

Hemagglutination and Structural Polypeptides of a New Coronavirus Associated with Diarrhea in Infant Mice

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With 4 Figures

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Summary

The hemagglutination (HA) and receptor destroying enzyme (RDE) activities of a newly isolated mouse enteric coronavirus (designated as DVIM) are described. DVIM agglutinates mouse or rat red blood cells (RBC) at 4° C. At 37° C the agglutination was rapidly reversed. The optimal pH for HA and for RDE activities using mouse red cells were shown to be 6.5 and 7.3 respectively. Hemagglutination by DVIM was not inhibited by pretreatment of RBCs with *Vibrio cholerae* filtrate or by pretreatment with Influenza-A neuraminidase. Therefore, the DVIM receptors on RBCs differ from the receptors of Influenza-A, and the RDE activity of DVIM acts specifically on this receptor.

In addition, an analysis of the DVIM polypeptides showed that the virions contain five major, VP1 (M. W. 139,000), VP2 (68,000), VP3 (53,000), VP4 (38,000), VP5 (22,000) and two minor, VP1a (110,000), VP1b (100,000) polypeptides. VP1 and VP1b were digested by bromelain, suggesting that they constitute the surface glycoproteins.

Introduction

Coronaviridae are a family of lipid-containing RNA viruses which exhibit a unique morphology. The virions have widely-spaced surface projections which form a radiating "corona" around the particles as shown by electron microscopy (18, 26). The diarrhea virus of infant mice (DVIM), a newly-isolated mouse enteric coronavirus, is antigenically related to other coronaviruses by complement fixation assay but clearly distinguishable by neutralization assay (21).

The existence of a receptor destroying enzyme (RDE)-like activity has not been reported for any of the coronaviruses. We report here a RDE activity associated with DVIM virions which differs from that of ortho- or paramyx-

viruses. The structural polypeptides of DVIM were analysed by polyacrylamide gel electrophoresis.

Materials and Methods

Virus and Purification

The mouse enteric coronavirus (DVIM) was kindly provided by Dr. Kozaburo Sato (Central Laboratory of Shionogi Pharm. Co., Osaka, Japan). DVIM was passaged in BALB/c-3T3 cell cultures in Eagle's minimal essential medium containing 10 percent fetal calf serum. Virus inoculation was carried out with 10^7 TCID₅₀ per Roux bottle. At 24 hours post-infection virus was harvested by three freeze-thaw cycles and stored at -70°C . Influenza virus strain A/RI/5, A/Victoria/3/75, A/NJ/8/76 and Hemagglutinating virus of Japan (HVJ or Sendai virus) strain Z were grown in the allantoic cavity of 10-day-old embryonated chicken eggs as previously described (24). The same purification processes were employed for each virus at 4°C . After clarification of culture fluid by centrifugation at $8,600 \times g$ for 30 minutes, virions were concentrated by centrifugation at $70,000 \times g$ for 90 minutes, resuspended in 0.1 M NTE buffer (0.05 M Tris-Cl, 0.1 M NaCl, 0.001 M EDTA), pH 7.3, layered onto a discontinuous gradient of sucrose (15:30:50 percent w/w), and centrifuged at $64,000 \times g$ for 2 hours. Virions were collected from the interphase between 30 and 50 percent sucrose solutions and were dialysed against NTE buffer. For polypeptide analysis, virions were re-purified by linear sucrose gradient (20 to 50 percent w/w) centrifugation at $77,000 \times g$ for 90 minutes.

Hemagglutination (HA) Assay

Routine HA tests were carried out as previously described (23), using 0.5 percent mouse RBCs in Dulbecco's PBS, containing 0.3 percent bovine serum albumin. Alternatively, 0.25 percent avian RBCs, or 0.5 percent other mammalian RBCs were used. The titer was recorded as the reciprocal of the highest virus dilution causing a detectable HA.

Hemadsorption

Hemadsorption tests were done on DVIM-infected BALB/c-3T3 cell monolayers grown on glass cover slips. A 0.5 percent suspension of mouse RBCs were adsorbed for 3 hours at 4°C . Cover slips were washed and prepared for analysis by light microscopy. The experiment for elution kinetics was performed on identical cell-monolayers at various incubation periods. To test for RDE-like activity, cells were incubated at indicated temperatures, prepared for microscopy and photographed. The elution ratio was expressed as the percentage of remaining RBCs enumerated per photographic field.

