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Physico-Chemical Properties of Mouse Hepatitis Virus (MHV-2) Grown on DBT Cell Culture

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Abstract Some properties of a strain of mouse hepatitis virus, MHV-2, grown on DBT cells were determined using a plaque assay on the cells. Viral growth was not inhibited by the presence of actinomycin D or 5-iodo-2-deoxyuridine. MHV-2 was completely inactivated by ether, chloroform, sodium deoxycholate or beta-propiolactone, but showed a moderate resistance to trypsin. Heating at 56 C for 30 min did not completely abolish the virus infectivity. The virus was stable after heating at 50 C for 15 min in 1M-$MgCl_2$ or 1M-$MgSO_4$ as well as at 37 C for 60 min at pH 3.0 to 9.0. Infectivity was decreased to 1/100 and 1/400 after storing at 4 C for 30 days and 37 C for 24 hr, respectively. The virus passed through a 200-nm but not a 50-nm Sartorius membrane filter. The buoyant density of MHV-2 was 1.183 g/cm^3 in sucrose gradient, and the fraction contained coronavirus-like particles measuring 70 to 130 nm in diameter. Survival rate was 10% after exposure to ultraviolet at 150 ergs/mm^2. Freezing and thawing or sonication at 20 kc for 3 min did not affect the virus titer. No hemagglutinin was demonstrable with red blood cells of the chicken, Japanese quail, mouse, rat, hamster, guinea pig, sheep, bovine or human.

Mouse hepatitis virus (MHV), avian infectious bronchitis virus (IBV) and human coronavirus (HCV) are main members of coronaviruses which have been classified on the basis of morphological characteristics of the virion having club-shaped surface projections as revealed by electron microscopy (45). Recently, transmissible gastroenteritis virus (TGEV) of swine (6, 18, 44), hemagglutinating encephalomyelitis virus of swine (HEV) (31, 35), rat coronavirus (34), sialodacryoadenitis virus of rat (SDAV) (1), canine coronavirus (3), bovine coronavirus (30), turkey bluecomb disease virus (37) and feline infectious peritonitis virus (49) were also included in this group of viruses (46).

While McIntosh (29) reviewed common characteristics of the coronaviruses, the physico-chemical natures of most viruses of this group are not completely clarified due to the difficulty to propagate in cultured cells. MHV can be grown in some cultured cells, but difficulty yet remains because available cell cultures are mostly primary ones. Thus, properties of MHV have been studied using virus from infected mouse livers without recourse to quantitative assays. Recently, the

authors (21, 22) reported a satisfactory assay system using a cell line, SR-CDFl-DBT (DBT), that is permissible to MHV-2 virus (32). By the use of this cell line, routine plaque assay can be readily made, and clearer results are obtained than by the use of other cell systems (19, 26). The plaque forming units (PFU) obtained with DBT cells are in agreement with mouse pathogenicity (21).

This paper deals with determination of several essential physico-chemical properties of MHV-2 passaged more than 50 times through DBT cells using the assay system on DBT cells.

MATERIALS AND METHODS

Cell culture. Established cell line of DBT (21, 22) and Vero as well as primary culture of chick embryo fibroblasts (CEF) were used. CEF were obtained from 11-day-old chick embryos. The cells were grown in growth medium (GM) consisting of 8 parts of modified Eagle's minimum essential medium (MEM, Nissui, Tokyo), 1 part of tryptose phosphate broth (TPB, Difco, U.S.A.), 1 part of inactivated calf serum (CS) and kanamycin 0.06 mg/ml. For maintenance of cells and harvesting of the virus was used a maintenance medium (MM) composed of 8.5 parts of MEM, 1 part of TPB and 0.5 part of CS. The cell line cultures were planted every 3 or 4 days.

Viruses. MHV-2 (32) passed 50 to 60 times through DBT cells was used, and DBT culture supernatant at 24 hr postinfection (p.i.) was stored at −20 C. As control viruses, herpes simplex type 2 (HS-2, Mori strain), poliovirus type 1 (Polio-1, Mahony strain) grown in Vero cells and Newcastle disease virus (NDV, Miyadera strain) grown in embryonated eggs were used. These viruses were kindly supplied by Dr. R. Hondo, Department of Viral Infection, Institute of Medical Science.

