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PHYSICO-CHEMICAL PROPERTIES OF CALF DIARRHEA CORONAVIRUS

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ABSTRACT

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Replication of calf diarrhea coronavirus was not inhibited by 5-iodo-2'-deoxyuridine, indicating that the virus is an RNA virus. Sensitivity to ether and chloroform indicated that the virus is enveloped, and this was confirmed by electron microscopic observation of the virion. The virus was readily inactivated by trypsin and sodium deoxycholate. The virus was labile at 50°C in diluted medium, but readily stabilized in the presence of $MgCl_2$. It was stable at pH 5 and 7, while a slight loss of infectivity was observed at pH 3. The virus was readily filtered through membrane filters of 200 and 100-nm pore sizes, but not through 50-nm filters. The buoyant density of the virion in CsCl was estimated to be 1.25 g/ml.

INTRODUCTION

Calf diarrhea coronavirus was recently recovered from the feces of calves with neonatal diarrhea and proved to be a causative agent of the disease (Stair et al., 1972; Mebus et al., 1972, 1973 a and b; Sharpee et al., 1976). The virus multiplied in bovine embryonic kidney cell cultures, but failed to induce readily recognizable cytopathic effect (Mebus et al., 1973 a). Recently we have observed that the virus readily replicates and induces a marked cytopathic effect in cultures of a continuous cell line, BEK-1, derived from bovine embryonic kidney. This observation has provided a sensitive, practical assay method (Inaba et al., 1976), whereby the virus and its disease can now be studied more systematically. The present study was undertaken to learn some of the physico-chemical properties of the virus.

MATERIALS AND METHODS

Cell cultures. — BEK-1 cells were grown as described previously (Inaba et al.,

1976). The growth medium used was Eagle's minimum essential medium (MEM) containing 10% tryptose phosphate broth (TPB), 10% calf serum and antibiotics, and the maintenance medium was MEM containing 10% TPB, 0.05% yeast extract, 0.5% sodium glutamate, 0.1% glucose and antibiotics.

Viruses. — The calf diarrhea coronavirus (Mebus et al., 1973 a), passaged in cultures of bovine embryonic kidney cells, was kindly supplied by Dr C.A. Mebus, University of Nebraska. The virus was passaged twice in calf kidney cell cultures and five times in BEK-1 cell cultures in our laboratory (Inaba et al., 1976) before use in the present study. Strain BF1 of bovine enterovirus (BE virus) (Inaba et al., 1962) and strain Los Angeles of infectious bovine rhinotracheitis (IBR) virus were also used for comparison. All these viruses were grown in BEK-1 cell cultures and the supernatant fluid of infected cultures was used as seed virus in the present study.

Infectivity assay. — Serial dilutions of the viral material were made with maintenance medium and each dilution was inoculated in 0.1-ml amounts into three tubes of BEK-1 cell culture. After virus adsorption at 37° C for 1 h, the inoculated cultures had 0.5-ml amounts of maintenance medium added and were then incubated at 34° C in a roller drum for 5 days. The titer was expressed in 50% tissue culture infectious doses ($TCID_{50}$) per 0.1 ml.

Hemagglutination (HA). — HA titer was measured by the method described previously (Sato et al., 1977) and was expressed as the reciprocal of the highest antigen dilution which showed complete hemagglutination.

RESULTS

Effect of 5-iodo-2'-deoxyuridine (IUDR)

Calf diarrhea coronavirus was tested along with IBR and BE viruses. A group of tube cultures of BEK-1 cells were inoculated with virus at an input multiplicity of 0.1 $TCID_{50}$/cell. After virus adsorption at 37° C for 1 h, one half of the inoculated cultures received 0.5-ml amounts of maintenance medium and the remaining half received maintenance medium containing 50 μg/ml of IUDR. The cultures were incubated in a roller drum at 34° C, and, at intervals, four cultures were taken from each group. The fluid phase of the cultures was pooled and stored at −60° C after clarification by low-speed centrifugation. All the specimens were assayed for infectivity simultaneously. The results are illustrated in Fig. 1. The replication of calf diarrhea coronavirus was not affected by IUDR, indicating that the virus is an RNA virus. The control viruses employed behaved as expected; BE virus was not affected by IUDR, but IBR virus did not multiply in the presence of IUDR.

