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EFFECT OF SODIUM DESOXYCHOLATE ON INFECTIOUS CANINE HEPATITIS VIRUS, AND AN ADDITIONAL DESCRIPTION OF A SMALL ASSOCIATED VIRUS*1

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INTRODUCTION

Existence of a soluble complement fixing (CF) antigen in dog kidney cell cultures infected with infectious canine hepatitis virus (ICH virus) separate from the virus particle, was suggested by FASTIER (1957), SHIMIZU et al. (1960) and substantiated by HIRATO et al. (1960). Another soluble antigen of ICH virus, hemagglutinating (HA) antigen, was described by KUNISHIGE et al. (1961) and SHIMIZU (1964).

In adenoviruses, 3 soluble antigens are known. PEREIRA et al. (1959), using immunoelectrophoresis, first separated these soluble antigens of adenovirus type 5, into 1) group specific A antigen, 2) type specific C antigen, and 3) B antigen which causes the cytopathic effect. WILCOX & GINSBERG (1961) distinguished the soluble antigens of adenovirus (types 4 & 5) as L antigen, E antigen and toxin, by using diethylaminoethyl (DEAE) cellulose column chromatography, and described the L antigen, E antigen and toxin were identical respectively with the A, C and B antigens designated by PEREIRA et al. (1959).

Morphologically, these soluble antigens of adenovirus type 5 were confirmed to be the subunits of the virus particles. L (A) antigen was the hexagonal capsomere, major subunits of the capsid (WILCOX et al., 1963; VALENTINE & PEREIRA, 1965), toxin (B antigen) was the morphological unit at each of the 12 corners of the icosahedral virion and was the complex of pentagonal capsomere and a fiber (VALENTINE & PEREIRA, 1965), and E (C) antigen was the fiber structure, a part of the toxin (B antigen) (WILCOX et al., 1963; VALENTINE & PEREIRA, 1965).

In antigenic specificity, L (A) antigen was a group-specific complement fixing

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antigen, E (C) antigen was a type-specific complement fixing antigen (WILCOX & GINSBERG, 1961), and E (C) antigen with toxin (B antigen) showed hemagglutinating activity (VALENTINE & PEREIRA, 1965). NORRBY (1966) reported that the soluble HA antigen of adenovirus type 3 consisted of 12 capsomere-like structures associated respectively with a thin club-shaped projection and that the HA antigen was morphologically similar to the aggregate of 12 B antigens. Such a relationship of soluble antigens to the virus particle has not yet been elucidated in ICH virus.

Attempts were made by the authors first, to fractionate CF and HA antigens of ICH virus, and second, to disrupt the ICH virus. Disruption of ICH virus was planned to discover whether the soluble antigens are the structural components of ICH virus particles, as in the case of adenoviruses. Sodium desoxycholate (SDC) was used for disruption of the virus. It was noticeable that the titer of the CF and HA antigens changed remarkably after disrupting the virus with SDC. The results, with electron microscopical findings, are described in this paper. Existence of ICH-associated or satellite virus was also described.

MATERIALS AND METHODS

Virus The Matsuda strain of ICH virus was used throughout the experiment. It was isolated in this department in 1954 and preserved by serial transfers in monolayer cultures of dog kidney cells (DKC). In this experiment the 33rd～40th passages were used.

Tissue culture and virus inoculation Minced kidney cortex from puppies was trypsinized with 0.25% trypsin (NBC). Cells were washed twice with HANKS' balanced salt solution and finally resuspended in growth medium to obtain 10^6 cells per ml. The growth medium used was HANKS' balanced salt solution supplemented with 10% calf or horse serum and 0.5% lactoalbumin hydrolysate. The medium was added to 100 u/ml of penicillin and 100 γ/ml of streptomycin. The cells, suspended in the growth medium, were dispensed 10 ml per bottle (4×10×4 cm) and 0.5 ml per tube (12×120 mm). Cell sheets grown in bottles were used for the propagation of the virus. Each bottle was inoculated with 1 ml of the virus material, after 2 hrs, for the adsorption of virus, maintenance medium was added. The maintenance medium used was HANKS' balanced salt solution containing 1% calf or horse serum and 0.5% lactoalubmin hydrolysate.

Soluble antigens and the virus particle preparations The infected tissue culture fluids and cells were harvested after complete a cytopathic effect was observed. The fluid and cell mixture harvested was treated by sonic vibration, described later, and centrifuged at 3,000 rpm for 30 min. The supernatant was isolated and centrifuged at 8×10^4 g, for 90 min in an ultracentrifuge (model 40 p, Hitachi). The supernatant was used as the soluble antigens. The pellet was resuspended in 1/10 original volume of HANKS' balanced salt solution, and centrifuged again at 4×10^3 g for 30 min, and the supernatant was obtained as virus particle preparation.

Infectivity titration Ten-fold serial dilutions of virus materials were made with

HANKS' balanced salt solution. The infectivity was determined by inoculation 0.1 ml of each ten-fold dilution into sets of 4 tubes containing DKC. Following a reincubation of 7 days, the 50% tissue culture infective doses ($TCID_{50}$) were calculated utilizing the method of BEHRENS-KÄRBER.

Treatment with sodium desoxycholate Two per cent SDC in distilled water was added to the virus material of the same volume. The SDC treatment was performed in a 37°C water bath.

Sonic vibration The sonic osillator (model KMS-100, Kubota) was employed for sonic vibration. The disruption of cells was carried out at 100 volts and 7 kilocycles for 10 min.

Column chromatography Thoroughly washed DEAE cellulose was used to make a column 1 cm in diameter and 10 cm in height, equilibrated with 0.01 M phosphate buffer (pH 7.2) prior to the addition of the sample. After the sample was adsorbed, a program of step-wise elution, employing a solution of constant pH and increasing NaCl molarity, each in an amount equivalent to 10 times the volume of the original sample, was carried out. Eluates so obtained were tested for the CF and HA antigens.

