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TECHNICAL MANUSCRIPT 130

PURIFICATION AND LIPID CONTENT OF VEE VIRUS

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U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 130

PURIFICATION AND LIPID CONTENT OF VEE VIRUS

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ABSTRACT

The virus of Venezuelan equine encephalitis (VEE) was purified from chick embryo suspensions by two methods, and the total lipid content and RNA content of the purified virus preparations were compared. The lipid content of VEE virus purified by sucrose gradient centrifugation was much lower (24 per cent) than that of VEE virus purified without gradient centrifugation (54 per cent). Virus of lower lipid was still fully infectious, the specific infectivity for purified virus being $10^{14.8}$ mouse intracerebral LD_{50} per gram of protein. With the decrease in lipid content of the virus there was a concomitant increase in RNA content.

Electron microscopic examination of purified VEE virus showed that the virus had an average diameter of 68 millimicrons and possessed a center core about 30 millimicrons in diameter.

I. INTRODUCTION

Studies on the chemical composition of the viruses of Eastern (EEE) and Western (WEE) equine encephalitis indicated that these viruses contained about 54 per cent fat-solvent extractable material and 4 per cent RNA when they were purified by ultracentrifugation.[1,2] Preparations of Venezuelan equine encephalitis (VEE) virus purified in our laboratory by the same method employed by Taylor et al[1] for EEE virus, or by variations of this method, also contained approximately the same percentages of lipid and RNA as had been reported for EEE and WEE viruses. Olitsky and Casals[3] predicted, however, that "the chemical results stated may need revision if more purified preparations become available." With VEE virus such has been the case when viral preparations of higher purity were obtained by including sucrose gradient centrifugation in the purification procedure, yielding virus of much lower lipid content and higher RNA content.

It is the purpose of this report to compare the lipid and RNA contents of VEE virus purified with and without the use of gradient centrifugation and to show that virus of lower lipid content remains fully infectious. A preliminary report of this work was made earlier.[4]

II. MATERIALS AND METHODS

A. VIRUS

The Trinidad strain of VEE virus described by Hardy[5] was used in all experiments. The working seed for these studies was a ten per cent chick embryo seed.

B. PREPARATION OF VIRUS SUSPENSIONS

VEE virus was propagated in 11-day embryonated eggs incubated at 37°C after inoculation by the allantoic route with approximately 500 egg LD_{50} of virus. At the peak time of death (18 to 20 hours after inoculation), decapitated embryos were homogenized for three minutes in a Waring Blendor with four parts by weight of Ringer-Locke solution. Following the procedure described by Taylor[1] for EEE virus, the 20 per cent embryo suspensions were held at pH 8.3 to 8.5 for four days at 4°C to minimize the effect of a normal embryo component that tended to sediment with the virus in the centrifugation sequence. Suspensions were then stored at a temperature of -45°C until needed for further processing.

C. INFECTIVITY ASSAY

Infectivity was determined by the inoculation of virus via the amniotic route into 14-day embryonated eggs and by intracerebral inoculation into 12- to 14-gram white mice.

D. PROTEIN DETERMINATION

Protein content of virus preparations was determined by the method of Lowry et al.[6] Crystalline bovine plasma albumin (Armour) was employed as a standard.

E. DIALYSIS AND LYOPHILIZATION

Samples of virus for analysis were prepared by dialyzing suspensions of the purified virus against distilled water, then concentrating them to smaller volume by pervaporation before lyophilization in ampoules or small bottles. Samples were further dried to constant weight over phosphorus pentoxide before extraction of lipids or nucleic acid.

F. LIPID DETERMINATION

Total lipid was determined as alcohol-ether-soluble lipid by (a) three extractions with a 3:1 mixture of ethanol-ether at 30°C in a closed tube, using a Cahn electro-balance to weigh the virus samples before and after extraction, and (b) by direct weighing of the extracted lipid, using a micro-extraction apparatus similar to that described by Shaffer et al[7] and the extraction procedure employed by Thomas[8] with Tipula irridescent virus. In this method, the lipid extracted by refluxing with a 1:1 mixture of methanol-diethyl ether was re-extracted with petroleum ether. The resulting petroleum ether-soluble and -insoluble fractions were then dried to constant weight. No additional lipid was demonstrated in the virus residues on repeated treatment with methanol-ether.