Polyacrylamide Gel Electrophoresis (PAGE)

Sodium dodecyl sulfate (SDS)-PAGE was performed on 7.5 percent polyacrylamide gels by the method of MAIZEL *et al.* (17), using 6 mm diameter tubular gels 60 mm long. Purified virus was solubilized in 1 percent SDS, 1 percent 2-mercaptoethanol, 20 percent glycerol, and 0.006 percent bromphenol blue, for 1.5 minutes at 100°C . After pre-electrophoresis for one hour at 5 mA/gel, samples were applied and electrophoresis was carried out at 5 mA/gel in 0.1 M sodium phosphate buffer, pH 7.2, for 6 hours, at room temperature.

Gels were fixed overnight with 20 percent sulfosalicylic acid and stained with 0.25 percent Coomassie brilliant blue in 50 percent methanol. The gels were destained with several changes of 15 percent methanol in 7 percent acetic acid. Gels were stained for carbohydrate and lipid with Schiff's reagent and oil-red-O, respectively, following the methods of HIERHOLZER *et al.* (8). Stained gels were scanned with a densitometer (Joko gel scanner) at 600 nm for peptides, 550 nm for carbohydrates, and 450 nm for lipids, respectively.

The approximate molecular weights of the polypeptides were determined by the method of SHAPIRO *et al.* (22). Percentage composition of each structural polypeptide was determined from the photometric scans using a Joko scanning planimeter.

Results

Characterization of Hemagglutination Activity of DVIM

Red blood cells of seven different species were tested for agglutinability with DVIM, and the results are summarized in Table 1. Only mouse and rat RBCs were agglutinated at 4° C, indicating a restricted receptor range for the HA activity.

Mouse RBCs agglutinated by DVIM at 4° C, could be liberated from agglutination by incubation at 20° C (Table 2). The DVIM sample had a titer of 4,096 HAU at 4° C, which decreased to 8 HAU after 60 minutes of incubation at 20° C. This indicates that in addition to its HA activity, DVIM virions appear to possess a RDE-like activity which differs from the RDE activity shown for paramyxoviruses, which is not active at 20° C.

Table 1. *DVIM hemagglutination with red blood cells of various species, at different temperatures*

Species of red cell	HA titers at		
	4° C	20° C	37° C
Chicken	<2	<2	<2
Goose	<2	<2	<2
Rabbit	<2	<2	<2
Guinea pig	<2	<2	<2
Human "0"	<2	<2	<2
Rat	128	<2	<2
Mouse	128	<2	<2

Avian red cells were prepared as 0.25 percent suspensions in PBS (pH 7.3), mammalian red cells were prepared as 0.5 percent in the same PBS

Table 2. *Reduction of HA-titers at 20° C*

Virus	HA titers at	
	4° C	20° C ^a
DVIM	4096	8
HVJ (Z st.)	512	512
Inf. A/RI/5	1024	1024
Inf. A/Victoria/3/75	32	32

^a Hemagglutination test were carried out at 4° C as usual, then the microplate was incubated at 20° C, for 60 minutes

The HA titer was stable at 4° C. However, HA appeared to be reversible only under some pH conditions, when incubated at 37° C, as shown in Table 3. The optimal pH for HA activity is relatively wide, whereas RDE-like activity appeared optimal at a pH of about 7.4 and exhibited no activity at pH 6.5, at 37° C. The HA activity was stable at 37° C, up to 24 hours, but was inactivated at 56° C, within 20 minutes (data not shown).

The stability of HA activity and infectivity of DVIM to various reagents is summarized in Table 4. Bromelain, a proteolytic enzyme which removes the club-shaped projections from the surface of other coronaviruses (4, 8, 25), affected both HA activity and infectivity of DVIM. Trypsin did not affect HA activity or infectivity, while pepsin affected only HA activity. The HA titer was reduced slightly by NP-40 treatment, and infectivity was destroyed. Ethyl ether destroyed both virus HA activity and infectivity.