Complement-fixation test The test was performed according to KOLMER's method. Dog sera were inactivated at 63°C for 15 min (HIRATO et al., 1960).

Hemagglutination test Hemagglutination test was performed as described by SHIMIZU et al. (1960). Human red blood cells (RBC) were washed 3 times with the HA medium, which was 0.06 M phosphate buffer (pH 7.5), containing 0.25 M dextrose and 0.02 M NaCl, and then suspended in the same solution to obtain 0.25% suspension.

Hemagglutination inhibition test The hemagglutination inhibition (HI) test was carried out in the manner described by SHIMIZU (1964). Dog sera usually showed non-specific inhibition in the virus hemagglutination and normal agglutination with human RBC. Accordingly, dog sera used in the experiment were treated with a receptor destroying enzyme (RDE, Takeda), heated at 65°C for 20 min in order to remove non-specific inhibition, adsorbed with human RBC then refrigerated overnight in order to avoid normal hemagglutination.

Agar gel diffusion precipitin test The agar gel diffusion technique employed was followed according to SCHRICKER & HANSON (1963) with a slight modification in that the agar gel was made in distilled water. All wells were cut 4.5 mm in diameter and the spacing between wells was 5 mm. Specific precipitates were visible within 24 to 48 hrs following incubation at room temperature.

Preparation of antisera Antiserum against both CF and HA antigens (serum D-20). A puppy, 1 month old, was infected intravenously with 3~5 ml of ICH virus 3 times at 7-day intervals. ICH virus used for the inoculation was the tissue culture fluid which contained 10^5 to 10^6 infectious virus per ml. A week after the last inoculation, CF and HI titers of the dog serum had reached to 1 : 128 and 1 : 1,024 respectively. At this time, the puppy was bled via cardiac puncture and the serum preserved at −20°C.

Anti-CF antigen serum (serum DS-1) CF antigen fractionated on DEAE cellulose column chromatograph was concentrated to 1/10 of the original volume by dialysis against

polyethylene glycol #4,000 (Nihon Rikagaku Yakuhin) and adsorbed with human RBC to remove the HA antigen contaminated with the CF antigen. A puppy was given an injection of the CF antigen with adjuvant intramuscularly. The adjuvant was a mixture of 1 volume of Aracel A (Atlas Chem. Indust.) and 4 volumes of Drakeol No. 6 (Pennsylvania Oil Co.). Two weeks later, the animal was intravenously injected with the CF antigen without the adjuvant twice at weekly intervals. The dog was bled 7 days after the last injection.

Elcetron microscopy The specimens were negatively stained with 1% phosphotungstic acid on the carbon-coated collodion membrane grid, and examined in a JEM-7 electron microscope at instrumental magnifications of × 50,000~80,000.

RESULTS

1 Analysis of the soluble antigens of ICH virus by the gel diffusion precepitin test

The soluble antigens (CF titer was 1 : 128, HA titer was 1 : 80, infectivity was not detectable in original concentration) concentrated by dialysis against polyethylene glycol to 1/10 volume, were used as the antigen for agar gel diffusion test. The concentrated soluble antigen (CF titer, 1028; HA titer, 320) was dialysed against HA medium, adsorbed with 10% human RBC at 4°C overnight. After centrifugation at 2,000 rpm for 10 min, the supernatant was used for precipitation as soluble CF antigens (CF titer, 512; HA titer, 5). Cells and fluids of non-infected dog kidney cell cultures were used as control and tested in the same manner.

Reactions of the soluble antigen, the soluble antigen adsorbed with human RBC and the control with serum D-20, antiserum against both CF and HA antigens (CF titer, 128; HI titer, 1024), are shown in figure 1. The soluble antigen formed two lines, one of them was identical with the line of the soluble antigen adsorbed with human RBC. Therefore, it is obvious that the line adsorbed with human RBC was the HA antigen reaction and the other was formed by CF antigen.

2 Separation of the soluble antigens of ICH virus on DEAE cellulose column chromatograph

The soluble antigens of ICH virus, were adsorbed with DEAE cellulose and were eluted by increasing the concentration of NaCl in 0.01 M phosphate buffer solution at pH 7.2 as described in Materials and methods. Complement-fixation and hemagglutination tests were undertaken with each eluate. Both dog immune serum (D-20) and anti-CF antigen serum (DS-1) were employed to detect the CF antigenicity of each eluate. The CF antigen was eluted with 0.05 M NaCl while HA antigen was eluted with 0.2 M NaCl, as shown in figure 2. It is noticeable, as shown in figure 3, that anti-CF antigen serum (DS-1) did not show a complement fixation reaction with the HA antigen which eluted maximally with 0.2 M NaCl. Thus it was indicated that the CF antigenicity shown by the HA antigen fraction differed from that of the CF antigen fraction.

3 The effect of sodium desoxycholate on CF, HA and infectivity titers of the virus particle

The virus suspension, in distilled water, was added to the SDC solution to give final

FIGURE 2 *Fractionation of ICH soluble antigens on DEAE cellulose column chromatograph—CF titer was measured with anti-virus particle serum D-20*