G. NUCLEIC ACID DETERMINATION

The ribonucleic acid content of the dry, purified virus samples was determined by the method of Ogur and Rosen.[9] Samples of VEE virus purified with and without gradient centrifugation, from which lipid had been extracted, were washed once with cold two per cent perchloric acid before being thoroughly mixed with cold ten per cent perchloric acid and placed at 4°C. After 44 hours, samples were centrifuged and the absorption spectra at 260 millimicrons of the supernatant fluids were determined in a Beckman DU spectrophotometer. By comparison with standards of purified RNA in perchloric acid solution, the amount of RNA present in the virus was estimated.

One preparation of VEE virus purified by gradient centrifugation was analyzed for nucleic acid by the procedure of Ogur and Rosen mentioned above and also by the hot sodium chloride method that Ada and Perry[10] employed to determine the nucleic acid content of influenza virus.

H. ELECTRON MICROSCOPY

Electron micrographs were taken with an RCA EMU-2 electron microscope. A droplet of purified virus, suspended in sucrose-Ringer-Locke, Ringer-Locke, or ammonium acetate solution (0.1M, pH 7.2), was allowed to stand for three to five minutes on a collodion film supported by a specimen screen. Most of the drop was withdrawn, and the specimen was rinsed two or three times with ammonium acetate solution before being allowed to dry in air. The preparations were shadowed with uranium at an angle of 30 degrees.

I. PARTIAL PURIFICATION PROCEDURE

VEE virus was partially purified from infected chick embryo suspensions by a modification of the method employed by Taylor and coworkers[1] for EEE virus. The original method, which consisted of filtration through a layer of Celite followed by three cycles of differential centrifugation, was changed in the following respects: (a) centrifugation speeds and times were modified slightly, and (b) a tryptic digestion step was introduced into the procedure; the sediment of the first high-speed centrifugation was suspended in a solution of ten milligrams per milliliter of trypsin (Difco 1:250) and incubated at 36°C for 10 minutes.

To obtain more highly purified virus for comparative analyses two methods were employed: (a) adsorption and elution of virus from glass filters, and (b) density gradient centrifugation.

J. ADSORPTION-ELUTION FROM GLASS

The partially purified virus was subjected to a modification of a procedure employed by Puck et al[11] for the concentration of bacteriophage. The virus suspension was filtered through a series of Pyrex sintered-glass filters of medium porosity that had been prerinsed with cold Ringer-Locke solution of pH 8.3. Virus was eluted with beef heart infusion broth (pH 7.2) that had been previously centrifuged at 105,000g for three hours to eliminate nonviral material that sediments from the broth under these conditions. Combined broth eluates were centrifuged at 105,000g for three hours to sediment the virus in the form of small, translucent, amber-colored pellets. To eliminate traces of broth, sediments were resuspended in five to ten volumes of Ringer-Locke solution and resedimented by centrifugation at 41,190g for one hour.

K. DENSITY GRADIENT CENTRIFUGATION

Following the third high-speed centrifugation the partially purified virus was resuspended in ten milliliters of Ringer-Locke - three per cent tryptose solution and then passed through a Millipore filter (pore size 0.45 micron). The filtrate was layered on a sucrose gradient of density 1.072 to 1.220 grams per milliliter that had been prepared by stepwise layering and held at 4°C overnight. After centrifugation at 90,000g for three hours in a Spinco SW-25 swinging bucket rotor, the visible virus zone was removed through the side of the tube with a modified trocar-cannula assembly.[12] The suspension was then dialyzed against Ringer-Locke solution to reduce the sucrose concentration and permit sedimentation of the virus by centrifugation at 105,000g for one hour. The small, clear pellet was resuspended in a minimal volume of ammonium acetate solution.

Highly purified virus suspensions produced by the adsorption-to-glass and the density gradient methods in a series of identical trials were respectively pooled, dialyzed against distilled water, lyophilized, and dried to constant weight.

