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PURIFICATION AND PHYSICO-CHEMICAL CHARACTERIZATION OF ECBOVIRUS TYPE SA-I

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INTRODUCTION

In the accompanying paper (Verwoerd, Oellermann, Broekman & Weiss, 1967) the serological properties and growth characteristics of ecbovirus SA-I have been described.

In the present paper studies on the purification, crystallization, stability and physico-chemical characteristics of the virus are reported. The results indicate that the virus, though serologically unrelated, is practically identical to poliovirus and other enteroviruses as far as morphology and physico-chemical properties are concerned.

MATERIAL AND METHODS

Virus production

The growth characteristics of ecbovirus SA-I are described in the accompanying paper (Verwoerd *et al.*, 1967). Bulk cultivation of virus was carried out in monolayers of BHK-21 cells in Roux-flasks, using Eagle's medium plus 10 per cent tryptose phosphate broth (TPB) without serum. The virus suspension consisting of lysed cells and culture fluid was harvested 18 to 24 hours after infection and centrifuged to remove cell debris.

Concentration of virus by co-precipitation with yeast-RNA

The cell lysate was cooled to 0° C and maintained at this temperature during the course of precipitation. Commercial yeast-RNA, dissolved in dilute NaOH, was added to a concentration of 200 mg per litre of lysate. The pH was then adjusted to 3·0 by the addition of N/1 HCl, with rapid stirring. After standing for at least 30 minutes at 4° C, the precipitate was sedimented by centrifugation at 3000 × g for 30 minutes.

Enzyme treatment

The acid precipitate was rapidly dissolved in 0·2 M sodium-pyrophosphate in veronal buffer containing 0·2 mg trypsin per ml, and incubated for 30 minutes at 37° C. Crystalline pancreatic RNase (5 × crystallized, Light & Co.) was added to a final concentration of 10 μgm/ml and incubated for a further period of 30 minutes at 37° C. Insoluble material was removed by low speed centrifugation.

Sedimentation

Low speed centrifugation for clarifying virus suspensions was performed in a refrigerated centrifuge at 2500 × g for 10 to 30 minutes. For preparative ultra-sedimentation the virus was centrifuged for three hours at 39,000 rpm (average 100,000 × g) in the 40 rotor of a Spinco Model L ultra-centrifuge.

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Isopycnic density gradient centrifugation in CsCl was carried out according to the method of Hausen & Schäfer (1962). To 3 ml virus suspension 1·73 ml of a saturated solution of CsCl in water was added. The mixture, with a refractive index of 1·3670 at 20° C as measured in an Abbé-refractometer, was overlayered with liquid paraffin and centrifuged for 18 hours at 39,000 rpm in the SW-39 rotor. Fractions of 5 to 10 drops were then collected after puncturing the bottom of the centrifuge tube. The CsCl was removed by dialysis against 0·1 M phosphate buffer.

For analytical purposes a Spinco Model E ultra-centrifuge was used.

Purification with fluorocarbon

The method of Hausen & Schäfer (1962) was followed employing three cycles of extraction in an ice bath with equal volumes of chilled virus suspension and Frigen 113 (Hoechst, 1,2,2—trichloro-trifluoro-ethane) in a Dounce-homogenizer. The emulsion was centrifuged each time to speed up separation.

Protein determination

The colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) was used.

Isolation of infectious RNA

RNA was extracted from infected cells with phenol according to the method of Moscarello (1965) with some minor modifications. Monolayers of L-strain fibroblasts in 12 to 15 Roux-flasks were infected with ecbovirus at an input multiplicity of about 20 pfu per cell. Seven hours after infection the cells were harvested with trypsin, washed twice with PBS and the centrifuged cell pellet stored at −20° C. For extraction, the pellet was thawed in 0·8 ml of a 0·15 M NaCl solution containing 10^{-2} M EDTA and 5 per cent sodium desoxycholate. The cells were disrupted by homogenizing in a Dounce-homogenizer and then extracted three times with equivalent amounts of 80 per cent phenol in the homogenizer. Excess phenol was removed by five extractions with ether, and ether was removed by bubbling nitrogen through the solution. The whole procedure was carried out at 4° C. The RNA was further purified by precipitation with an excess of ice-cold ethanol.

