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# PURIFICATION STUDIES ON THE INFECTIOUS CANINE HEPATITIS VIRUS

## II. VIRAL PARTICLES AND THEIR CHARACTERISTICS REVEALED BY MODIFICATION OF THE PURIFICATION PROCEDURE

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### INTRODUCTION

A previous publication<sup>6)</sup> has described one method of purification of the ICH virus from infectious tissue culture fluid for electron microscopy, and demonstrated that these particles are an average 85 m $\mu$  (non-shadow), 96 m $\mu$  (Pd-shadow) in diameter. The purpose of the present communication is to report with respect to the fine structure of virus particles revealed by a slightly altered procedure in comparison with the previously reported one and electron stain or formalin treatment of purified virus particle forms extracted from infectious tissue culture fluid and cells, and furthermore the several characteristics of purified virus.

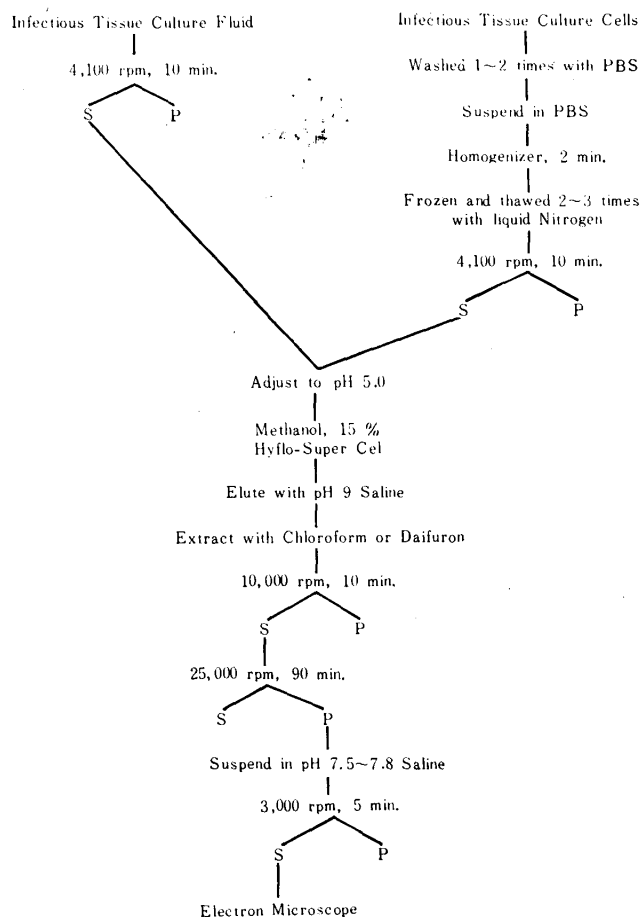
### MATERIALS AND METHODS

**Virus:** The "MATSUDA" strain of ICH virus was used throughout. It had been transferred serially 5 to 10 times in monolayer cultures of dog kidney epithelial cells before the yields were subjected to purification.

**Virus purification:** The procedure for the purification and concentration of the virus is in part a modification of the methods used in the purification of poliomyelitis and foot-and-mouth disease virus as was described in the previous report. That is to say, the extraction with organic solvent was performed using chloroform or Daifuron-S3 (Osaka Metal Industry Co.) and the concentration of virus was accomplished by means of differential centrifugation for 10 min. at 10,000 rpm, 90 min. at 25,000 rpm in a rotor No. 40 of the Hitachi Model-40P ultracentrifuge as is outlined diagrammatically in table 1.

**Electron staining method:** Electron staining procedure based on that of BRENNER & HORN (1959) was used. To 1.0 ml of virus suspension in 4% ammonium acetate was added 1.0 ml of 1 to 2% solution of phosphotungstic acid or 1% sodium phosphotungstate. The solutions were then sprayed directly onto the carbon-coated collodion membrane grids with a Vaponefrin atomiser spray gun.

**Formalin treatment:** The final purified virus (pellets of the centrifugation for 90 min.

TABLE 1. *Purification of ICH Virus*

at 25,000 rpm) was resuspended in 2% neutral formalin saline and was prepared for electron microscopy by the agar pseudo-replica method.

**Electron microscopy:** The specimens were examined in a JEM-4CHD electron microscope at instrumental magnifications of  $\times 4,500 \sim \times 5,000$  to  $\times 10,000$  using single condenser illumination.