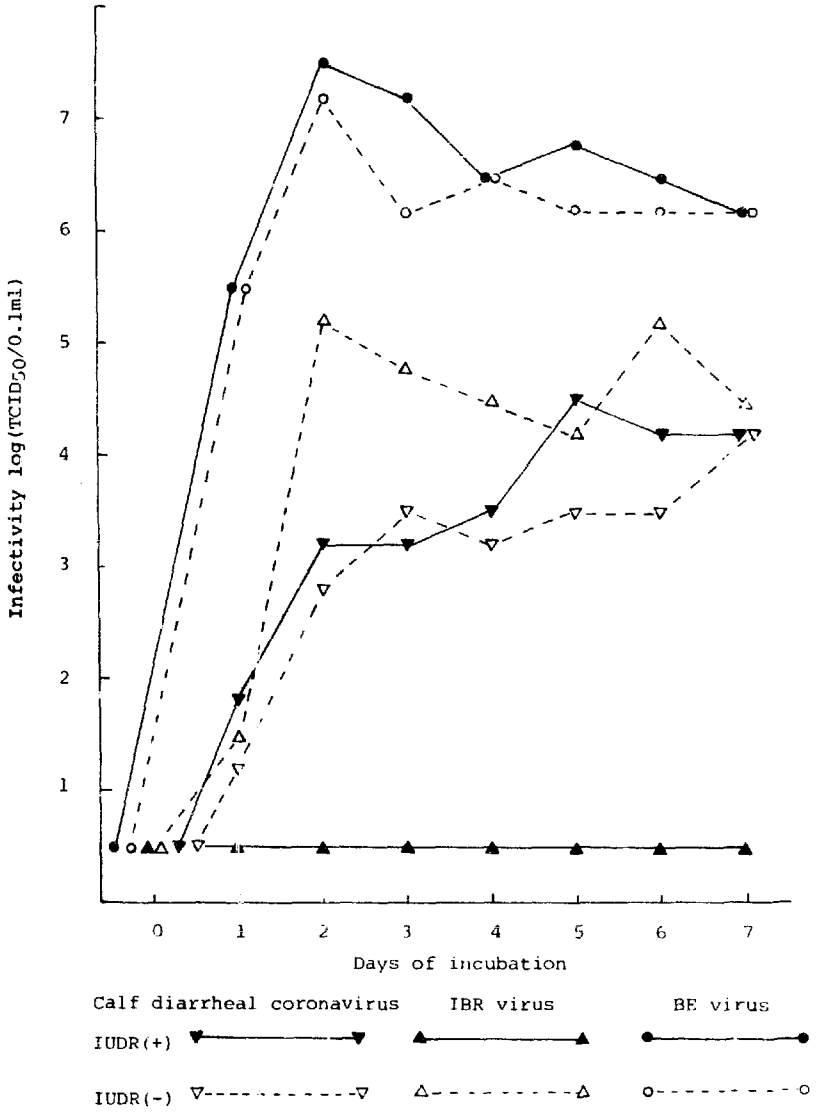

Fig. 1. Effect of 5-iodo-2'-deoxyuridine (IUDR) on replication of calf diarrhea coronavirus.

Sensitivity to ether and chloroform

In a test tube fitted with a rubber stopper 1.6 ml of infectious culture fluid and 0.4 ml of anesthetic ether were mixed, kept at room temperature for 1 h, and centrifuged to separate the water phase, which was used for infectivity assay after evaporation of the residual ether under reduced pressure. The treatment with chloroform was carried out by mixing 1.9 ml of infectious culture fluid and 0.1 ml of chloroform. The mixture was kept at room temperature for 1 h, centrifuged, and the resulting water phase was used for infectivity assay. As shown in Table I, calf diarrhea coronavirus was

sensitive to ether and chloroform. Of the viruses used as controls, IBR virus was completely inactivated but BE virus was resistant to these treatments.

Sensitivity to deoxycholate (DOC)

One milliliter of infectious culture fluid and 1 ml of 0.2% DOC in PBS were mixed and the mixtures were kept at room temperature for 1 h and assayed for infectivity. As shown in Table I, calf diarrhea coronavirus was completely inactivated by DOC. IBR virus was also inactivated by DOC, but BE virus was not.

Effect of molar magnesium chloride

One milliliter of infectious culture fluid, diluted ten-fold with distilled water, was mixed with 1 ml of 2.0 M $MgCl_2$ solution, the maintenance medium or distilled water, and the mixtures were incubated in a water bath at 50°C for 1 h and assayed for infectivity. As shown in Table II, calf diarrhea coronavirus, like BE virus, was stabilized by M $MgCl_2$, but readily inactivated in diluted medium at 50°C within 1 h, while IBR virus in diluted medium was as stable as in the maintenance medium but was rapidly (within 1 h) inactivated in M $MgCl_2$ solution.

Effect of pH

Two tenths of a milliliter of infectious culture fluid and 1.8 ml of McIlvain's buffer solutions at pH 3.0, 5.0 or 7.0, or PBS (pH 7.2) were mixed in test tubes fitted with rubber stoppers. The mixtures were incubated at room temperature (22°C) for 1 h and assayed for infectivity. At pH 3 a slight loss of infectivity was shown, while at pH 5 and 7 no loss in infectivity was shown (Table III).