Assay of infectious RNA

Monolayers of L-strain fibroblasts were prepared by seeding 60 mm petri dishes 24 hours before use with 5 ml cell suspension at a concentration of 6×10^5/ml. Before use the plates were washed twice with Ca^{++} and Mg^{++}-free PBS.

Indicator cells were harvested from Roux-flasks by trypsinization and washing in the usual manner. After standing for one hour at 42° C at a concentration of $1{\cdot}2 \times 10^6$/ml the cells were centrifuged and resuspended at a concentration of 5 to 6×10^7/ml in Eagle's medium without serum. Fivefold dilutions of RNA were made in PBS. To 0·2 ml of indicator cell suspension 0·1 ml of RNA dilutions plus 0·1 ml of a 10^{-2} M EDTA and 0·05 ml of a 0·25 per cent kaolin suspension was added. This mixture was incubated for 10 minutes at 37° C and 0·1 ml added per plate. After standing for one hour to allow the indicator cells to settle down, the medium was removed carefully and replaced by 5 ml of 0·6 per cent agarose in Eagle's minimal medium. Plaques were counted on the third day in the usual way after staining with neutral red.

Electron microscopy

Specimen grids were prepared according to the method described by Huxley & Zubay (1961), except that perforated films were prepared from a 0·1 per cent solution of formvar in 1,2-dichlorethane. The formvar was not dissolved after a thin carbon layer had been evaporated onto the coated grids. Simultaneously with the carbon coating on the glass slide containing the formvar-coated grids, a thin layer of carbon was also deposited on a glass slide containing a perforated formvar film prepared from a 0·05 per cent solution. This second film was floated off on a water surface and lifted onto the array of grids which thus contained two perforated films of different thickness.

A variation of the negative staining technique of De-Thé & O'Connor (1966) was used. A drop of 0·4 per cent potassium phosphotungstate (PTA) solution at pH 7·0 was put into a hollow porcelain block and a small drop of virus suspension was gently placed on the PTA surface. About two to three grids were then floated on the drop for about 10 seconds, with the carbon in contact with the PTA-virus mixture. The grids were drained and rinsed twice in distilled water.

Electron microscopy was carried out in a Sienens Elmiscope IA electron microscope, operating at 80 kv with the double condenser system and using a combination of 200 μ and 20 μ apertures. Contamination of the object was minimized by utilizing a liquid nitrogen-cooled anti-decontamination device. The grids were inserted in the electron microscope with the specimen side away from the electron source. Photographic enlargements of the electron microscope plates were made by inserting them in the enlarger with the emulsion side away from the bromide paper.

RESULTS

Purification

Purification of ecbovirus SA-I was carried out by the procedure summarized below and which was developed from methods described by different authors for the purification of other enteroviruses.

Summary of purification procedure

I *Cell lysate*
1. Removal of cell debris by low speed centrifugation
2. Co-precipitation with yeast RNA at pH 3·0
3. Treatment with pyrophosphate, trypsin and RNase

II *Crude concentrate*
1. Clarification by low speed centrifugation
2. Ultra-sedimentation

III *Pellet*
1. CsCl-equilibrium density gradient centrifugation
2. Dialysis

IV *Purified Virus*

The first step consisted of concentration of the virus by acid precipitation from cell lysates after sedimentation of cell debris by low speed centrifugation. A better recovery was obtained when cell debris was not removed, but the latter interfered

with subsequent steps in the purification procedure. Other means of improving precipitation were, therefore, investigated. The best results were obtained by co-precipitation of the virus with added yeast nucleic acid as recommended by Charney, Machlowitz, Tytell, Sagin & Spicer (1961). Preceipitation with methanol and protamine sulphate according to Kaighn, Moscarello & Fuerst (1964) was also successful but gave less reproducible results.