Table 3. *Effect of pH on the HA-titers at 4° C and reduction of titers post incubation at 37° C*

pH (at 25° C)	HA-titer		Reduction rate (a/b)
	4° C ^a	Post incubation ^b at 37° C	
5.40	64	8	8
5.55	64	8	8
5.90	64	16	4
6.20	128	128	1
6.50	256	256	1
6.82	256	128	2
7.27	128	16	8
7.43	128	8	16
7.57	8	8	1
7.87	4	4	1

HA-titrations were carried out with 0.5 percent mouse red blood cells in indicated pH, at 4° C, thereafter the microplate was incubated at 37° C for 60 minutes

Table 4. *The effect of chemical and proteolytic modification on HA and infectivity of DVIM*

Treatment	HA-titer	Viral infectivity
None	128	+
Trypsin	128	+
Pepsin	32	+
Bromelain	< 2	—
NP-40	96	—
Ethyl-ether	< 2	—

Ethyl ether treatment of virus was carried out by mixing with an equal volume of ethyl ether at 4° C for 1 hour, and following removal of ethyl ether HA activity and infectivity were assayed. Enzyme treatment of purified virions was carried out by incubating purified virions at 37° C in PBS containing 50 mM dithiothreitol with trypsin, pepsin, or bromelain at 1 mg/ml, respectively. After enzyme treatment, virions were pelleted through 30 percent (w/w) sucrose cushion and assayed for HA activity and infectivity. Nonidet P-40 (NP-40) treatment was carried out in the same manner as enzyme treatments, in PBS containing 1 percent NP-40 and 50 mM dithiothreitol. Infectivities were expressed as positive: +, and negative: —

Further Characterization of Receptor Destroying Activity

The data in Tables 2 and 3 indicate that DVIM virions agglutinate mouse RBCs at 4° C, and the process was reversed at 37° C. The present experiments were designed to characterize further the virus associated RDE-like activity which might have an effect on the receptor sites of RCBs. DVIM infection of BALB/c-3T3 cells exhibits a cytopathic effect evidenced by the formation of syncytia.

The infected cells exhibit virus-specific enzyme activities associated with these syncytia. Fig. 1 shows the elution kinetics of red blood cells from the syncytia, induced by DVIM 10 hours after infection, at 20° and 37° C respectively. Approximately 70 to 80 per cent of RBCs were liberated within 30 minutes at 37° C while the liberation at 20° C progressed slowly. Therefore, the cell surfaces of DVIM-induced syncytia exhibit the same HA and RDE-like activities associated with virions.

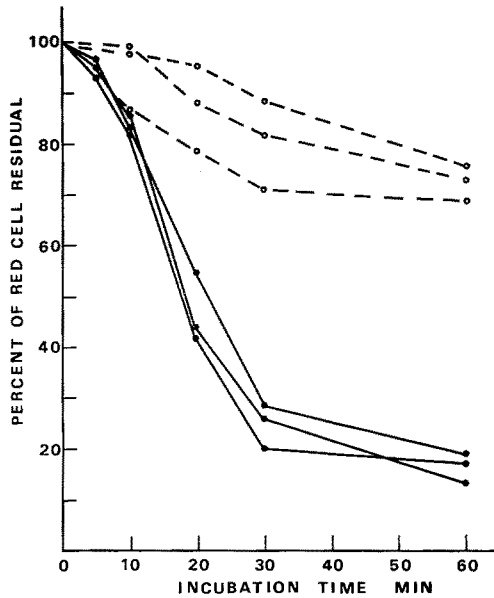


Fig. 1. The liberation of mouse red blood cells from the hemadsorbed syncytium, formed at 10 hours after inoculation with DVIM. Hemadsorbed syncytia were incubated at 37° C (●—●) or 20° C, (○—○). The liberation rate of red cells was expressed as the percentage of remaining cells on the syncytia, counted in the same photographic field at various incubation periods

If liberated RBCs are reexposed to DVIM-infected monolayers, no hemadsorption is observed. The same syncytia could again hemadsorb freshly prepared RBCs (Fig. 2). This implies that hemadsorption of DVIM does not correspond to "pseudo-hemadsorption" described by BUCKNALL *et al.* (3). Furthermore, the elution of red blood cells from syncytia appears to be a result of enzymatic digestion of RBC receptors by a RDE-like activity situated on the syncytia surface. This activity was dependent upon incubation temperature, similar to RDE-like activity of DVIM virions.