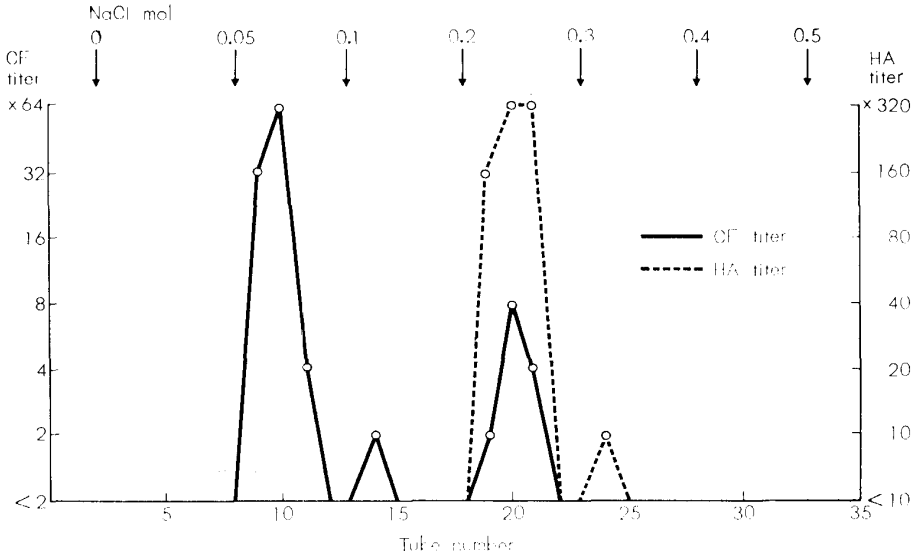
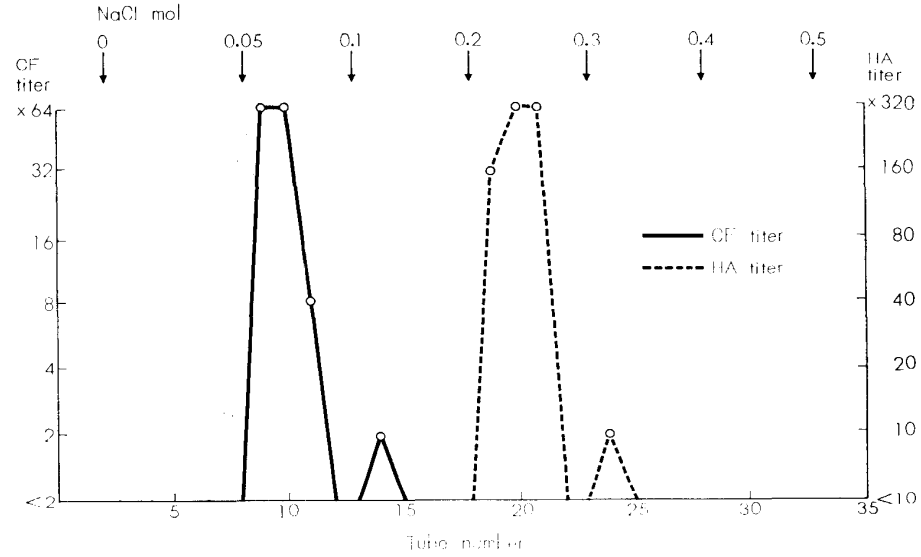


FIGURE 3 *Fractionation of ICH soluble antigens on DEAE cellulose column chromatograph—CF titer was measured by anti-CF antigen serum DS-1*



concentrations of 1.0, 0.5, 0.25, 0.125 and 0.0625%. CF, HA and infectivity titers were measured at 30, 60 and 120 min after the addition of SDC and are shown in table 1. CF titer was 1 : 16 in the control, but increased to 1 : 64 after treatment with 1% SDC for 30~120 min. After treatment with 0.5, 0.25 and 0.125% SDC for 60 or 120 min, CF titer increased to 1 : 32. On the contrary, HA titer, 1 : 320 in the control was decreased to

TABLE 1 *The CF, HA and infectivity titers of ICH virus after treatment with SDC*

SDC	SDC TREATMENT FOR								
	30 min			60 min			120 min		
	CF titer	HA titer	Infectivity*1 titer	CF titer	HA titer	Infectivity titer	CF titer	HA titer	Infectivity titer
%									
1.0	64*2	320	— *3	64	160	—	64	40	N. D.*4
0.5	32	320	—	32	160	—	32	160	N. D.
0.25	16	320	—	32	160	—	32	160	N. D.
0.125	16	320	2.75	16	160	2.50	32	160	N. D.
0.0625	16	320	$\geq$ 5.50	16	320	$\geq$ 5.50	16	320	N. D.
control (no SDC)	16	320	4.75	16	320	5.25	16	320	N. D.

*1 Expressed as $TCID_{50}$/ml
*2 CF and HA titers were expressed with reciprocals.
*3 The virus was not detectable at 1 : 100, 1 : 10 and 1 : 1 dilutions, due to cell degeneration caused by SDC.
*4 N. D. not done

1 : 160 after treatment with 0.125~1% SDC for 60 min and to 1 : 40 after treatment with 1% SDC for 120 min. Infectivity titer was decreased to 1/100 after treatment with 0.125% SDC for 30 min, and could not be detected after treatment with more than 0.25% SDC. CF and HA titers after treatment with 1% SDC, which was most effective in the experiment shown in table 1, were measured from 15 to 180 min with and without shaking (tab. 2). An

TABLE 2 *The CF and HA titers of ICH virus particle after treatment with 1% SDC*

TIME FOR SDC TREATMENT*1	SDC TREATMENT			
	With aggitation		Stationary	
	CF titer	HA titer	CF titer	HA titer
min				
15	64	320	64	320
30	64	320	64	160
60	64	80	64	160
120	64	40	64	160
180	64	$\leq$ 20	64	40
Control (no SDC)*2				
30	16	320	16	320
120	16	320	16	320

*1 SDC treatment was performed in a 37°C water bath.
*2 Distilled water was used instead of 1% solution of SDC.

increase in CF titer, from 1 : 16 to 1 : 64, was noticeable even after a 15 min treatment with SDC. The effect of shaking was obvious in the decrease of HA titer. With shaking, HA titer decreased to 1 : 80 after 60 min, and to less than 1 : 20 after 180 min, while without shaking the decrease in HA titer was not so remarkable. CF titer was not influenced by the shaking. Regardless of shaking, change in CF titer occurred more rapidly than the change in HA titer and the rate of decrease in HA titer was more marked than the rate of increase of CF titer.