Acid precipitates were dissolved in 0·2 molar sodium pyrophosphate containing 0·2 mg of trypsin per ml. According to Kaighn *et al.* (1964) pyrophosphate is very effective in the fractionation and removal of contaminating host protein and nucleic acid from the virus, and, therefore, enhances subsequent digestion of these materials. It also serves as an ideal solvent for the acid precipitate, immediately adjusting the pH to a physiological level.

After the enzyme treatment described under methods, the clarified crude concentrate virus was sedimented by ultra-centrifugation. The pellet was resuspended in phosphate buffer, and undissolved solids again sedimented by low speed centrifugation. Resuspension of the pellet was facilitated by adding trypsin to the buffer.

Final purification was attained by either three fluoro-carbon extractions or by equilibrium density gradient centrifugation in CsCl. The two methods gave comparable degrees of purification, but the yields were more consistent with CsCl centrifugation. Virus was collected from the fractions with a refractive index between 1·3655 and 1·3670, corresponding to a density of 1·348 at 4° C, when using the latter method. A second band of lower density but also absorbing strongly at 260 mμ was usually found. According to several authors this band corresponds to incomplete or " empty " virus particles (Frommhagen, 1965).

The results of a typical purification experiment are summarized in Table 1. Reproducible yields of highly purified virus in milligram quantities were obtained by the simple procedure outlined above.

TABLE 1.—*Purification of ecbovirus SA-I*

Fraction	Volume	pfu/ml	Total pfu	% Recovery	mg prot./ml	Pfu/mg prot.	Purification
I Lysate....	1500 ml	$1·4\times10^{8}$	$1·96\times10^{11}$	—	3·36	$3·90\times10^{7}$	—
II Concentrate...	40 ml	$4·5\times10^{9}$	$1·8\times10^{11}$	92	8·90	$5·10\times10^{8}$	13×
III Pellet.....	3·0 ml	$5·7\times10^{10}$	$1·7\times10^{11}$	87	5·49	$1·14\times10^{10}$	286×
IV Purified virus...	1·0 ml	$1·13\times10^{11}$	$1·13\times10^{11}$	60	0·99	$1·10\times10^{11}$	2860×

Criteria of purity

The final product gave a single boundary in the analytical centrifuge (Plate 1) and was essentially free of contaminating materials when examined in the electron microscope (Plate 3). Furthermore the amount of virus in the above preparation calculated from the protein content corresponds closely to the amount calculated from absorption data. Taking 30 per cent as the mean protein content of enteroviruses, 0·99 mg protein is equivalent to 1·41 mg virus. The absorbance at 260 mμ (A_{260}) of the purified virus diluted 1: 9 in 0·1 M NaCl was found to be 1·03. This

corresponds to a 0·015 per cent virus suspension utilizing the A_{260} value of 67 reported for a 1 per cent poliovirus suspension (Schwert & Schaffer, 1955). Nine ml of suspension at this concentration, therefore, contains 1·35 mg of pure virus.

Crystallization

Dialysis of the purified virus preparation against distilled water for three to four days leads to the deposition of small crystals, just visible to the naked eye. The crystals were sedimented by low speed centrifugation and washed once with distilled water. Infectivity was mainly found in the sediment. Examination of the crystalline sediment and the first supernatant in the electron microscope demonstrated clearly that almost all the virus in the original preparation was present in the former fraction, clearly in regular crystalline array (Plate 3).

Chemical composition

The first indication that the viral nucleic acid is indeed RNA, was derived from the histochemical investigation of the intracellular site of virus replication, reported in the previous paper (Verwoerd *et al.*, 1967). Cytoplasmic accumulation of material with acridine orange and Schiff methylene blue staining reactions similar to RNA, but refractory to Feulgen and fluorescent Feulgen staining was observed.