III. RESULTS

At successive steps in the purification procedures samples were taken for assay of infectivity and total protein. From these values the specific infectivities (mouse or egg LD_{50} per gram of protein) of the samples were calculated. The average specific infectivities of the final purified viral suspensions obtained in both the glass adsorption and the sucrose gradient methods were the same: $10^{14.8}$ mouse intracerebral LD_{50} per gram of protein. The reduction in protein content and concomitant increase in specific infectivity at several stages of the gradient centrifugation procedure are shown in Table I. There was an over-all increase in specific infectivity from $10^{11.7}$ to $10^{14.8}$ mouse intracerebral LD_{50} per gram of protein. This increase resulted from a reduction in protein from 7.5 to 0.0033 milligram per milliliter (considering all samples on an unconcentrated basis), or a reduction of greater than 99.9 per cent. Although a sharp decrease in the lipid content of the virus occurred during gradient centrifugation (as shown in Table II), note that a gain rather than a loss in specific infectivity was produced at this step.

Average recovery of purified infectious virus in both methods was about 25 per cent. The yield of dry purified virus ranged from 0.014 to 0.021 milligram per gram of starting embryo.

TABLE I. PURIFICATION OF VEE VIRUS FROM THE CHICK EMBRYO

Fraction	Protein, mg/ml	Mouse $ICLD_{50}$ per gram protein
Starting 20% embryo	7.6	$10^{14.7}$
After Celite filtration	3.7	$10^{15.0}$
After 3 centrifugation cycles	0.012	$10^{14.0}$
After gradient centrifugation and final sedimentation	0.0033	$10^{14.0}$

TABLE II. EFFECT OF SUCROSE GRADIENT CENTRIFUGATION ON THE LIPID CONTENT OF VEE VIRUS

Sample	Lipid Content, per cent			Ratio of Pet. Ether-Soluble to Pet. Ether-Insoluble Lipid
	Total Lipid[a]	Pet. Ether-Soluble	Pet. Ether-Insoluble	
Virus suspension before gradient centrifugation	53.1	40.2	12.9	3:1
Virus suspension after gradient centrifugation	24.9	15.2	9.7	1.6:1
Upper zone of gradient	58.0	51.5	6.5	8:1

a. Methanol-ether-soluble.

Sucrose gradient centrifugation resulted in a specific separation of VEE virus from contaminating nonviral components. In the gradient derived from virus-infected embryos a narrow, opalescent band was present near the center of the gradient, although no zone was visible at this location in the gradient derived from normal embryos that were processed in parallel with the infected material. Futhermore, if viral infectivity was completely lost in an occasional trial, no band corresponding to the virus band was visible.

An electron micrograph of VEE virus sampled directly from the virus zone of the sucrose gradient is shown in Figure 1. The particles have an average diameter of 68 millimicrons, and consist of a center core about 30 millimicrons in diameter, which is surrounded by an outer ring about 15 millimicrons in width, with a depression between core and outer ring. In some electron micrographs numerous coreless particles or "doughnuts" have been seen. For comparison, an electron micrograph of VEE virus purified by the adsorption-to-glass method is shown in Figure 2. These particles also have an average diameter of 68 millimicrons but do not show the center core and ring.

The pronounced difference in the lipid content of the VEE virus purified by (sintered glass) adsorption-elution and by density gradient centrifugation is shown in Table III. The average content of alcohol-ether soluble lipid of the samples purified by gradient centrifugation was 24.3 per cent, less than half the average value of 53.6 per cent for four preparations obtained without gradient centrifugation. The total lipid of the filtered preparations and first two preparations of virus purified by gradient centrifugation was determined by three extractions with ethanol-ether at 30°C, using the difference in weight before and after extraction for calculation. The lipid contents of the last two preparations were determined both by weight difference and by direct weighing of the extracted lipid, using micro-extraction method of Thomas.[8]

A comparison of the lipid content of virus from the third preparation before and after gradient centrifugation with that of the upper, cloudy zone that remained at the top of the gradient is shown in Table II. The total lipid content of the virus suspension was reduced 30 per cent; at the same time, lipid was concentrated at the top of the gradient, as indicated by the 58 per cent lipid content of the upper zone. Considering the ratio of petroleum ether-soluble to petroleum ether-insoluble lipid in these samples, it appears that more petroleum ether-soluble material is removed from the virus suspension by density gradient centrifugation. Based on preliminary tests, the petroleum ether-insoluble fraction appears to be largely phospholipid; the petroleum ether-soluble fraction appears to contain all the cholesterol found in the virus.