4 The effect of sodium desoxycholate on CF, and HA titers of the soluble antigen

SDC solution was added to the soluble antigen in a concentration of 1% and CF and HA titers were measured as described above. The result of this experiment is shown in table 3. CF titer was 1 : 16 in control, but increased to 1 : 32 after 1 min and to 1 : 64

TABLE 3 *The CF and HA titers of ICH soluble antigen treated with SDC*

SDC	TIME FOR TREATMENT	CF TITER	HA TITER
	min		
	1	32	40
	15	64	40
1%	30	64	40
	60	64	20
	120	64	10
	0	16	40
Control	30	16	40
(no SDC)	120	16	40

SDC treatment was performed in a 37°C water bath

after more than 15 min treatment with SDC. On the contrary, HA titer which was 1 : 40 in control decreased to 1 : 20 after 60 min and to 1 : 10 after 120 min treatment with SDC. Thus, the soluble antigen showed the change in antigenic titer after SDC treatment similar to the virus particle.

5 Recovery of CF and HA titers after SDC was removed by dialysis

The virus particles treated with 1~0.0625% SDC for 120 min were dialysed against saline for 24 hrs to remove SDC. The CF and HA titers before and after dialysis are shown in table 4. The CF titer which increased from 1 : 16 to 1 : 64 by treatment with 1% SDC reverted to the original 1 : 16 after dialysis. Also, the HA titer which decreased to 1 : 40 from 1 : 320 by treatment with 1% SDC reverted to the original 1 : 320 after dialysis. Therefore, it was clear that the CF and HA titers of the virus changed by SDC treatment reverted to the original titer after removing SDC by dialysis.

TABLE 4 *The CF and HA titers of SDC treated virus before and after dialysis*

SDC	CF TITER		HA TITER	
	Before dialysis	After dialysis	Before dialysis	After dialysis
%				
1.0	64	16	40	320
0.5	32	16	160	160
0.25	32	16	160	160
0.125	32	16	160	160
0.0625	16	16	320	160
control (no SDC)	16	16	320	320

SDC treatment was performed in a 37°C water bath for 120 minutes. Dialysis was done against saline for 24 hrs.

6 Morphological study of virus particle treated with SDC

From the results described above, it is obvious that SDC treatment drastically affected the infectivity, CF and HA titers of ICH virus. This phenomenon was studied morphologically. The normal ICH virus particles are shown in figure 4. When the virus particles were treated with 0.5% SDC in test tubes, the virus could not be observed as particles under the electron microscope. It appears that the virus particles were destroyed. For the purpose of observing the destroying virus particle, a small drop of virus particle suspension was placed on a carbon-coated collodion membrane grid, to which was added, one drop of 0.5% SDC. In brief, virus particles were treated with SDC on the grid. One min later, the specimen on the grid was reduced by absorbing with filter paper and negatively stained.

In figure 5, many virus particles destroyed by SDC are seen, normal particles are not seen at all. The particles were swollen and in various forms, the arrangement of capsomeres was irregular and rough. There were some particles whose capsomeres were arranged like a limited membrane.

Figures 6 and 7 show the virus material which were treated sufficiently with 0.5% SDC but the SDC had been removed by dialysis. The capsomeres which were once released and became free after the treatment with SDC again aggregated. The aggregates of capsomeres were, however, irregular in size, and usually did not recover the original form of the virus particles.

7 Existence of small virus particles associated with ICH virus

Small virus particles different from ICH virus were observed electron microscopically in the negatively stained specimens of ICH virus. These particles are presented in figures 8, 9 and 10. Figures 8 and 10 show both ICH virus and the small virus particles, the latter were about 25 mμ, 1/3 of ICH virus particle size in diameter. These particles possessed capsomere-like structures (fig. 10), were regular in size and included empty particles (fig. 9). From these findings the authors consider that the small particles are virus particles.

DISCUSSION

It was reported by FURMINGER (1964), and DARBYSHIRE & PEREIRA (1964) that agar gel diffusion precipitin reaction of ICH virus against the homologous immune serum showed two precipitin lines. In this experiment, two precipitin lines were also obtained between ICH soluble antigen and immunized dog serum. The line near the antiserm was adsorbed by human RBC and were accordingly, considered to be HA antigen, the other the CF antigen. There is no doubt that the soluble antigen of ICH virus contains at least two different antigens, CF and HA antigens.

These soluble antigens were fractionated by DEAE cellulose column chromatography, CF antigen was eluted at 0.05 M NaCl and HA antigen was eluted at 0.2 M NaCl in 0.01 M phosphate buffer solution. Separation of the CF and the HA antigens of ICH virus was thus achieved. HA antigen which was eluted at 0.2 M NaCl was not detected by the anti-CF antigen serum (DS–1), but was detected by dog immune serum against ICH virus. This finding also indicates the antigenic difference between CF and HA antigens.

Toxin which has been known to be present in adenovirus soluble antigen was not detected in ICH virus soluble antigen. This coincided with the fact that the cells infected with ICH virus of high multiplicity of infection did not show any cytotoxic effect. CPE was, however, observed as a result of the multiplication of ICH virus. CPE caused by adenovirus is known to be due to toxin (PEREIRA et al., 1958; EVERETT & GINSBERG, 1958; ROWE et al., 1958). Toxin like material was not found in the soluble antigen of ICH virus either as a biologically active or antigenically active material. Further investigation is needed in respect of the mechanism of CPE of the ICH virus.