**Complement fixation test:** The technique used was essentially identical with that of the modification of KOLMER's method. For the determination of antigen titers, two exact units of complement (0.5 ml) and 0.25 ml of hyperimmune dog serum (4 antibody units) were added to the serial dilution of the antigen to be tested. Tubes containing the above mixtures were stored at 4°C for 16 to 18 hrs. and 0.5 ml of hemolytic system was added the next day.

The tests were read after incubation in a water bath of 37°C for 30 min.

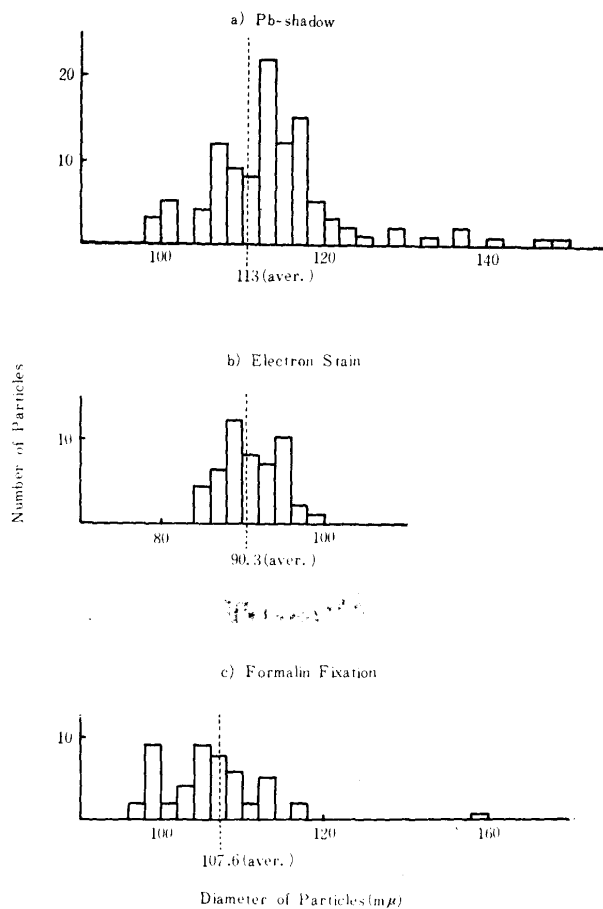
**Hemagglutination test:** Hemagglutination titrations were carried out in the manner described by SHIMIZU et al. (1960).

**Ultraviolet absorption:** The ultraviolet absorption spectrum of the virus suspension before and after purification procedure was estimated with a Hitachi-EPU-2 spectrophotometer.

RESULTS AND DISCUSSION

1. **Electron Micrograph** Electron micrographs of agar pseudo-replica of the virus particles purified from infectious tissue culture fluid and cells revealed a uniform-sized, spherical particle 113 m $\mu$  (Pd-shadow) in diameter (Figs. 1 a, 3).

FIGURE 1. *Distribution of Diameter of the Virus Particle in Purified Sample*



The flattened particles containing a central protuberance revealed in a previous purification procedure were not observed in this purified sample at all. In the purified virus suspension derived from cells 24 hrs. after infection there were revealed a small number of intact virus particles, smaller amorphous particles and their aggregates (Fig. 4).

With the lapse of time after infection these intact virus particles derived from cells increased gradually in number.

By 48 hrs. the number of these intact virus particles had increased slightly, but the greater part of the particles showed flattened and amorphous feature (Fig. 5). At 72 to 96 hrs. after infection there could be observed only the same particles as revealed in infectious tissue culture

fluid (Fig. 6). Particles of this kind have never been observed from non-infected cells (Fig. 7). Figures 8 and 9 are micrographs of ICH virus stained with sodium phosphotungstate. They are classified into three kinds of particles, viz., empty shells (a), uniform less dense particles (b) and particles including a dense core of various sizes (c).

It appears that each particle is composed of several subunits (Fig. 10).

Figures 11 and 12 are micrographs of the virus stained with phosphotungstic acid. It was noted that the outside of the particles is markedly surrounded by a halo structure. In general, the profile of particles was rendered clearer as a result of treatment of phosphotungstic acid compared with that of sodium phosphotungstate but the latter is superior to the former in spreading out the sample. Particle-size is within the range of 86 to 100  $m\mu$  diameter (average 90.3  $m\mu$ ) (Fig. 1 b). The virus particles when fixed with 2% neutral formalin have a clearly hexagonal or pentagonal image and the diameter of the particles varies from 98 to 160  $m\mu$  (average 107.6  $m\mu$ ) (Figs. 1 c, 13).