TABLE I

Effect of ether, chloroform and sodium deoxycholate (DOC) on calf diarrhea coronavirus

Virus	Control	Infectivity (log_{10} $TCID_{50}$ 0.1 ml) after treatment with		
		Ether	Chloroform	DOC
Calf diarrhea coronavirus	3.5[c]	<0.5	<0.5	<0.5
BE virus[a]	7.5	6.8	6.5	6.2
IBR virus[b]	6.5	<0.5	<0.5	<0.5

[a] Bovine enterovirus (strain BF1).
[b] Infectious bovine rhinotracheitis virus (strain Los Angeles).

TABLE II

Effect of 1 M $MgCl_2$ on calf diarrhea coronavirus

Virus	Infectivity (log_{10} $TCID_{50}$ 0.1 ml)			
	Before heating	After heating at 50°C for 1 h in		
		1-M $MgCl_2$	MM[c]	H_2O
Calf diarrhea coronavirus	3.5	2.5	1.5	<0.5
BE virus[a]	7.5	6.2	5.5	4.5
IBR virus[b]	6.5	<0.5	4.8	4.5

See footnotes to Table I.
[c]Maintenance medium.

TABLE III

Effect of pH on calf diarrhea coronavirus

pH	Infectivity (log_{10} $TCID_{50}$ 0.1 ml)
3.0	2.8
5.0	3.8
7.0	3.8
7.2 (PBS control)	3.8

Effect of trypsin

One milliliter of infectious culture fluid and 1 ml of 2, 1, 0.5 or 0.25% trypsin solution (Difco, 1 : 250) in PBS (0.8% NaCl, M/150 phosphate buffer, pH 7.4) were mixed. The mixtures were incubated at 37°C for 1 h and assayed for infectivity after addition of 2 ml of trypsin inhibitor solution (1 mg/ml). As shown in Table IV, calf diarrhea coronavirus was sensitive to trypsin but somewhat less sensitive than IBR virus, whereas BE virus was resistant to trypsin.

Filtration

The virus was readily filtered through Sartorius membrane filters of 200 and 100-nm pore sizes, but not through 50-nm filters (Table V).

Caesium chloride equilibrium density gradient centrifugation

Infectious culture fluid was centrifuged at 3 000 rpm for 30 min to remove coarse debris and virus was sedimented by centrifugation at 100 000 × *g* for

TABLE IV

Effect of trypsin on calf diarrhea coronavirus

Virus	Infectivity (log_{10} $TCID_{50}$ 0.1 ml)				
	Control (PBS, pH 7.4)	After treatment with trypsin			
		1%	0.5%	0.25%	0.125%
Calf diarrhea coronavirus	3.5	<0.5	<0.5	3.2	3.5
BE virus[a]	5.8	5.8	5.8	5.8	6.2
IBR virus[b]	5.8	<0.5	<0.5	1.5	1.2

See footnotes to Table I.

TABLE V

Filtration of calf diarrhea coronavirus

Filtration, pore size (nm)	Infectivity (log_{10} $TCID_{50}$ 0.1 ml)
Before	3.8
200	3.5
100	2.2
50	<0.5

2 h, and resuspended in 0.01 volume of PBS. The resulting virus suspension was mixed with a CsCl solution to a density of 1.25 g/ml and centrifuged in a Spinco SW 50.1 rotor at 300 000 × *g* for 20 h. Fractions were obtained by puncturing the tube bottom, and assayed for infectivity and hemagglutinin (Fig. 2). Infectivity showed a peak which coincided in position with the peak of hemagglutinin. The density of the peak fractions was estimated to be 1.24 g/ml. Electron microscopic examination of these peak fractions by the phosphotungstic negative staining technique revealed numerous spherical virions. Although most particles were more or less damaged, they were shown to have an envelope covered with widely spaced club-shaped projections about 20 nm long (Fig. 3). The size of the virions ranged from 110 to 160 nm, the average diameter including surface projections was estimated to be about 130 nm.

DISCUSSION

The present knowledge concerning the basic properties of calf diarrhea coronavirus is limited. The results presented in this report contribute some information on the physical and chemical properties of the virus, and support