Attempts were made by the authors to disrupt ICH virus using SDC, and to investigate the relationship of the soluble antigen to the virus particle. Sodium dodecylsulfate has been used to disrupt adenovirus (SMITH et al., 1965). SDC is a surface active agent similar to sodium dodecylsulfate and has been used for differentiating viruses by their sensitivity to SDC (THEILER, 1957). It was found in this study that the ICH virus particle treated with SDC lost infectivity and that, at the same time, CF antigen titer was increased and HA antigen titer was decreased.

The reason for the change of the antigenic titers are surmised as follows. CF and HA antigens are the components of the virus particle. The increase in CF antigen titer might be due to the fact that CF antigens (capsomeres) of virus particles are released by treatment with SDC from the virus particle, consequently, antigenic sites to combine with antibodies are increased. The decrease of HA antigen titer was presumed as follows. The sites of ICH virus particle combining

with RBC are polyvalent. When disrupted with SDC, HA antigens were released from the virus particle each possessing only one site to combine with RBC, and consequently, they could not agglutinate RBC.

The above speculations were made on the basis that the soluble antigens were the components of the virus particle.

It was also found that not only the virus particle but also the soluble antigens showed an increase in CF titer and a decrease in HA titer after treatment with SDC. The finding indicates that the soluble antigens are also composed of units of antigens which might have been released by the SDC treatment, resulting in the change of antigen titers. NORRBY (1966) reported that the soluble HA antigen of adenovirus type 3 consisted of 12 subunits.

Removal of SDC by dialysis resulted in the recovery of the original antigen titers from those changed by SDC treatment. This is an interesting observation which gives us the suggestion that the CF and HA antigens might have a tendency to aggregate each other after removal of the SDC by dialysis. This was morphologically proved and is discussed below.

Electron microscopic observation showed that ICH virus particles were no longer found after treatment with more than 0.5 % SDC. Virus particles would be completely disrupted. This was in accordance with the fact that infectivity was not detectable after SDC treatment. As the result of the virus disruption, capsomeres were completely free. This is morphological support for the reason given for the change of CF and HA titers. It is also interesting that the free capsomeres were aggregated after SDC was removed by dialysis. The recovery of CF and HA titers after dialysis was thus morphologically proved. The aggregation of capsomeres after removing SDC by dialysis did not mean reconstitution of the true virus particle, because infectivity was not recovered. However, the results will show the tendency of the capsomeres to aggregate, and might be useful in the study of the process of ICH virus assembly.

Recently small virus particle associated with human and simian adenovirus strains were reported (MAYOR et al., 1965; ATCHISON et al., 1965, 1966; ARCHETTI et al., 1966; SMITH et al., 1966). ACHISON et al. (1966) reported that the small virus particle contaminated in SV_{11}, designated adenovirus associated virus (AAV), was about 240 Å in size, contained DNA as nucleic acid and required adenovirus for its multiplication. MAYOR et al. (1965) described the antigenic relationship of the adenovirus to its associated virus using fluorescent antibody technique.

In ICH virus, this small associated virus was not reported. In this experiment, however, the small virus particles were found often in the ICH virus specimens under the electron microscope. They were morphologically identical with the small associated virus reported in adenovirus, possessed capsomere-like structure

and included empty particles. Existence of ICH-associated or satellite virus was thus proved in this paper.

SUMMARY

The soluble antigen of ICH virus was fractionated by DEAE cellulose column chromatography and two antigens, CF and HA antigens, were separated. CF antigen was eluted at 0.05M NaCl, HA antigen was eluted at 0.2 M NaCl, both in 0.01 M phosphate buffer solution. The CF antigenicity shown by the HA antigen fraction differed from that of the CF antigen fraction.

The ICH virus particle could be destroyed by treatment with 0.5% sodium desoxycholate (SDC), consequently its infectivity was lost, CF titer was increased and HA titer was decreased. When SDC was removed by dialysis, the changed CF and HA titers returned to the original levels. The destruction of the virus particle by SDC was shown electron microscopically. Also the aggregation of free capsomeres was morphologically observed after SDC was removed. Discussion was carried out on the change due to SDC, the recovery due to removal of SDC, of the CF and HA antigen titers. The results obtained agreed in that CF and HA antigens are the structural components of ICH virus.

Existence of ICH-associated or satellite virus was proved electron microscopically.

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PLATE I

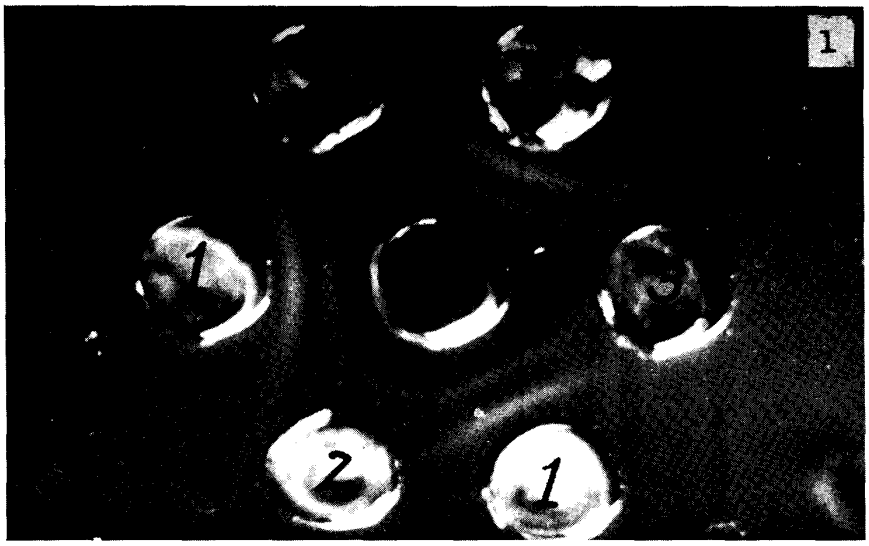
Fig. 1 Agar gel precipitation pattern of ICH virus soluble antigen to dog immune serum