To confirm the receptor destroying activity of DVIM, the effect of the purified virus on red blood cells was examined. Mouse RBCs treated with DVIM were reagglutinated by Influenza-A viruses or HVJ, but could not be reagglutinated by DVIM virions (Table 5). In contrast, RDE treated mouse RBCs could still be fully agglutinated by DVIM (Table 6). Influenza-A hemagglutination of RBCs was reduced in proportion to the concentration of RDE.

Table 5. *HA-titers determined with virus-treated mouse red blood cells*

Virus	HA-titers		
	Red blood cell pretreatment		Control
	Inf. A/RI/5	DVIM	
DVIM	2048	128	2048
Inf. A/RI/5	32	4096	1024
Inf. A/NJ/8/76	8	256	64
HVJ	512	1024	256

Each virus (> 5000 HA) was mixed with mouse RBCs (2.5 percent final conc.). Each mixture was incubated at 4° C for 3 hours, followed by incubation at 37° C for 4 hours. These preincubated RBCs were thoroughly washed with PBS and resuspended at 0.5 percent and then used for HA titration. Control RBCs were not pretreated with virions

Table 6. *HA-titers determined with RDE-treated mouse red blood cells*

Virus	RDE concentration (mg/ml)			Control
	0.12	0.06	0.03	
DVIM	256	256	256	256
Inf. A/RI/5	4	8	32	64
Inf. A/Vic./3/75	< 4	< 4	< 4	32
HVJ	64	32	32	32

Mouse red blood cells were treated with RDE at indicated concentration, dissolved in 0.01 M Ca-Borate buffer (pH 7.2), at 37° C for 60 minutes, after which the cells were washed and resuspended as a 0.5 percent suspensions in PBS and used for HA titration. RDE was obtained from *Vibrio cholerae* variant of 558 strain filtrate (Takeda Pharm. Co. Osaka, Japan). Control RBCs were not treated with the RDE

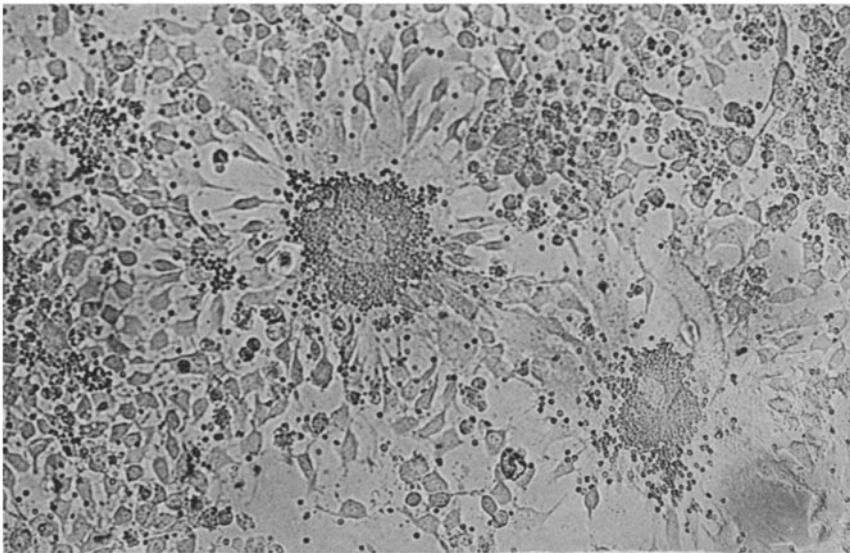


Fig. 2. Readsorption of freshly prepared RBC to previously exposed syncytia. Hemadsorbed syncytium which were incubated at 37° C to release RBCs for 60 minutes, were exposed to freshly prepared RBCs and photographed

Above results strongly suggest that DVIM possesses an enzymatic activity which reacts with surface components of RBCs. However, this enzymatic action appears to differ from the bacterial neuraminidase and that of Influenza-A virions.