Plaque assay. Plaque assay of MHV-2 was carried out as reported previously (21, 22). After DBT cells were dispersed from culture bottles with phosphate buffered saline (PBS, pH 7.2) containing EDTA (1:10,000), a cell suspension (2.5×10^5 cells/ml) was prepared in GM and placed into 60 mm Petri dishes in the amount of 5 ml. The cultures were incubated at 37 C in 5% CO_2 atmosphere for 2 to 3 days. Then, the resulting confluent cell monolayers were washed once with PBS and 0.2 ml of virus material was inoculated. After incubation at 37 C for 1.5 hr for virus adsorption, the inoculated cultures were overlaid with 5 ml of MM containing 1% Bacto agar (Difco, U.S.A.). After 36 to 40 hr of incubation at 37 C in 5% CO_2 atmosphere the inoculated cultures were overlayed with 5 ml of agar medium containing neutral red (1:10,000), and plaques were counted. Plaque assay for HS-2 and Polio-1 viruses was performed on Vero cells according to modified methods of Taniguchi and Yoshino (44) and of Dulbecco and Vogt (12), respectively. Titration of NDV was made using CEF (25).

Actinomycin D and 5-iodo-2-deoxyuridine (IUDR) treatment. Before inoculation of MHV-2 and HS-2 viruses onto DBT and Vero cells, respectively, the cell cultures were treated for 2 hr with actinomycin D (Merck-Banyu, Tokyo) at a concentration of 0.5 μg per ml in MM. In assay for NDV on CEF, the drug concentration was

reduced to 0.2 μg per ml because of higher sensitivity of the cells to this drug. IUDR (Sigma, U.S.A.) treatment was made for 2 hr at a concentration of 50 μg per ml in MM before virus inoculation. After virus inoculation with 1 PFU per cell of MHV-2, NDV and HS-2 onto the treated DBT, CEF and Vero cells, respectively, the inoculated cultures were maintained with media containing each drug and harvested at 24, 48 and 72 hr p.i., respectively.

Ether and chloroform treatment. Five ml of a virus material was mixed with an equal volume of ether or chloroform and the mixture was incubated at room temperature for 10 min. After centrifugation and evaporation with nitrogen gas, the water phase was assayed for infectivity.

Sodium deoxycholate (SDC) treatment. This test was performed by the method of Studdert (41). Two-tenths ml of SDC (Difco, U.S.A.) solution in PBS was added to 1.8 ml of a virus material and the mixture was incubated at 37 C for 60 min.

Trypsin treatment. One ml of a virus material and 1 ml of a trypsin (Difco 1:250, U.S.A.) solution in PBS were mixed and dilutions with MEM containing DS at 2% was incubated at 37 C for 60 min without inactivation of trypsin.

Beta-propiolactone (BPL) treatment. Two-tenths ml of a fresh BPL (Wako Pure Chem, Tokyo) solution in PBS was added to 1.8 ml of a virus sample so as to make the final concentration of BPL 0.4%. After an incubation at 37 C for 1 hr the mixture was diluted 10-fold in 0.2 M phosphate buffer (pH 7.2) and assayed for infectivity.

pH stability. The test pH solutions were as follows: pH 2.0 (0.01N HCl in 0.85% NaCl solution), pH 3.0 to 5.0 (McIlavaine buffer, 0.1M citrate acid 0.2M phosphate buffer), pH 7.0 (MEM), and pH 8.0 to 12.0 (0.1M glycine–0.1N NaOH). To 1.8 ml of these buffers or other solutions was added 0.2 ml of a virus material and the mixtures were incubated at 37 C for 60 min. After the incubation, 0.2 ml of each mixture was added to 1.8 ml of 0.2M phosphate buffer (pH 7.2) and was assayed for infectivity.