1: soluble antigen
2: soluble antigen adsorbed with human red cell
3: antigen from normal dog kidney cells
4: dog immune serum against ICH virus

Fig. 4 Normal ICH virus particle

Fig. 5 SDC treated virus

Figs. 4 and 5 were the preparations negatively stained with 1% phosphotungstic acid.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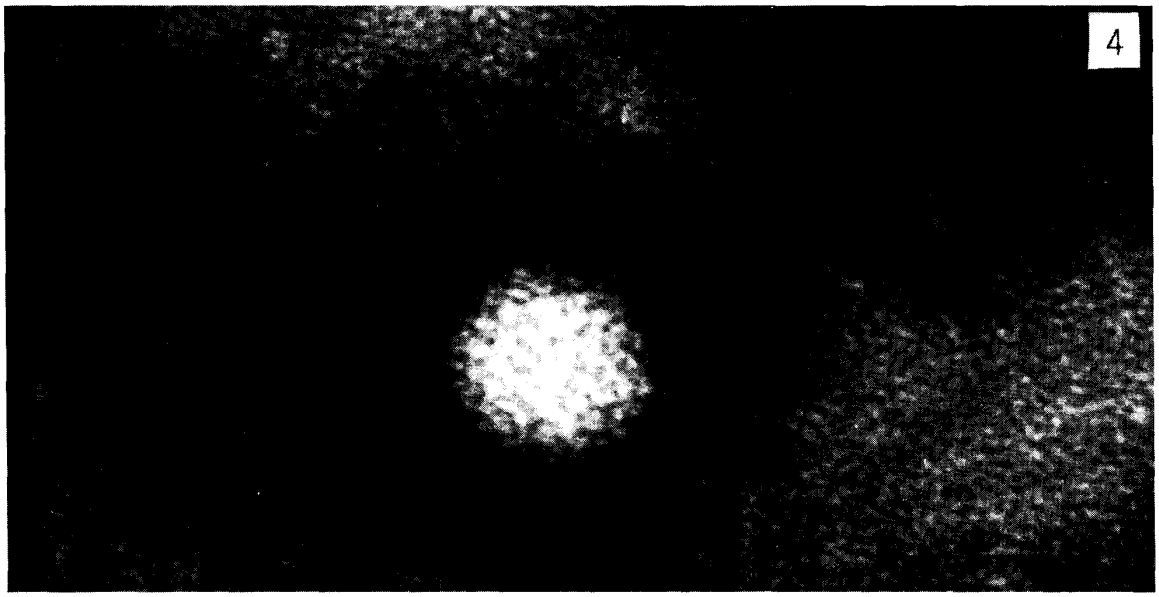
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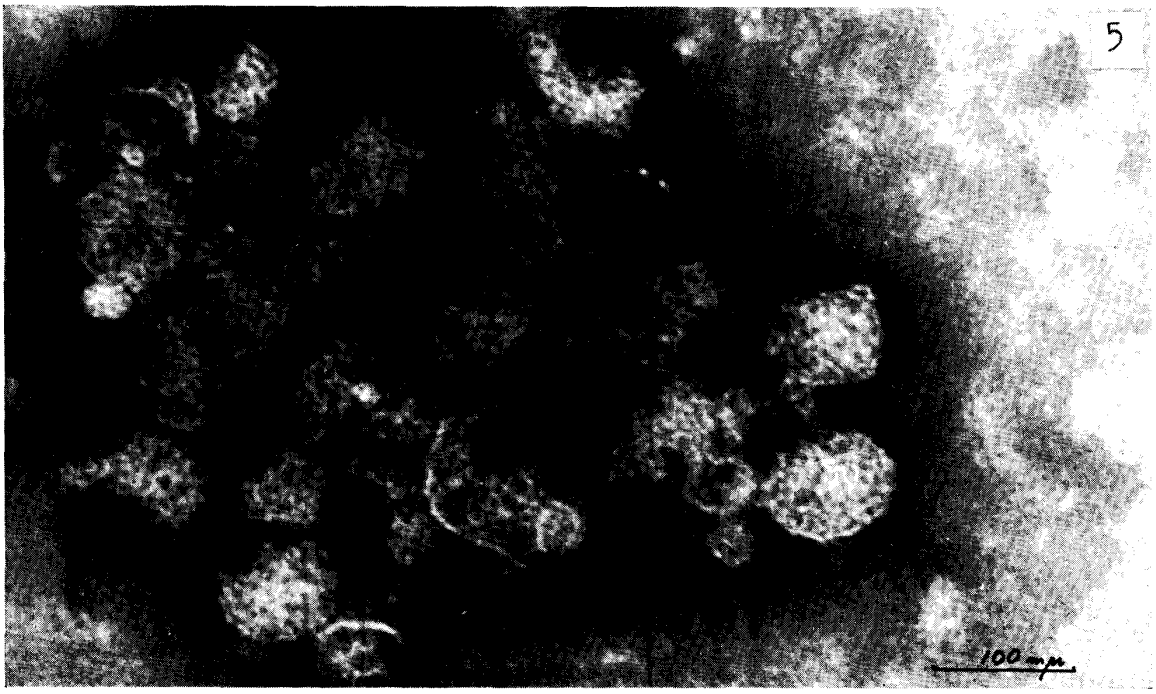
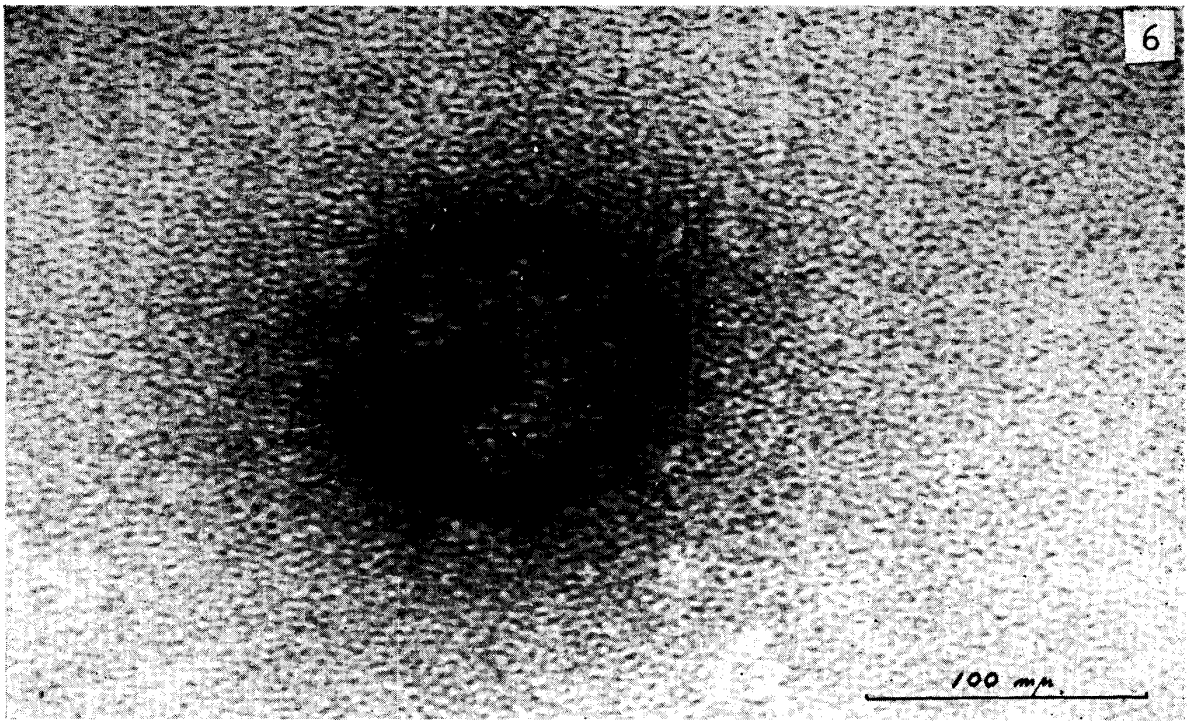

