

Biochemical and Biophysical Characteristics of Diarrhea Viruses of Human and Calf Origin

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Polyacrylamide gel electrophoretic analysis of purified preparations of human and calf diarrhea viruses indicated eight polypeptide components, or possibly nine in the case of the calf diarrhea virus. Thermal denaturation and analytical studies of the calf diarrhea virus genome showed it to consist of 11 double-stranded segments of RNA. The placing of the human and calf diarrhea viruses together with other similar viruses into a genus separate from reovirus and orbivirus, but within the family *Reoviridae*, is discussed.

The family *Reoviridae* at present includes two recognized genera, reovirus and orbivirus, and a number of plant and insect viruses (cytoplasmic polyhedrosis) whose classification has not been formalized (21). Recent work in various parts of the world has implicated a new type of virus in causing acute infantile enteritis (3, 9, 13). This virus appears to be extremely common, usually infects children at an early age, and has previously escaped detection because it is not cultivable by routine diagnostic procedures (7). A similar virus has been shown to be associated with diarrhea in neonatal calves (18). This virus is also widespread (21, 32) and has been adapted, with difficulty, to cell culture (19, 32). On the grounds of morphology and morphogenesis, these two newly detected morphologically indistinguishable viruses have features in common with both reoviruses and orbiviruses (12) but more closely resemble the virus of epizootic diarrhea of infant mice (1), and a simian virus SA-11 (8). We have investigated some biochemical and biophysical characteristics of the diarrhea viruses of human and calf origin and present data on the polypeptides of both agents and the nucleic acid of the latter. These results further justify their tentative classification within the family *Reoviridae*, but together in a new genus for which the names duovirus (6) or rotavirus (10) have previously been suggested.

MATERIALS AND METHODS

Viruses. The human diarrhea virus was extracted from stools collected from infants with acute enteritis. The material was kindly supplied by R. Bishop and G. Davidson, Royal Childrens Hospital, Melbourne, Victoria.

The calf diarrhea virus was extracted from stools

collected from calves with diarrhea and supplied by courtesy of the Victorian Department of Agriculture, "Attwood" Veterinary Research Laboratory.

Reovirus type 3 (Abney strain), research reference reagent, was obtained from the National Institutes of Health, Bethesda, Md.

Electron microscopy. Virus preparations were negatively stained with one-tenth saturated ammonium molybdate using the loop drop technique. Specimens were examined with a Hitachi HU11A electron microscope operating at 50 kV with an instrument magnification of 20,000.

Purification of diarrhea viruses and buoyant density determination. The following purification procedure was applied to both human and bovine stools. Virus was extracted to a preliminary stage, suitable for electron microscopic examination, according to the method described by Bishop et al. (4). Briefly, this procedure involved extraction with fluorocarbon and differential centrifugation. For the analysis of viral polypeptides and nucleic acid, this partially purified virus from individual or pooled stools was further purified by rate zonal centrifugation in 20 to 35% (wt/wt) sucrose gradients in 0.002 M Tris-hydrochloride, pH 7.5. Gradients were centrifuged for 70 min at $75,000 \times g$ in a Spinco SW41 rotor at 4 C. The resultant virus-containing bands visible in the upper half of the gradient were harvested separately and, after concentration by pelleting, examined by electron microscopy. Preparations showing large numbers of virus particles by negative staining were layered onto preformed 40 to 55% (wt/vol) cesium chloride gradients in 0.2 M Tris-hydrochloride, pH 7.5. After centrifugation at 4 C for 18 h at $180,000 \times g$, the various bands observed were harvested and dialyzed overnight against 0.002 M Tris-hydrochloride, pH 7.5. The particles in the bands were then pelleted and examined by electron microscopy. Suitably purified virus preparations were then selected for nucleic acid or polypeptide analysis on the basis of visible debris content.

The density of the virus particles was obtained from measurements of the positions of the bands

obtained by isopycnic ultracentrifugation in relation to those taken by plastic beads of accurately known density (Beckman Instruments, Inc., Fullerton, Calif.) included in the gradients.

Reovirus, propagated in Vero cells, was purified similarly to the diarrhea viruses.

Polyacrylamide gel electrophoresis of diarrhea virus polypeptides and molecular weight determination. Cylindrical 8.75% polyacrylamide gels were prepared according to the method of Laemmli (14). Virus preparations were dissociated by treating in Laemmli sample buffer at 90 C for 2 min. Polypeptides were stacked at 1 mA/gel and then electrophoresis was continued at 2 mA/gel. After staining with 0.5% Coomassie blue in methanol-acetic acid-water (5:1:5) and destaining in 7.5% acetic acid, gels were photographed according to the method of Oliver and Chalkley (23).