Temperature sensitivity. Rubber-stoppered test tubes containing 2 ml of a virus material were kept in water baths at 4, 37 and 56 C for various intervals. Thermostability in 1M-$MgCl_2$ and 1M-$MgSO_4$ was tested by the method described by Wallis et al (48). A 1:10 dilution of infected culture fluid in 1M-$MgCl_2$ and 1M-$MgSO_4$ was heated at 50 C for 15 min.

Filtration. This test was made by using membrane filters (Sartorius, West Germany) with pore sizes of 200, 100 and 50 nm. The filters were pretreated with MM, as described by Ver et al (47).

Determination of buoyant density. For virus labeling, DBT cell cultures were infected with about 1 PFU per cell. After virus adsorption at 37 C for 1 hr, the cultures were incubated at 37 C for 12 hr in MM containing 5 mCi/ml uridine-5-^{3}H (Daiichi Pure Chem., Tokyo). After low-speed centrifugation, the infectious culture fluid was mixed with an equal volume of saturated ammonium sulfate. The mixture was stored at 4 C for 1 hr, and spun down at 10,000 × *g* for 15 min. The pellet was resuspended in 1/50 to 1/100 the original volume of PBS, and the suspension was applied to a 20–60% (w/v) sucrose density gradient in TES buffer (0.05M Tris-HCl, 0.001M EDTA and 0.1M NaCl, pH 7.2) in a Spinco SW 27.1 rotor and

spun at 25,000 rpm for 6, 16, and 24 hr at 4 C. After the centrifugation, fractions were obtained by puncturing the tube bottom and assayed for virus infectivity with DBT cells and for ^{3}H-uridine radioactivity and density by a scintillation counter and a refractometer, respectively.

Electron microscopy. A virus preparation was placed onto a carbon coated grid and stained with 2% phosphotungstic acid (pH 6.0). The grid was examined with a JEM 100B electron microscope.

Sensitivity to ultraviolet (UV) irradiation. UV irradiation was performed by the method of Yoshikura (51). Two ml of infected culture fluid in 60 mm Petri dishes was irradiated with 10 ergs/mm^2 sec using a Latarget's dosemeter.

Hemagglutination (HA): HA test was carried out at 4, 22 and 37 C by a microtiter system (39).

RESULTS

Effects of Actinomycin D and IUDR on Virus Growth

After inoculation at a multiplicity of 1 PFU per cell of MHV-2, NDV and HS-2 to drug-treated DBT, CEF and Vero cells, respectively, the cultures were maintained with media containing each drug. Infectivity assays for each virus were made at 24, 48 and 72 hr p.i. As shown in Table 1, the infectivity of MHV-2 and NDV, as determined on DBT cells and CEF, respectively, was not affected by actinomycin D nor by IUDR, whereas the growth of HS-2 in Vero cells was clearly inhibited by IUDR but not by actinomycin D.

Sensitivity to Ether and Chloroform

After treatment with the lipid solvents, the infectivity became undetectable even with undiluted culture supernatants of MHV-2 and NDV, while the infectivity of Polio-1 was not affected at all (Table 2).

Table 1. Effects of actinomycin D and IUDR

Virus[a)]	Infectivity titer (log PFU/ml)		
	Control	Actinomycin D	IUDR
MHV-2	8.6	7.9	8.3
NDV	6.1	6.1[b)]	6.0
HS-2	5.3	4.1	2.0

Cells were treated with actinomycin D (0.5 μg/ml) or IUDR (50 μg/ml) for 2 hr, and then inoculation was made at a multiplicity of 1 PFU per cell. After virus adsorption for 1.5 hr at 37 C, the cultures were maintained in media containing each drug.

[a)] MHV-2 was sampled at 24 hr postinfection (p.i.) and assayed on DBT cells. NDV was sampled at 48 hr p.i. and assayed on CEF. HS-2 was sampled at 72 hr p.i. and assayed on Vero cells.

[b)] The concentration of actinomycin D was reduced to 0.2 μg/ml because of its high sensitivity of CEF.