5
100 mμ

PLATE II All the preparations were negatively stained with 1 % phosphotungstic acid.

Figs. 6 & 7 ICH virus material treated with 0.5 % SDC, then dialyzed against saline


6
100 mμ.

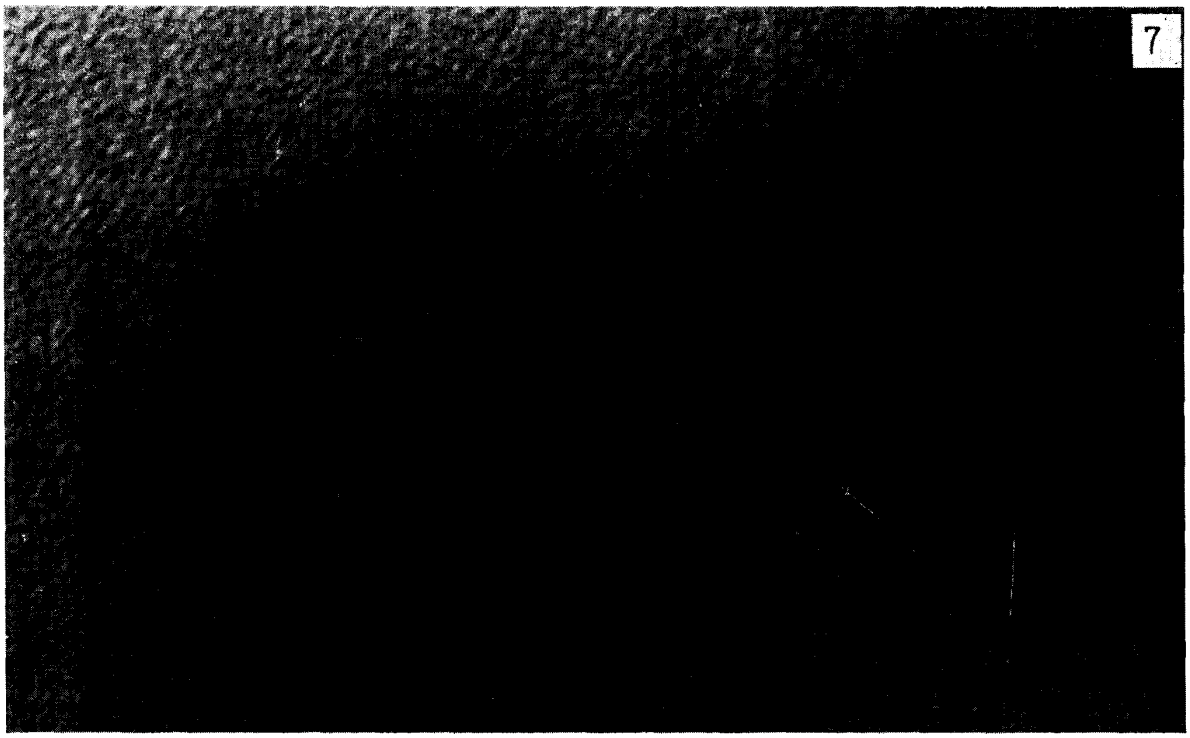
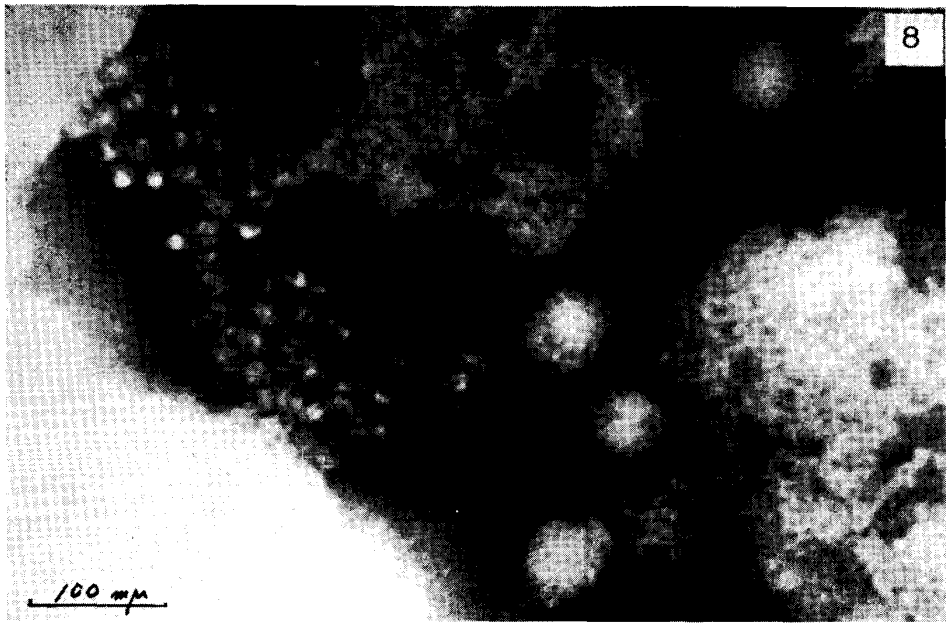