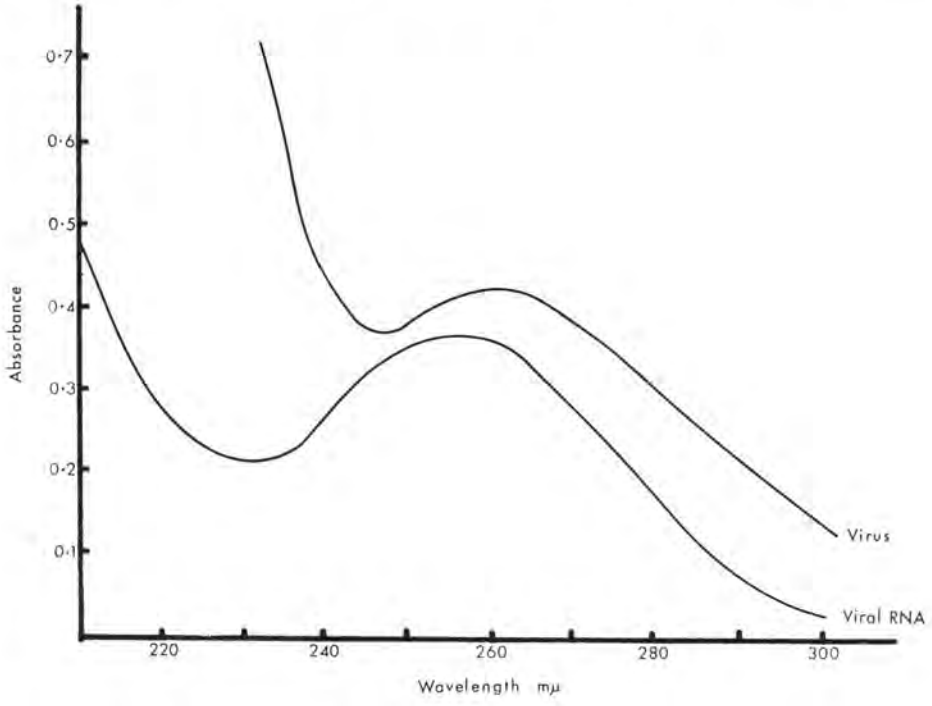

FIG. 1.—Ultra-violet absorption-spectrum of purified ecbovirus type SA-1 in 0·1 M NaCl

The ultra-violet spectrum of the purified virus, together with that of its RNA, can be seen in Fig. 1. According to the method of Warburg & Christian (1942), it can be estimated from the A_{260}/A_{280} and A_{260}/A_{272} ratios that the virus contains approximately 30 per cent protein and 70 per cent nucleic acid. All attempts to isolate infectious RNA from purified virus preparations failed. Biologically active RNA was subsequently isolated by the method of Moscarello *et al.* (1965) from infected cells at a stage just before the first completed virus could be detected. Infectivity was determined by plaque assay described under methods. Destruction of all infectivity by incubation with pancreatic RNase proved both the nature of the nucleic acid and the absence of intact virus in the RNA extract.

Inactivation Studies

The stability of the virus under different circumstances is summarized in Table 2. For these tests the virus was produced in ascites cells and used as an unpurified suspension in Earle's saline.

At 4° C, about 10 per cent of the virus particles were still viable after eight months.

The virus was inactivated by heat but not by ether. Partial stabilization against the effect of heat was afforded by the presence of magnesium ions. Considerable loss in viable virus occurs during freeze-drying in buffered-lactose-peptone. (Final concentration: 10 per cent lactose, 2 per cent peptone in 0·86 M phosphate buffer.) The virus is not very resistant to freeze-drying.

These characteristics are all in close agreement to those described for other enteroviruses (Andrewes, 1964).