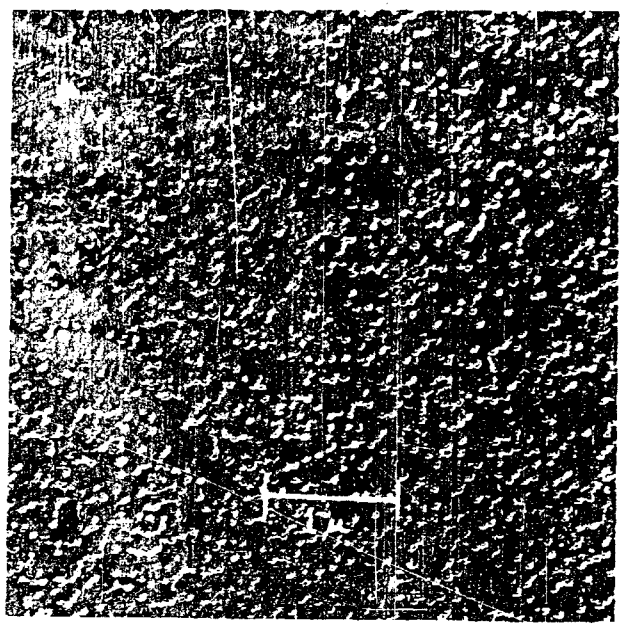

Figure 1. Electron Micrograph of VEE Virus Purified by Sucrose Gradient Centrifugation. Shadowed with uranium. Magnification 30,000X.

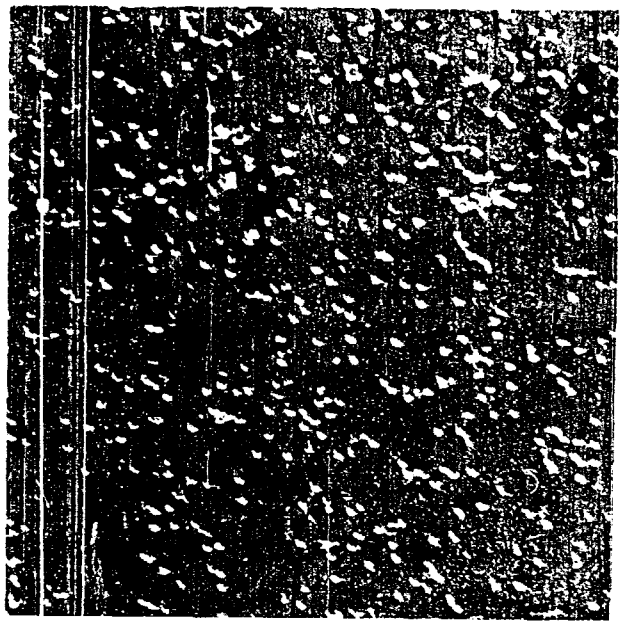

Figure 2. Electron Micrograph of VEE Virus Purified by Adsorption-to-Glass Method. Shadowed with uranium. Magnification 40,000X.

TABLE III. LIPID CONTENT OF VEE VIRUS PURIFIED WITH AND WITHOUT DENSITY GRADIENT CENTRIFUGATION

Treatment	Sample[a]	Total Lipid,[b] per cent
Purified without gradient centrifugation	1	55.0
	2	55.0
	3	51.5
	4	52.8
	Mean	53.6
Purified with gradient centrifugation	1	22.6
	2	25.5
	3	24.9
	4	25.2
	Mean	24.3

a. Each sample consisted of purified virus pooled from a series of identical trials.
b. Alcohol-ether-soluble lipid.

Table IV lists the RNA content of samples of VEE virus purified by sucrose gradient centrifugation and by adsorption and elution from glass. RNA was determined by a perchloric acid extraction method. The average RNA content of five samples of VEE virus purified by gradient centrifugation was 6.4 per cent, as compared with 3.2 per cent for two samples purified by adsorption and elution from glass filters.