Table 2. Effects of ether and chloroform on MHV-2

Virus	Infectivity after treatment with		
	Ether[a]	Chloroform	None
MHV-2	<1.0[b]	<1.0	7.8
NDV	<1.0	<1.0	7.5
Polio-1	8.0	8.0	7.8

[a] Five ml of each virus material and an equal volume of a lipid solvent were mixed and incubated at room temperature for 10 min, and the water phase was assayed for infectivity after centrifugation and gas evaporation.
[b] PFU/ml.

Table 3. Effect of SDC on MHV-2

Virus	Infectivity, after treatment with		
	SDC[a] 0.1%	SDC 0.01%	PBS control
MHV-2	<1.0[b]	7.7	8.0
Polio-1	8.0	8.0	8.1

[a] To 1.8 ml of virus material was added 0.2 ml of SDC solution in PBS and the mixture was incubated at 37 C for 60 min.
[b] PFU/ml.

Table 4. Effect of trypsin on MHV-2

Trypsin[a] %	Infectivity (PFU/ml)	
	MHV-2	Polio-1
1.0	3.5	8.0
0.5	4.0	Not tested
0.25	4.9	Not tested
0.125	6.2	Not tested
PBS	7.2	7.8

[a] One ml of each virus material and one ml of trypsin (Difco, 1:250) in PBS were mixed and dilutions made with MEM containing CS at 2% were incubated at 37 C for 60 min, for infectivity assay.

Sensitivity to SDC

After incubation with 0.1% SDC at 37 C for 60 min, the infectivity of Polio-1 was not affected but MHV-2 was completely inactivated. However, MHV-2 showed a moderate resistance to 0.01% SDC (Table 3).

Effect of Trypsin

After treatment with 0.125 to 1.0% trypsin at 37 C for 60 min the decrease in infectivity of MHV-2 was 1.5 to 4.2 log PFU. The infectivity of Polio-1 tested as a control was not affected by 1.0% trypsin (Table 4).

Table 5. Effect of BPL on MHV-2

Virus	Infectivity, treatment with 0.4% BPL[a]	
	Before	After
MHV-2	7.7[b]	<1.0
Polio-1	8.0	<1.0

[a] To 1.8 ml of virus material was added 0.2 ml of a 4.0% BPL solution in PBS and the mixture was incubated at 37 C for 60 min. After incubation the mixture was diluted 10-fold in 0.2 M phosphate buffer at pH 7.2 and assayed for infectivity.

[b] PFU/ml.

Table 6. Effect of pH on MHV-2

pH[a]	Decrease in infectivity after treatment (log)
2.0	>5.9[b]
3.0	0.4
5.0	0
7.0	0
8.0	0.1
9.0	0.9
10.0	>5.9
12.0	>5.9

Original titer was 7.3(log PFU/ml). After incubation at 37 C for 60 min, the mixture was diluted 10-fold in 0.2M phosphate buffer (pH 7.2) and assayed for infectivity on DBT cells.

[a] Test pH solutions were the following: 2.0 (0.01N HCl in 0.85% NaCl solution), 3.0 to 5.0(1M citrate acid–0.2M phosphate buffer), 7.0 (MEM), 8.0 to 12.0 (0.1M glycine–0.1N NaOH).

[b] Difference from the value at pH 7.0.

Treatment with BPL

By treatment with 0.4% BPL at 37 C for 60 min, the infectivity of MHV-2 and Polio-1 was completely destroyed, as shown in Table 5.

pH Stability

To 1.8 ml of buffers or test pH solutions was added 0.2 ml of a virus material and the mixture was incubated at 37 C for 60 min. After the incubation, 0.2 ml of the mixture was added to 1.8 ml of 0.2 M phosphate buffer (pH 7.2) and assayed for infectivity. As shown in Table 6, the decrease in titer within a pH range of 3.0 to 9.0 was not as marked as described for MHV-3 by Piazza (36), but the titer decreased remarkably at pH 2.0, 10.0 and 12.0.