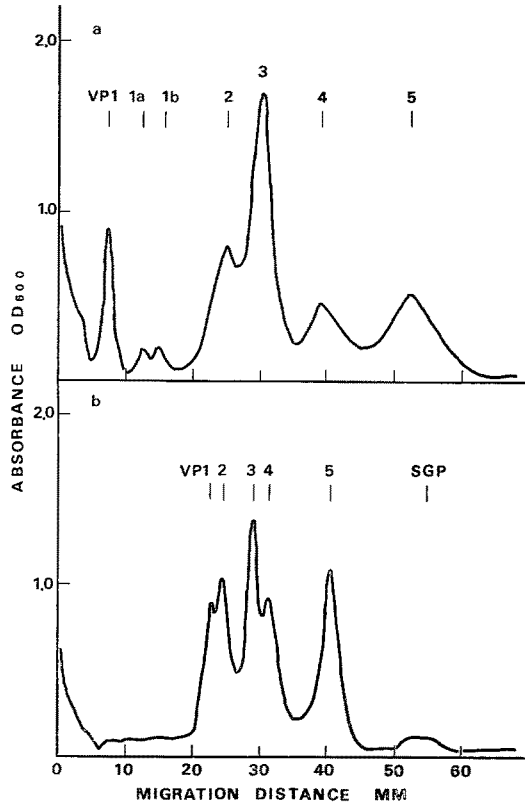


Fig. 3. Polypeptides of DVIM separated by 7.5 percent polyacrylamide gel electrophoresis. Gels were stained with Coomassie brilliant blue and scanned at 600 nm. *a* purified DVIM, *b* purified HVJ

Virus Polypeptide Analysis

Since DVIM is a newly isolated coronavirus, its structural polypeptides have not been characterized. Therefore we analysed the virion polypeptides by SDS-PAGE. Electrophoresed polypeptides, when stained with Coomassie brilliant blue, revealed a minimum of seven bands. A densitometer scan of stained polypeptides of DVIM and HVJ are shown in Figs. 3A and 3B, respectively. The five major polypeptides of DVIM were designated as VP1 to VP5 in decreasing order of electrophoretic mobility, as shown in Fig. 3A. Two additional minor bands VP1a and VP1b were observed between VP1 and VP2. The approximate molecular weight and molar ratios of the structural polypeptides, are presented

in Table 7. Preliminary analyses indicated that three polypeptides, VP1, VP1b and VP2 are glycoproteins, while VP2 reacted with oil-red-0, suggesting it may be a lipid-containing protein.

Table 7. *Structural polypeptides of mouse enteric coronavirus (DVIM)*

Polypeptide	Approximate MW ($\times 10^3$) ^a		Relative amount (%)	Relative molar ^b amount
	Mean	Range		
VP1	139	126—147	7	2.1
VP1a	110	100—116	4	1.5
VP1b	100	88—105	4	1.7
VP2	68	65—72	18	10.9
VP3	53	51—59	26	20.3
VP4	38	34—41	17	18.5
VP5	22	21—24	24	45.1

^a Mean of minimum for 20 gels from 7 electrophoresis runs

^b Relative amount divided by corresponding molecular weight and normalized to a percentage

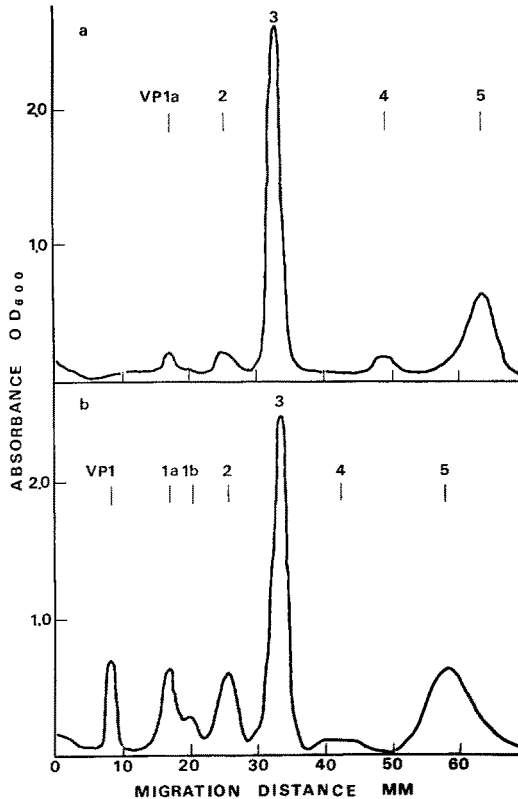


Fig. 4. Densitometer tracing of bromelain treated and untreated DVIM. Purified virions were treated with 0.1 percent bromelain at 37° C for 15 minutes and repurified as described in Materials and Methods. *a* repurified DVIM, *b* control preparation