7

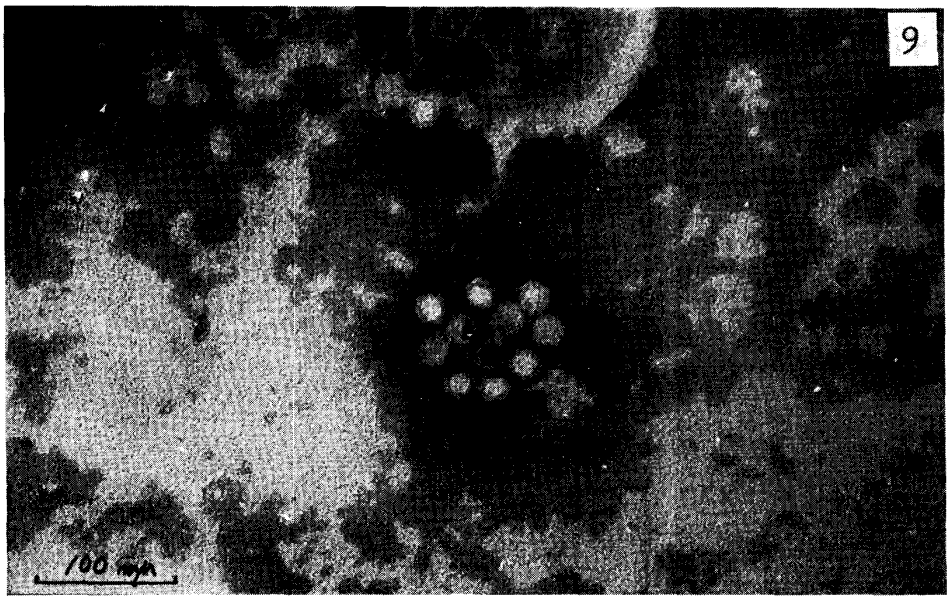
PLATE III All the preparations were negatively stained with 1 % phosphotungstic acid.

Fig. 8 ICH virus and many small virus particles

Fig. 9 Small virus particles

Fig. 10 ICH virus and a small virus showing capsomere-like structure


8
100 mμ


9
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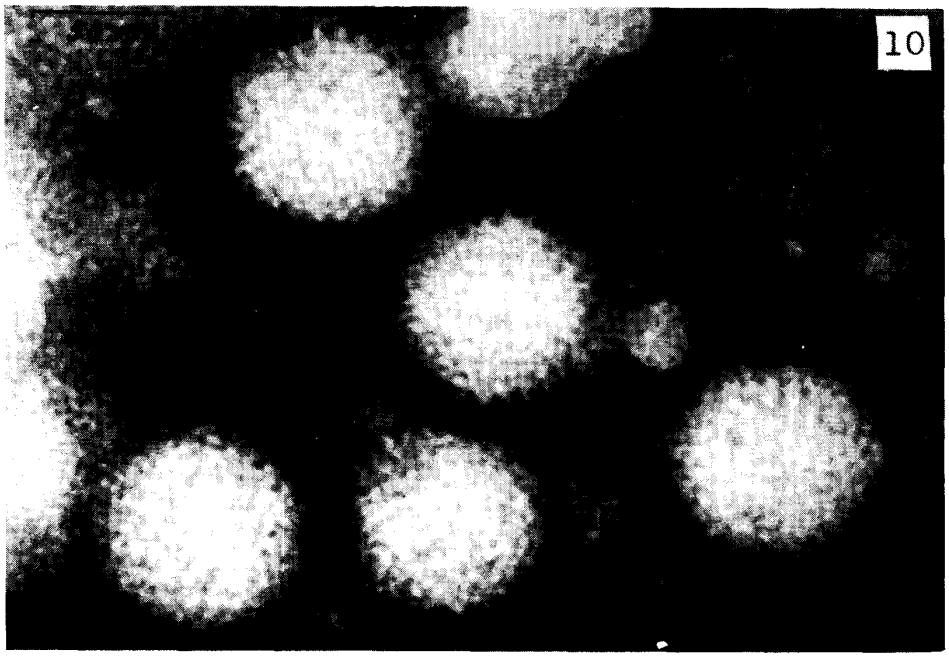
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