As regards the polyhedral shape, such virus particles were reported for the such a plant virus turnip yellow mosaic<sup>5)</sup> and for an insect virus<sup>12)</sup>.

As for the animal virus, VALENTINE & HOPPER (1957) reported that adenovirus particles exhibited spherical image when fixed with osmium tetroxide and metal-shadowed but had a clearly hexagonal image as a result of treating of osmium tetroxide-fixed virus particles further with 1 per cent phosphotungstic acid or by making carbon replicas of the surface of formalin-fixed particles. They therefore assume that the adenovirus particles have a symmetrical polyhedral form. Accordingly, it may be seen that ICH virus particles have a rhombic dodecahedral or icosahedral form. However, the need of further studies may be felt as regards the above matter. REAGAN et al. (1953) reported that the size of virus particles derived from fox encephalitis virus-bearing dog liver had a diameter in the 80 to 85  $m\mu$  range. From the present experiments the particle-size showed various values depending upon the treatment of sample as is shown in table 2.

TABLE 2. *Diameter of the ICH Virus Particles* ( $m\mu$ )

NON-SHADOW	ELECTRON STAIN	FORMALIN *	
		Unfixed	Fixed
87.9	90.3	113	107.6

\* Pd-shadow

At any rate the size of ICH virus particles may be grossly estimated as  $100 \pm 10 m\mu$ .

## 2. Characteristics of Purified Virus

1) **Cytopathogenic effect:** Cytopathogenic effect of purified virus upon dog kidney epithelial cell sheets was entirely the same as that of crude virus suspension.

The inclusion body was observed in nucleus (Figs. 14~17).

2) **Complement fixing and hemagglutinating activity:** In the process of methanol precipitation and Daifuron extraction the complement fixing and hemagglutinating titers increased in parallel with viral infectivity. The infectious agents were sedimented by centrifu-

gation at 25,000 rpm for 90 min. and the infectivity of supernate became very low but complement fixing titer of still remained high (Table 3).

TABLE 3. *Infectivity and CF and HA Titer of Each Fraction Resultant from Purification Procedure*

FRACTIONS	EXP. 1			EXP. 2		
	TCD <sub>50</sub>	CFT	HAT	TCD <sub>50</sub>	CFT	HAT
Original TCF	4.5	128	256	3.7	32	128
Soluble part of Methanol ppt.	5.5	512	2048	4.5	128	256
Aqueous phase after extraction with Daifuron	5.5	512	1024	3.5	64	256
Supernate	< 1.0	256	1024	< 1.0	64	32
25,000 rpm 90 min.						
Pellet	3.5	256	128	5.5	32	2048

Treatment of purified virus suspension with ether resulted in complete or almost complete loss of infectivity. However, such treatment did not decrease the complement fixing and hemagglutinating titers but occasionally caused their increase over the corresponding values of original suspension (Table 4).

TABLE 4. *Infectivity and CF and HA Titer of Purified Virus before and after Treatment with Ethyl Ether*

ETHER TREATMENT	EXP. 1			EXP. 2			EXP. 3		
	TCD <sub>50</sub>	CFT	HAT	TCD <sub>50</sub>	CFT	HAT	TCD <sub>50</sub>	CFT	HAT
Before	3.5	256	128	6.0	128	512	5.5	512	1024
After	< 1.0	256	64	2.0	128	1024	< 1.0	2048	2048