TABLE 2.—*Stability of Ecbovirus Type SA-I*

Treatment	Titre before treatment (pfu/ml)	Titre after treatment (pfu/ml)
18 hours in 20% ether at 4° C	$1·9\times10^{9}$	$1·0\times10^{9}$
2 hours at 50° C	$1·9\times10^{9}$	$2·0\times10^{5}$
2 hours at 50° C in I M $MgCl_2$	$1·9\times10^{9}$	$2·0\times10^{7}$
Freeze-drying	$1·9\times10^{9}$	$1·5\times10^{7}$
1 day at 4° C	$2·8\times10^{8}$	$2·8\times10^{8}$
7 days at 4° C	$2·8\times10^{8}$	$2·5\times10^{8}$
40 days at 4° C	$2·8\times10^{8}$	$0·75\times10^{8}$
80 days at 4° C	$2·8\times10^{8}$	$0·36\times10^{8}$
250 days at 4° C	$2·8\times10^{8}$	$0·30\times10^{8}$

Physico-chemical characteristics

(*a*) *Sedimentation data*

Sedimentation velocity measurements were performed in a Spinco Model E analytical ultra-centrifuge with ultra-violet optics. Highly purified virus at different concentrations in 0·1 M NaCl was sedimented at 12–20,000 rpm. In Plate 1 one of the UV-absorption photographs is shown to demonstrate the sharp boundary formed. A mean sedimentation constant of 152 Svedberg units at 20° C was found. This value is not dependent on virus concentration, indicating that the particles are spherical in shape.

PLATE 1.—Sedimentation pattern of ecbovirus type SA-I in an ultra-centrifuge with UV-absorption optics. The solvent is 0·1 M NaCl, $A_{254} = 0{\cdot}57$, speed: 19,160 rpm and interval between photographs: 4 minutes

Density gradient centrifugation of the RNA extracted from infected cells was carried out in a linear sucrose gradient of 5 to 20 per cent at a pH of 4·8 (Pons, 1964). Of the 0·2 ml fractions collected after completion of the run, 0·1 ml was diluted with distilled water for determination of the absorption at 260 mμ, the rest being used for determining infectivity by the plaque assay method previously described. Five absorption peaks were usually observed (Fig. 2), three of which corresponded to the normal nucleic acid components of the cell with sedimentation constants of 4 to 6S, 16 to 18S and 28 to 30S respectively. Two smaller peaks, one of which was absent in some experiments, preceded the 28S peak.

The infectivity seemed to be associated mainly with the small peak constantly present, with a sedimentation constant of approximately 35 to 37S, though some spreading usually occurred, possibly due to absorption effects. A minor infectivity peak, almost overlapping the 18S ribosomal fraction, was constantly present. The infectivity of this fraction was relatively insensitive to incubation with 0·1 mg pancreatic RNase for 30 minutes, in contrast to the 35S fraction which was completely inactivated by this treatment. Both RNase resistance and the sedimentation constant suggest that the 18S infectivity peak represents the double stranded replicative form described by several authors for other enteroviruses (Montagnier & Sanders, 1963; Baltimore, Becker & Darnell, 1964; Dalgarno, Martin, Liu & Work, 1966; Hausen, 1965).