Temperature Sensitivity

After storage at 4 C for 14 and 30 days, reduction rates in virus titer were 1.4

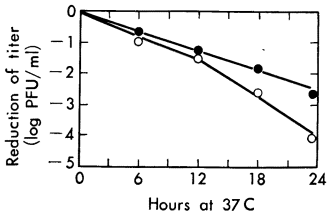

Fig. 1. Effect of incubation at 37 C. ●, original sample; ○, sample diluted 10-fold in MEM.

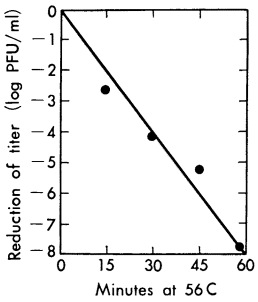

Fig. 2. Effect of heating at 56 C.

Table 7. Effect of heating in 1M-$MgCl_2$, 1M-$MgSO_4$ and H_2O

Virus	Decrease of infectivity (PFU/ml)		
	1M-$MgCl_2$	1M-$MgSO_4$	H_2O
MHV-2	0.1	0.1	2.5
Polio-1	0.1	0.8	5.0
NDV	1.9	1.1	1.9

A 10-fold dilution in each solution was tested after heating at 50 C for 15 min and assayed for infectivity.

and 2.1 log, respectively. At 37 C the infectivity was lost gradually during the first 12 hr, and the titer decreased more rapidly in samples diluted 1:10 with MEM (Fig. 1). After incubation at 37 C for 24 hr the diluted and undiluted virus samples showed decreases of 2.6 and 4.0 log in titer, respectively. The difference of virus titer between the diluted and undiluted samples may have resulted from a greater change of pH because the diluted one changed in color from orange to purple-scarlet after incubation for 24 hr. By heating at 56 C, a linear decrease in infectivity was seen as shown in Fig. 2, revealing a 4-log decrease after 30 min and complete loss after 60 min.

Thermostability in 1M-$MgCl_2$ and 1M-$MgSO_4$

A 1:10 dilution of infected culture fluid was made in 1M-$MgCl_2$ and in 1M-$MgSO_4$. These were heated at 50 C for 15 min, and infectivity was assayed. After heating in the presence of the salts, the infectivity of MHV-2 as well as Polio-1 was

Table 8. Infectivity of MHV-2 after filtration

Filter size[a)]	Titer of filtrate (PFU/ml)
No filtration	2.0×10^8
200 nm	9.0×10^7
100 nm	1.0×10^6
50 nm	<10

[a)] Sartorius membrane filter pretreated with Eagle's MEM containing 5% calf serum.