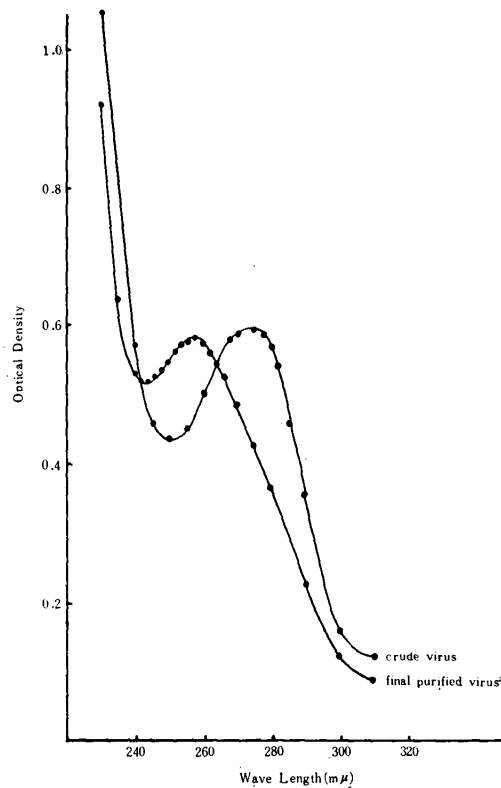
Therefore, it is assumed that there is a soluble antigen in ICH virus as suggested by HIRATO et al. (1960). However, the present authors can not support the opinion of TAKATORI (1960) that the virus particles are as inactive as complement fixing antigen. In this experiment, purified infectious virus particles keep their complement fixing and hemagglutinating activities. HOYLE (1952) has speculated on the basis of his results with ether treatment of virus suspensions that elementary body of influenza virus has a lipid membrane envelope which contains S components and small hemagglutinating particles. Ether dissolves the membrane, releasing S antigens and hemagglutinins. The present authors released S component and hemagglutinin with ether treatment of purified ICH virus using the method of HOYLE (1952). However, if the time of ether treatment is too long continued, complement fixing and hemagglutinating titers decrease in parallel with infectivity. Regarding such fact, MIZUTANI (1958) reported a similar phenomenon in influenza virus. HENLE & WIENER (1944) estimated the particle-size

of the soluble antigen of influenza virus at 100~150 Å whilst HOYLE (1952) showed that the soluble antigen derived from the elementary body by disintegration with ether had a particle-size of 120 Å.

In this experiment, the present authors could not observe by electron microscopy the size of S component of ICH virus.

3) Ultraviolet absorption spectrum: The ultraviolet absorption spectrum of the suspension of ICH virus particles before and after purification procedure was as graphed in figure 2.

FIGURE 2. *Ultraviolet Absorption Spectrum of Crude and Purified ICH Virus Suspension*



The ultraviolet spectrum of crude virus suspension before purification is a maximum at 275 mμ and a minimum at 250 mμ. However, the spectrum of purified virus particles is one characteristic of a nucleoprotein with a maximum at 258 mμ and a minimum at 240 mμ.

#### SUMMARY

The picture of the infectious canine hepatitis virus particle derived from infectious tissue culture fluid and cells has been clearly demonstrated by a modified purification procedure. It was able to observe the fine structure of the particle when the virus was stained with phosphotungstic acid or sodium phosphotungstate.

Fixing the purified virus particles with 2% neutral formalin resulted in the revelation of a clearly hexagonal or pentagonal image. Purified ICH virus particles keep infectivity and complement fixing and hemagglutinating activities and can be disintegrated by treatment with ether with the liberation of smaller particle, soluble antigen and hemagglutinin. The ultraviolet absorption spectrum of final purified virus particles shows a maximum at 258 m $\mu$  and a minimum at 240 m $\mu$ .

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## EXPLANATION OF PLATES

## PLATE I.

- Fig. 3. Electron micrograph of final purified ICH virus particles.  
Pd-shadow  $\times 50,000$

## PLATE II.

- Fig. 4. Purified sample derived from cells 24 hrs. after infection. Arrows indicate intact virus particles. Pd-shadow  $\times 22,500$

- Fig. 5. 48 hrs. after infection.  
Arrows indicate intact virus particles; most of the particles show flattened and amorphous features. Pd-shadow  $\times 22,500$

## PLATE III.

- Fig. 6. 72 hrs. after infection. Pd-shadow  $\times 22,500$

- Fig. 7. Purified sample derived from non-infected cells. Pd-shadow  $\times 22,500$

## PLATE IV. Purified virus particles stained with sodium phosphotungstate

- Fig. 8.  $\times 50,000$

- Fig. 9. a) empty shell b) uniform slightly dense particle  
c) particles including dense core of various sizes  $\times 100,000$

## PLATE V.

- Fig. 10. Virus particles stained with sodium phosphotungstate. Arrows indicate subunits.  $\times 140,000$

- Figs. 11, 12. Purified virus particles stained with phosphotungstic acid. Arrows indicate halo structure  $\times 140,000$

- Fig. 13. Purified virus particles fixed with 2% neutral formalin  
a) hexagonal form b) pentagonal form  $\times 50,000$

PLATE VI. Cytopathogenic effect of purified virus upon dog kidney epithelial cell sheets Giemsa stain  $\times 480$ 

- Fig. 14. Original infectious tissue culture fluid

- Fig. 15. Methanol precipitation fraction

- Fig. 16. Fraction after extraction with Daifuron

- Fig. 17. Final purified fraction