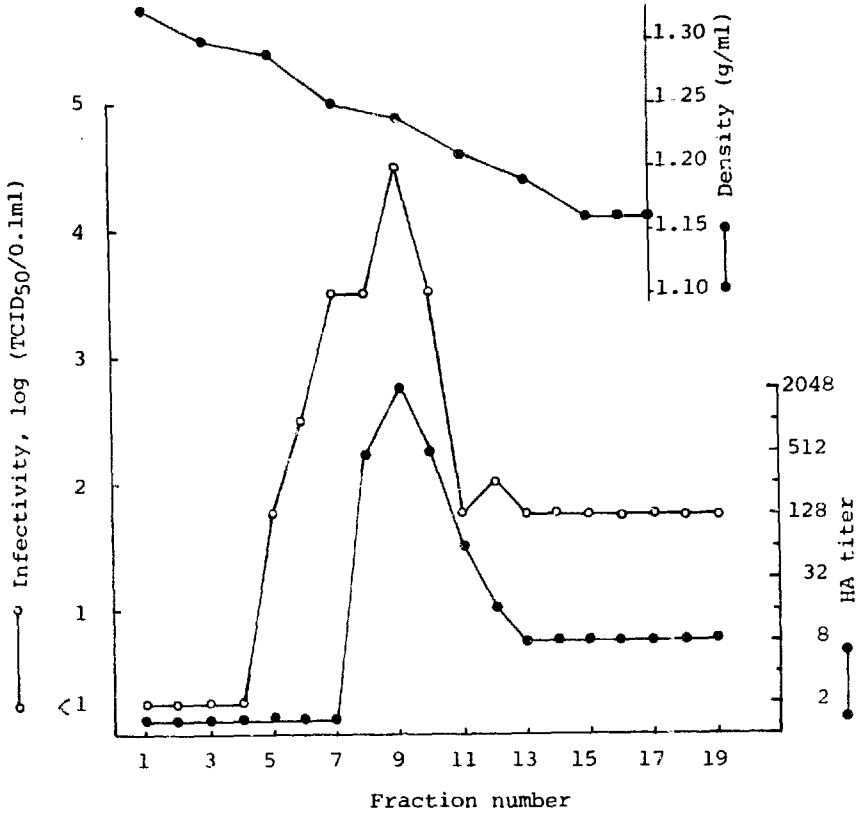

Fig. 2. CsCl equilibrium density gradient centrifugation of calf diarrhea coronavirus.

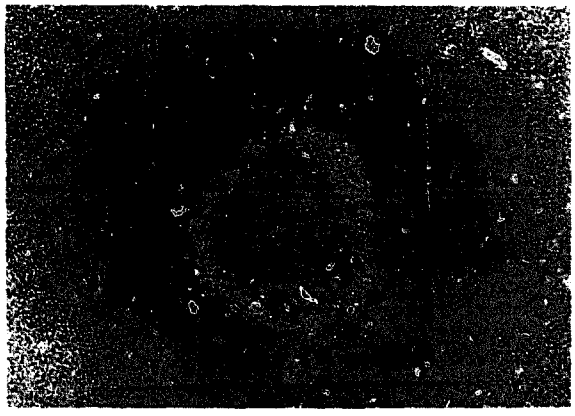

Fig. 3. Electron micrograph of negatively stained calf diarrhea coronavirus (× 200 000).

the classification of the virus as a coronavirus (McIntosh, 1974).

Lack of inhibition of the multiplication of calf diarrhea coronavirus by 5-iodo-2′-deoxyuridine indicates that it is an RNA virus. This finding confirms the recent report by Sharpee et al. (1976), and is in agreement with the fact that the growth of the coronaviruses so far tested has been found to be insensitive to the action of inhibitors of DNA metabolism (McIntosh,

1974). This finding should be further confirmed and extended by direct extraction and analysis of the nucleic acid from highly purified virions, including determination of whether it is single or double stranded.

Sensitivity to lipid solvents, ether and chloroform, indicates that the virus is enveloped, confirming the electron microscopic observation of the virion reported in this paper and by previous workers (Stair et al., 1972; Sharpee et al., 1976). The virus was readily inactivated by trypsin and sodium deoxycholate. The virus was labile at 50° C in diluted medium, but readily stabilized in the presence of 1 M $MgCl_2$, agreeing with previous results (Sharpee et al., 1976). Similarly, stabilization to heat by divalent cations has been reported for avian infectious bronchitis virus (Hopkins, 1967).

The virus was stable at pH 5 and 7, while a slight loss of infectivity was observed at pH 3. Previous reports disagree regarding the stability of various coronaviruses at acid pH (McIntosh, 1974). Recently, calf diarrhea coronavirus was reported to be as stable as transmissible gastro-enteritis virus at acid pH, while avian infectious bronchitis virus was acid labile (Sharpee et al., 1976).

The virus was readily filtered through membrane filters with 200 or 100-nm pore size, but not through 50-nm filters. On the other hand, the size of the virion including surface projections, as determined by electron microscopy, ranged from 110 to 160 nm with an average of 130 nm, substantiating the reports of Stair et al. (1972) and Sharpee et al. (1976). These measurements seem to agree with the present results of filtration, because virions are pleomorphic and may be collapsed and flattened when negatively stained.

The buoyant density of the virion in CsCl was estimated to be 1.24 g/ml, confirming the previous report by Stair et al. (1972), while that in sucrose has been reported to be 1.18 g/ml (Sharpee et al., 1976).

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