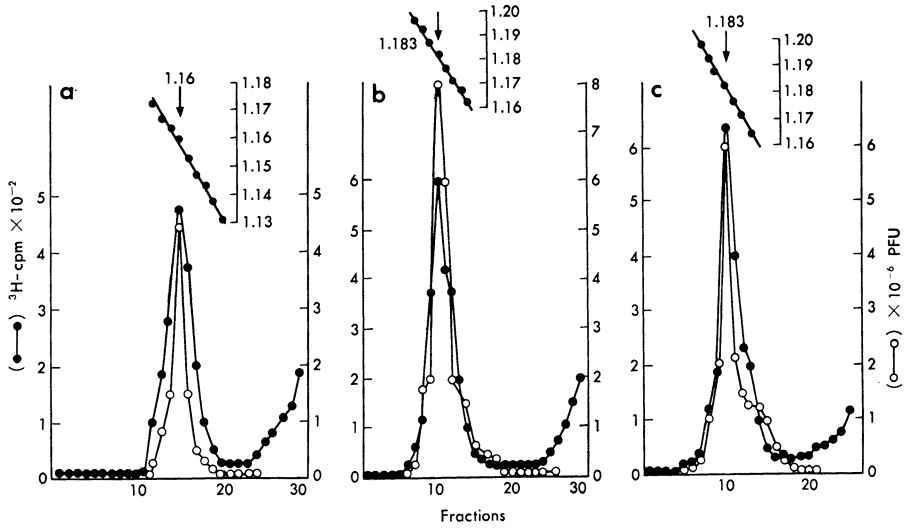

Fig. 3a–c. Determination of the buoyant density of MHV-2. ³H-uridine labeled MHV-2 was applied to a 20 to 60% (w/v) sucrose density gradient in TES buffer (0.05M Tris-HCl, 0.001M EDTA and 0.1M NaCl, pH 7.2) in a Spinco SW 27.1 rotor spun at 25,000 rpm at 4 C. After centrifugation for 6 hr (Fig. 3a), 16 hr (Fig. 3b) and 24 hr (Fig. 3c), fractions were obtained by puncturing the tube bottom and assayed for virus infectivity and incorporation of ³H-uridine into virion RNA.

retained as presented in Table 7, while 2.5 and 5 log reductions were observed with MHV-2 and Polio-1 viruses, respectively, after heating in distilled water. NDV showed a reduction of 1.9 and 1.1 log in titer in 1M-$MgCl_2$ and 1M-$MgSO_4$, respectively.

Filtration

As shown in Table 8, a 50% decrease of infectivity titer was seen after passing through a 200-nm filter. After filtration through a 100-nm filter the decrease was 2 log and no more infective viruses were detected from filtrates of a 50-nm filter.

Buoyant Density

³H-labeled MHV-2 was centrifuged in a sucrose density gradient at 25,000 rpm

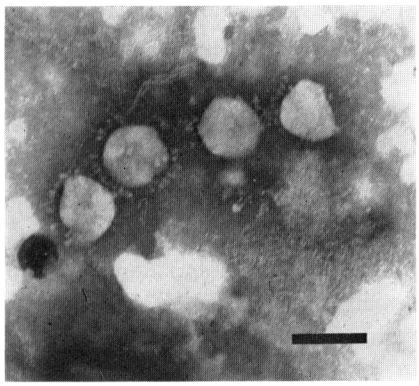

Fig. 4. Mouse hepatitis virus, MHV-2 strain, prepared from infected DBT cells. Virus samples obtained from the peak fractions after centrifugation at 25,000 rpm for 16 hr (Fig. 3b) were stained with 2% phosphotungstic acid at pH 6.0. Bar represents 100 nm.

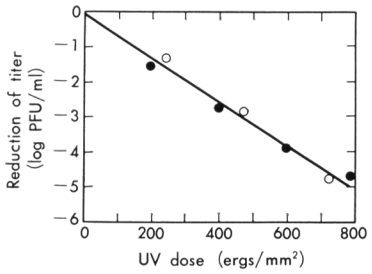

Fig. 5. Effect of UV. 2.0 ml of each virus sample (●, MHV-2; ○, NDV) in an open 60 mm Petri dish was irradiated at 10 ergs/mm²/sec with continuous agitation.

for 6, 16 and 24 hr, and the samples were collected and assayed for virus infectivity and radioactivity by liquid scintillation counting. As shown in Fig. 3a, after 6 hr-centrifugation a single peak of radioactivity was found in fraction 15, coinciding exactly with the peak of virus infectivity, whose buoyant density was estimated at 1.16 g/cm³. After centrifugation for 16 hr peaks of radioactivity and infectivity were found in the same fraction which moved to No. 11 (1.183 g/cm³) as shown in Fig. 3b, and they remained at the same density after centrifugation for 24 hr (Fig. 3c). These findings indicate that ³H-uridine was incorporated into the virion RNA, and MHV-2 virions reached an equilibrium in sucrose gradient at 1.183 g/cm³ under the condition of spinning at 25,000 rpm for 16 hr. Electron microscopy of negatively stained preparations revealed many particles of 70 to 130 nm in diameter having corona-like projections, 20 nm in length, in the top fraction obtained by 16 hr centrifugation (Fig. 4).

UV-Irradiation

Irradiation of virus samples was done with 10 ergs/mm²/sec of UV, as shown in Fig. 5. A linear decrease of residual active virus was shown with both MHV-2 and NDV when irradiated at 100 to 800 ergs/mm². The 10% survival dose after UV-irradiation was 150 ergs/mm² in both viruses.