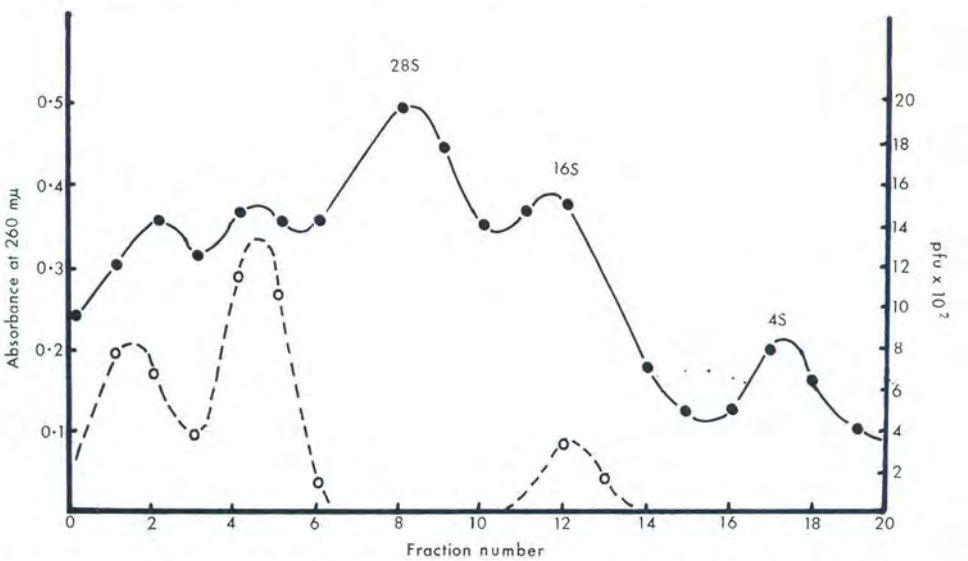

FIG. 2.—Sucrose density-gradient sedimentation pattern of RNA extracted from ecbovirus-infected L-cells. Curve 1 (unbroken line): UV-absorbance. Curve 2 (broken line): infectivity

PLATE 2.—Gel diffusion pattern in agarose with ecbovirus SA-I as antigen and homologous antiserum in slots at right angles to each other

(*b*) *Diffusion constant*

An estimation of the diffusion constant of the virus was made by the gel-diffusion technique of Allison & Humphrey (1960). Highly purified virus was used as antigen and allowed to diffuse against hyperimmune monospecific serum prepared in a rabbit against the purified virus. Antigen and antiserum were contained in slots at right angles to each other in a 0·5 per cent agarose gel in 0·02 M phosphate buffer at pH 7·2. The diffusion constant is calculated from the angle between the precipitation line and the slots (Plate 2). Reproducible results could not be obtained by this method, and values for the diffusion constant of the virus ranging from $1 \cdot 2$ to $2 \cdot 4 \times 10^{-7}$ cm^2 sec^{-1} were found. The mean value of $1 \cdot 7 \times 10^{-7}$, however, corresponds closely to that ($1 \cdot 8 \times 10$-^{7}) derived by means of ultra-centrifugation for ME-virus (Hausen & Schäfer, 1962).

(*c*) *Particle weight*

From the sedimentation and diffusion constants obtained, the particle weight of the virus was calculated using the partial specific volume for poliovirus of 0·639 cm^3 as determined by Schwert & Schaffer (1955). A value of $6 \cdot 1 \times 10^6$ daltons was arrived at.

(*d*) *Morphology*

The morphology of ecbovirus SA-I was studied by electron microscopy of highly purified crystalline preparations using the negative staining technique. Measurements of a large number of particles from different electron-micrographs indicated a mean particle diameter of 23 mμ.

The substructure seen in a few particles was very similar to that found in other enteroviruses (Horne & Nagington, 1959; Hausen & Schäfer, 1962; Mahnelt & Pette, 1965). The protein coat seemed to consist of 32 hollow capsomeres, arranged in a 5: 3: 2 symmetry (Caspar & Klug, 1962). A small proportion of the particles in all preparations were " empty ", that is, contained no inner core of nucleic acid. Due to penetration of the staining material, these particles had a typical ringlike structure in negatively stained preparations.

Discussion and Summary

Ecbovirus type SA-I, a bovine enterovirus, has been purified and crystallized and its chemical composition, physical characteristics and morphology studied. The virus particles have a mean diameter of 230Å, a sedimentation constant of 152S, a diffusion constant of approximately $1 \cdot 7 \times 10^{-7}$ cm^2 sec^{-1} and a particle weight of about $6 \cdot 1 \times 10^6$ daltons. Its capsid consists of a number of identical subunits, probably 32, arranged in icosahedral symmetry and enclosing the nucleic acid component which comprises about 30 per cent of the total weight.

Infective ribonucleic acid could only be isolated from infected cells. It has a sedimentation constant of approximately 37S corresponding to a molecular weight of about 2×10^6, and consists of single-stranded RNA. The presence of a small amount of infective double-stranded RNA could also be demonstrated.

In its physical characteristics and appearance, therefore, the virus is practically indistinguishable from polio- and other enteroviruses. The small differences in size found by different authors for various enteroviruses are probably insignificant and due to experimental error.