TABLE IV. RNA CONTENT OF VEE VIRUS

Type of Preparation		RNA Content, % of whole virus
Purified by gradient centrifugation		5.8
		6.7
		6.9
		6.2
		6.2
	Mean	6.4
Purified adsorption and elution from glass		2.9
		3.5
	Mean	3.2

One preparation of VEE virus purified by sucrose gradient centrifugation was analyzed for nucleic acid by two methods: the cold perchloric procedure of Ogur and Rosen[9] and the hot sodium chloride method of Ada and Perry.[10] Approximately equal weights of a lyophilized preparation of VEE virus from which the lipid had not been extracted were analyzed. In the latter method, a 1.53-milligram portion was extracted four times with ten per cent sodium chloride solution at 100°C for 30 minutes. The supernatant liquid of the centrifugation that followed each extraction was examined in a Beckman spectrophotometer for absorption at 260 millimicrons. A 1.58-milligram portion of the virus sample was washed with cold two per cent perchloric acid, then treated overnight at 4°C with ten per cent perchloric acid. With the perchloric acid method extractions were made three times. A bovine serum albumin control for each method was treated in the same manner. Results of the sodium chloride and perchloric acid extractions are compared in Table V. Hot sodium chloride extraction did not remove RNA as rapidly as cold perchloric acid extraction, and in four extractions gave a total of 6.07 per cent RNA. The first extraction with ten per cent perchloric acid yielded 5.7 per cent RNA, with the second and third extractions contributing an additional 0.8 per cent for a total of 6.5 per cent. Since a significant amount of RNA was released by the fourth sodium chloride extraction, further extraction with sodium chloride might have raised the value for total RNA content by this method closer to that given by the perchloric acid method. In any case, results obtained with either method were not significantly different.

TABLE V. COMPARISON OF TWO EXTRACTION METHODS FOR THE DETERMINATION OF NUCLEIC ACID CONTENT OF VEE VIRUS

Treatment	Percentage RNA Extracted				
	First Extraction	Second Extraction	Third Extraction	Fourth Extraction	Total
Hot NaCl (10%)	3.4	1.18	1.0	0.49	6.07
Cold $HClO_4$ (10%)	5.7	0.65	0.19	-	6.54

On the basis of a single comparison, the lipid content of EEE virus purified by gradient centrifugation is lower than that of EEE virus purified without gradient centrifugation. The total lipid content of a small sample of EEE virus purified by sucrose gradient centrifugation was 36.0 per cent; that of a preparation purified by the method of Taylor et al[1] was 53.1 per cent.

IV. DISCUSSION

Preparations of VEE virus purified by sucrose gradient centrifugation were characterized as having a much lower lipid content than preparations purified without gradient centrifugation. The specific infectivity (LD_{50} per gram of protein), however, remained the same with both methods of purification.

The formation of a virus band localized in the same relative position in many individual density gradient trials suggests that the virus particles that concentrate at this level represent particles with the minimal amount of lipid, i.e., particles from which loosely bound lipids have been removed and that contain only lipids that are an integral part of the viral structure.

Sindbis virus, which like VEE virus is a member of the group A arboviruses, has been reported by Pfefferkorn and Hunter[13] to have a total lipid content quite similar to that of VEE virus. Sindbis virus, derived from the extra-cellular fluid of chick fibroblast monolayers, was purified by a procedure based on adsorption to aluminum phosphate gel and differential centrifugation. Work is in progress to determine the lipid content of VEE virus produced in a tissue culture system and the effect of gradient centrifugation on the lipid content of this type of virus.

Electron microscopic evidence of an average diameter of 68 millimicrons for VEE virus purified by the methods outlined in this report agrees closely with the range of 65 to 75 millimicrons reported by Mussgay and Weibel[14] for VEE virus purified by treatment with protamine sulfate and differential centrifugation. The 80-millimicron diameter reported by these authors for virus purified by an alternate method, and their observation of a 45-millimicron core for VEE virus, might be related to inactivation associated with dialysis against demineralized water or resuspension of purified virus in demineralized water preparatory to electron microscopic examination. A diameter of 30 millimicrons for the core of VEE virus, as indicated in this paper, suggests a similarity to the 30-millimicron core reported by Morgan et al[18] for WEE virus as seen in thin sections.

From the results we have obtained with VEE virus, it seems likely that the application of gradient centrifugation to the purification of EEE and WEE viruses will yield virus of lower lipid content than the 54 per cent originally reported. Our initial data with EEE virus support this hypothesis.

Studies to compare the lipid contents of VEE virus propagated in two hosts, suckling mouse brain and the chick embryo, indicate a similar, if not identical, quantity of lipid present in both that corresponds to the low value reported in this paper. This tends to strengthen the hypothesis that lipid remaining in the VEE virus particle subsequent to gradient centrifugation represents lipid that is an integral part of the viral structure.

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