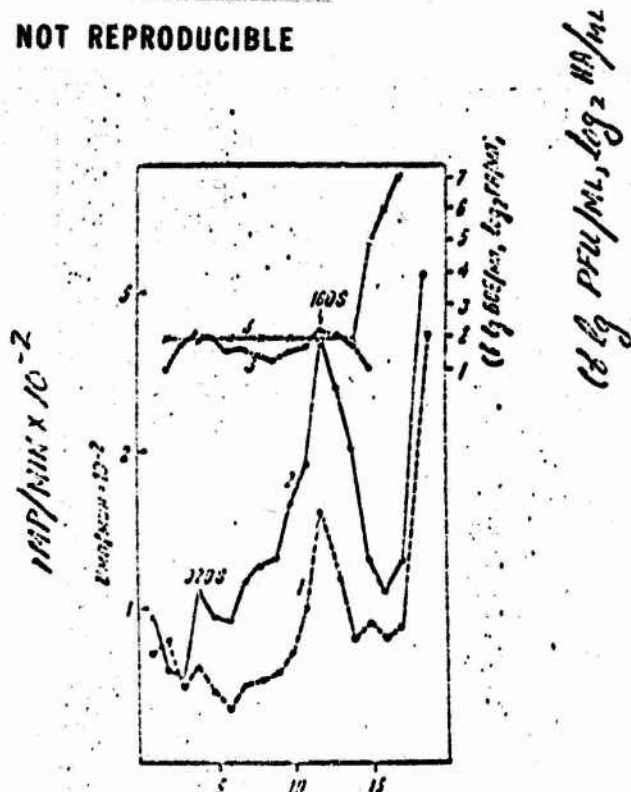
| TREATMENT Обработка                                | 5-MIN. TREATMENT     |                  | 30-MINUTE TREATMENT   |                   |
|--|----------------------|------------------|-----------------------|-------------------|
|  | 5-минутная обработка |                  | 30-минутная обработка |                   |
|  | ГAE/мл               | БОЕ/мл           | ГAE/мл                | БОЕ/мл            |
| Эфир ETHER   | 640                  | $6.0 \cdot 10^7$ | 640                   | $2.45 \cdot 10^4$ |
| Эфир+твин ETHER+TWIGEN                             | 640                  | $2.5 \cdot 10^8$ | 1280-2560             | $5 \cdot 10^6$    |
| 0.5% дезоксихолат натрия SODIUM DESOXYCHOLATE 0.5% | 1024                 | $2.7 \cdot 10^6$ | 160                   | $< 10^3$          |

Sketch 1. Dispersion of  $H^3$  (1) and  $C^{14}$  (2) markers, of infectious (3) and HA (4) activity of the purified VEE virus during centrifuging in a density gradient of saccharose 15 - 60% (25,000 rev/man, 3 1/2 hours)

Sketch 2. Dispersion of radioactivity and biological activity of the VEE virus with preliminary treatment with tween and ether, in a density gradient of saccharose 15 - 60% (25,000 rev/man, 2 1/2 hours. Note: designations the same as in sketch k.

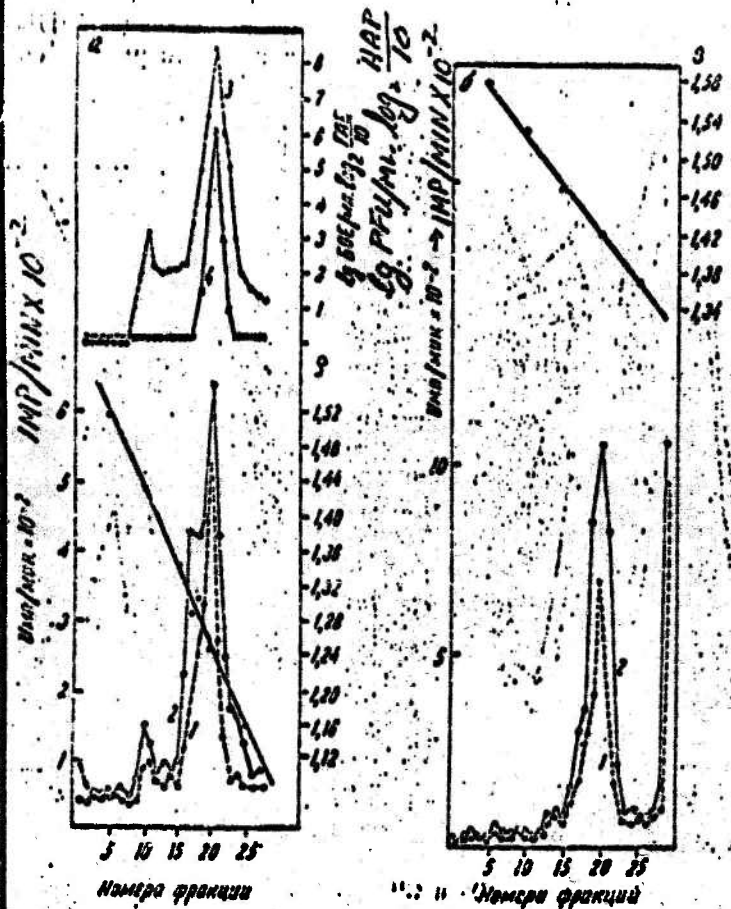


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Sketch 3. Distribution of purified virus in fractions of the density gradient of cesium chloride (a) and distribution of radioactivity in fractions of the density gradient of cesium chloride during centrifuging of the  $10^7$  of the VEE virus (b)

Remaining designations same as in Sketch 1.



NUMBERS OF FRACTIONS      NUMBERS OF FRACTIONS

Sketch 4. Electron Microscope picture of virions (a) and ribonucleoproteids of the virus (b), received from various fractions of the gradient. See text for conditions for receipt of the fractions.