Effect of Bromelain on the Structural Polypeptides

A densitometer scan of a stained polyacrylamide gel of the polypeptides of virus particles treated with bromelain (1.0 mg/ml at 37° C for 15 minutes) is shown in Fig. 4. As can be seen from this figure, VP1 and VP1b were digested by the bromelain treatment, suggesting that these polypeptides are found on the surface of the virion. Polypeptides VP1a and VP2 were also reduced, and only trace amounts of them were still observed, suggesting these polypeptides may also be surface polypeptides. VP3 was unaffected by bromelain treatment, suggesting that it represents an "inner core" protein.

Discussion

Direct HA (i.e. without pretreatment of the virus) and hemadsorption have been described for several coronaviruses, OC-43 (11, 12), HEV (6, 20), IBV (1), and bovine coronavirus (2). The hemagglutinating ability of OC-43 required several passages in suckling mouse brain (12), and Massachusetts strain of IBV (1) required both sucrose gradient purification and digestion with phospholipase C for activation. The HA of DVIM, reported here, was detectable directly in the culture fluid. The ratio of infectivity to HA varied between 1.2 and 3.1×10^4 TCID₅₀/0.1 ml/HA unit. DVIM hemagglutinated the RBCs of only two species tested, while other coronaviruses, OC-43, HEV and IBV, were less specific. Accordingly, HA of DVIM was expressed only using two types of red blood cell species, mouse and rat, and was also detectable at low temperatures in a stable pattern.

The formation of "prozone" described by BINGHAM *et al.* (1) was not observed for DVIM. The instability of HA and hemadsorption at 20° C suggests that a viral enzyme may be responsible for uncoupling the virus from the red blood cell receptor. The possible enzymatic activity of DVIM was demonstrated on homologous virus receptors of mouse RBCs without any effect on receptors for heterologous viruses, Influenza-A and HVJ. Furthermore, it was also shown that bacterial neuraminidase (RDE) had no effect at all on the receptor for DVIM; nevertheless, the receptors for influenza-A viruses were destroyed by the same treatment. Additionally, even after purification through a sucrose density gradient, DVIM retains its receptor-destroying activity, thereby showing that this activity is viral-specific and associated with virions. Our preliminary results strongly suggest the existence of some type of receptor-destroying enzyme such as that of Influenza C virus (10, 13).

The optimal pH of DVIM HA activity was distinct from that of the receptor-destroying activity. It has not been determined whether these activities may reside on a single polypeptide or on two different polypeptides. Because the surface projections of DVIM are somewhat different morphologically compared to other coronaviruses, it will be of interest to study the exact nature of the hemagglutinating and receptor-destroying activity of DVIM. The receptor-destroying activity of DVIM appears to be unique, when compared to that of ortho- and para-myxoviruses.

Purified DVIM contains seven species of polypeptides, five major and possibly two minor polypeptides. These results compare favorably to those obtained for human coronavirus OC 43 (8), IBV (14), bovine coronavirus LY-138 (7) and other

murine coronaviruses MHV-3 (16) and JHM (27). The classification of the polypeptides of DVIM as glyco- or glycolipoprotein must be considered as tentative at this point since the chemical nature of these complexes cannot be precisely defined by differential staining techniques. The number and size of glycoproteins of coronaviruses which have been reported previously by many investigators, were shown to vary. It appears that our VPI (MW 139,000) corresponds to a large polypeptide (MW 150,000—200,000) reported for other coronaviruses (4, 8, 9, 15, 16, 19, 24, 25, 27), because it is glycosylated and appears to be situated on the surface of the virion. Other DVIM virion polypeptides also appear to be surface glycoproteins. Furthermore, some of the investigators agree that coronaviruses contain a polypeptide having an approximate molecular weight of 50,000, corresponding to our VP3, which is not glycosylated and tends to comprise a large proportion of the virus protein (4, 15, 19, 25). Polypeptide VP3 represents 26 percent of virus protein and is not affected by bromelain digestion at all. This suggests that VP3 is also located in the "inner core" (5, 19, 25) of the virion. Although a considerable amount of VP4 was not observed in Fig. 4, the possibility that the VP4 could be an artifact produced by different conditions of reduction (24), still remains unexplained.

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