Freezing–Thawing and Sonication

Two ml of a virus material was subjected to 10 cycles of rapid freezing and thawing by dry-ice acetone and water. Then, the infectivity was assayed with the result that it remained unchanged. The infectivity was not affected either when 5 ml of a chilled virus material was sonicated at 20 kc for 180 sec.

HA Test

By mixing 0.025 ml of a MHV-2 material with 0.025 ml of a 0.5% suspension of red blood cells (RBCs) obtained from either the chicken, Japanese quail, mouse, rat, hamster, guinea pig, sheep, bovine or human O subject, no HA activity of MHV-2 was detectable with any of the RBCs tested.

The properties of MHV-2 revealed in the present studies are summarized in Table 9.

Table 9. Summarized properties of MHV-2

Treatment	Effect
Actinomycin D	No growth inhibition
IUDR	No growth inhibition
Ether and chloroform	Inactivated
0.1% sodium deoxycholate	Inactivated
1.0% trypsin	Sensitive
0.4% beta-propiolactone	Inactivated
pH 3.0 to 9.0 at 37 C for 60 min	Slight reduction
56 C for 30 min	Mostly inactivated
50 C for 15 min in H_2O	Moderate reduction
50 C for 15 min in 1M-$MgCl_2$ or $MgSO_4$	Slight reduction
Virion size	70 to 130 nm in diameter
200 nm filtrate	Infectivity retained
50 nm	No infectivity
Buoyant density in sucrose	1.183 g/cm^3
UV-irradiation (D_{10}=150 ergs/mm^2)	Sensitive
Sonication at 20 kc for 180 sec	Not affected
HA activity with erythrocytes of chicken, Japanese quail, mouse, hamster, rat, guinea pig, sheep, bovine and human O	Not detected

DISCUSSION

Recently, coronaviruses have been grouped according to morphology of virus particles characterized by large petal-shaped projections (46). They are shown

to be single-stranded RNA viruses measuring 70 to 120 nm in diameter with a lipoprotein envelope and to multiply by budding into intracytoplasmic spaces. However, informations on the physico-chemical properties of this group of viruses remain incomplete because there is much difficulty in propagating many of them in cultured cells (28). While MHV has been grouped as a main member of this group and it can be propagated on mouse macrophage primary cultures, its properties have remained still unclear since the growth in macrophages *in vitro* is not always enough to evaluate exactly the virus titers.

We have reported previously the usefulness of the DBT cell culture for growth and titration of MHV (21, 22). Then, attempts were made to see physico-chemical properties of MHV-2 propagated in this line of cells.

Mallucci (26) observed that the replication and cytopathogenicity of MHV-3 in mouse peritoneal macrophages were not affected by actinomycin D or 5-fluorodeoxyuridine. In the present study, actinomycin D and IUDR did not inhibit the multiplication and cytopathic effect of MHV-2 in DBT cells. In addition, ^{3}H-uridine was incorporated into the virion RNA because of the distribution of infectivity coinciding exactly with the uridine label.

MHV-2 was sensitive to ether and chloroform as reported with other strains of MHV (7, 11, 14, 15,27). Rowe et al (38) showed that a low-virulent strain, MHV-S, was isolated from feces of mice, indicating that enteric infection is possible, and this was supported by Calisher and Rowe (5) revealing a moderate resistance of MHV-S to SDC. Our results on MHV-2 were not compatible with those because MHV-2 was completely inactivated by 0.1% SDC. However, 0.01% SDC did not affect the infectivity of MHV-2. As MHV-S virus is more resistant to SDC than MHV-2 (unpublished observation), the difference in resistance to SDC seems to concern with the enteric or hepatic pathogenicity of MHV strains. MHV-2 was completely inactivated by 0.4% BPL as reported by Hercules and Von Kaemel (20). Some infectivity was retained after treatment with 1.0% trypsin at 37 C for 60 min.