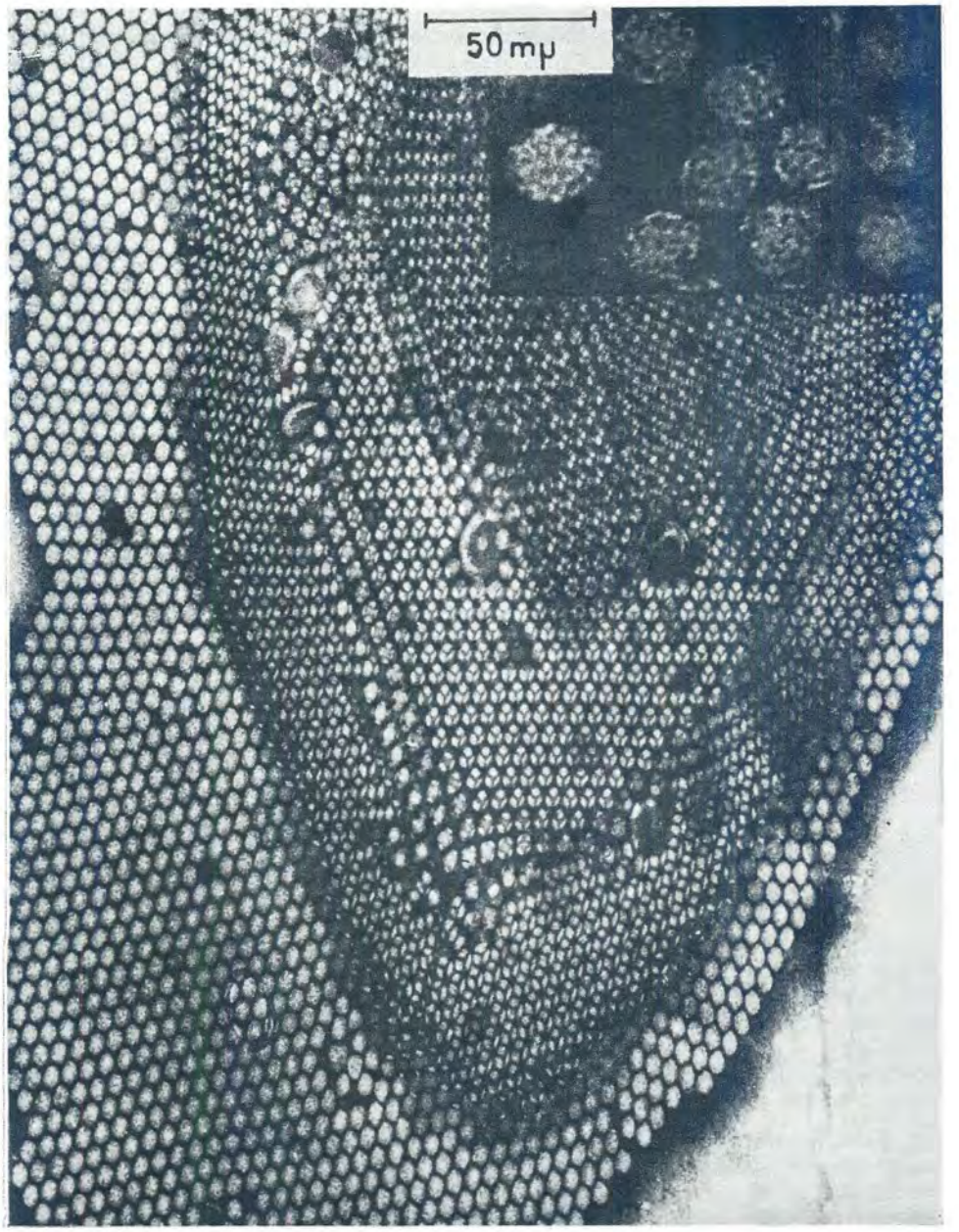

PLATE 3.—Electron micrograph of crystalline ecbovirus type SA-I, negatively stained with PTA (Final magnification × 130,000)
Inset: Higher magnification of virus particles. (Final magnification × 430,000)

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