Hamparian et al (16) described that the stability of viruses at pH 3.0 provides another criterion for classification of animal viruses. In general, many of enveloped-viruses are labile at pH 3.0, while non-enveloped viruses, except for rhinovirus, are mostly stable. It is very interesting that enveloped MHV-2 was shown to be stable at pH 3.0 retaining 90% of infectivity even after incubation at 37 C for 60 min. This is compatible with the report of Piazza (36) who described that the infectivity of MHV-3 was not affected after storing at 37 C for 60 min at pH 2.5 to 9.5. Cartwright et al (6) demonstrated that the infectivity of a strain of TGEV was not lost after incubation at 37 C for 3 hr at pH 3.0 and Furuuchi et al (13) reported on the pH 3.0 stability of TGEV stating that the attenuated strain was inactivated completely within 2 hr but residual infectivity of the virulent strain was detected at 37 C up to 8 hr. Pensaert and Callebaut (34) also reported that HEV was stable at pH 3.0 at room temperature. In the case of IBV, Stinski and Cunninghum (40) reported that 92 to 96% of infectivity of Beaudette strain was still recovered after being allowed to stand at 4 C for 30 min at pH 3.0 and Cowen and Hitchner (8) demonstrated that stability at pH 3.0 is variable depending upon each strain of this

virus. According to Bhatt et al (1), at least a 1-log reduction was seen in infectivity of SDAV after incubation at pH 3.0 at 25 C for 3 hr. From those data the stability at pH 3.0 might be considered as one of important criteria for difining coronaviruses.

While other strains of MHV were reported to be heat-labile (7, 11, 14, 15, 27), the present study demonstrated that MHV-2 was not completely inactivated after heating at 56 C for 30 min although there was a 4-log reduction in titer. Such discrepancy would be attributed to the difference in virus materials to be examined, because the data of MHV-1 or MHV-3 were obtained with infected liver emulsions giving some unfavorable effects of host cell origin.

Wallis et al (48) stated that thermostability in 1M-$MgCl_2$ and 1M-$MgSO_4$ at 50 C for 15 min was important as criteria for animal virus classification. Our results showed that MHV-2 in 1M-$MgCl_2$ and 1M-$MgSO_4$ is not inactivated after heating at 50 C for 15 min as Coria (9) reported with aivan IBV in 1M-$MgSO_4$. While Harada et al (18) and McClurkin and Norman (28) observed that TGEV was inactivated in 1M-$MgCl_2$ after heating at 50 C for 60 min. In addition, Harada et al (17) described that some mutant strains of TGEV showed a resistance to 1M-$MgCl_2$ at 50 C.

The sizes of MHV-1 and MHV-3 virions were estimated to be 80 to 120 nm and 115 to 172 nm according to the filtration test by Gledhill and Andrewes (14) and Dick et al (11), respectively, while Svoboda et al (42) stated that both MHV-2 and MHV-3 have a diameter of 90 nm by electron microscopic studies. In accordance with these, our results revealed that most MHV-2 virions have sizes between 100 to 200 nm as determined by filtration, and that, by negative staining, they were estimated to be 70 to 130 nm in diameter having projections about 20 nm in length. These finding were similar to those of other coronaviruses reported previously (33, 35, 37, 43, 45).

The buoyant density of MHV-2 was estimated to be 1.183 g/cm^3 in a sucrose gradient in our study. The density of MHV-2 agrees with that reported previously for some coronaviruses such as OC 43 strain (1.19 g/cm^3) (24) and 229E strain (1.18 g/cm^3) (4) of HCV, IBV (1.19 g/cm^3) (2, 10) and TGEV (1.19 c/cm^3) (50).

MHV-2 was shown to be sensitive to UV like NDV, showing a 10% survival dose of 150 ergs. Manaker et al (27) observed that MHV-3 was resistant to freezing and thawing, and we found that MHV-2 also retained its infectivity after freezing and thawing as well as after sonication at 20 kc.

The present results may provide additional informations in physico-chemical properties of MHV-2, a typical coronavirus, based upon exact quantitative assays for virus titers.

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