The molecular weights of the viral polypeptides were determined by comparing their electrophoretic mobilities with those of polypeptides of known molecular weights. The polypeptides used for reference were β -galactosidase (mol wt 130,000) grade IV (Sigma Ltd.), bovine serum albumin (mol wt 67,000) fraction V (C.S.L. Australia), ovalbumin (monomer mol wt 43,000, dimer mol wt 86,000) 5 \times crystallized, B grade (Calbiochem), and pepsin (mol wt 35,000) 3 \times crystallized, B grade (Calbiochem). For the virus of calf origin, the reference polypeptides and viral polypeptides were co-electrophoresed in the same gel as well as being electrophoresed in separate gels run at the same time. Both procedures gave very similar estimates of molecular weights. For the virus of human origin, the reference polypeptides and viral polypeptides were electrophoresed under the same conditions but in separate gels.

Deproteinization of purified calf diarrhea virus for nucleic acid determination. Deproteinization by phenol extraction of purified calf diarrhea virus was carried out at room temperature according to the method of Nonoyama et al. (22).

Determination of nucleic acid type of the calf diarrhea virus. The type of nucleic acid present in the calf diarrhea virus was determined by carrying out the diphenylamine reaction for DNA (5) and the orcinol reaction for RNA (20). Salmon sperm DNA, A grade (Calbiochem), and RNA from bakers' yeast, type XI (Sigma Chemical Co.), were used as reference reagents.

Thermal denaturation characteristics of the nucleic acid of the calf diarrhea virus. A thermal denaturation curve was constructed by heating the calf diarrhea virus nucleic acid dissolved in 0.1 \times SSC (0.015 M NaCl + 0.0015 M sodium citrate, pH 7.2) in a special heating attachment fitted to a Pye Unicam spectrophotometer. The procedure used was similar to that of Bellamy et al. (2).

Polyacrylamide gel electrophoresis of the nucleic acid of calf diarrhea virus and molecular weight determination. Cylindrical 7.5% polyacrylamide gels were prepared as described by Martin and Zweerink (17). The deproteinized viral nucleic acid preparation was suspended in Loening (15) buffer containing 6 M urea, 1% sodium dodecyl sulfate and

0.1% 2-mercaptoethanol, applied directly to the gels, and electrophoresis was carried out for 48 h at 2 mA/gel. After staining with 0.2% methylene blue in 0.4 M acetic acid-acetate buffer and destaining in doubly distilled water, gels were scanned at 610 nm using a Canalco model J11 microdensitometer and photographed according to the method of Oliver and Chalkley (23). Scans were photographed directly from the densitometer tracings.

The molecular weights of the genome segments were calculated according to the method used by Verwoerd et al. (28) after co-electrophoresis of nucleic acid preparations of reovirus and calf diarrhea virus. The molecular weights of reovirus type 3 (Abney strain) published by Shatkin et al. (25) were used as reference.

RESULTS

Purity, morphology, and buoyant density. Various bands were obtained from CsCl gradients of both human and calf diarrhea virus preparations. The major bands occurred at buoyant densities of 1.30 g/ml, 1.36 g/ml, and 1.37 g/ml, and a minor band occurred at a buoyant density of 1.29 g/ml. By electron microscopy, all these bands were observed to contain virus particles. The bands at densities 1.29 and 1.30 contained particles with single- or double-shelled capsids which were always penetrated to varying degrees by negative stain (Fig. 1a). They resemble the top component of reovirus (26). Large amounts of amorphous debris, membrane-bound vesicles, and some bacterial debris including flagella were usually associated with these bands. The particles of highest buoyant densities (1.36 and 1.37) are shown in Fig. 1 (b and c). Bands at both these densities contained particles with single- or double-shelled capsids, but bands at density 1.37 contained a higher percentage of particles without the outer shell than did bands at density 1.36. Very little or no debris of the kind observed contaminating the top component was associated with these bands. The human and calf diarrhea viruses observed in bands of corresponding buoyant density were morphologically indistinguishable.

Electrophoresis of polypeptides and molecular weight determination. Preparations in which minimal or no debris was observed by electron microscopy were selected for polyacrylamide gel electrophoresis. Preliminary electrophoretic studies indicated that, if debris was obvious by electron microscope examination, then a number of extra bands of varying intensities were inconsistently obtained in addition to those which were consistently present and which were considered to be of viral origin. In view of the large number of different virus preparations obtained from different humans

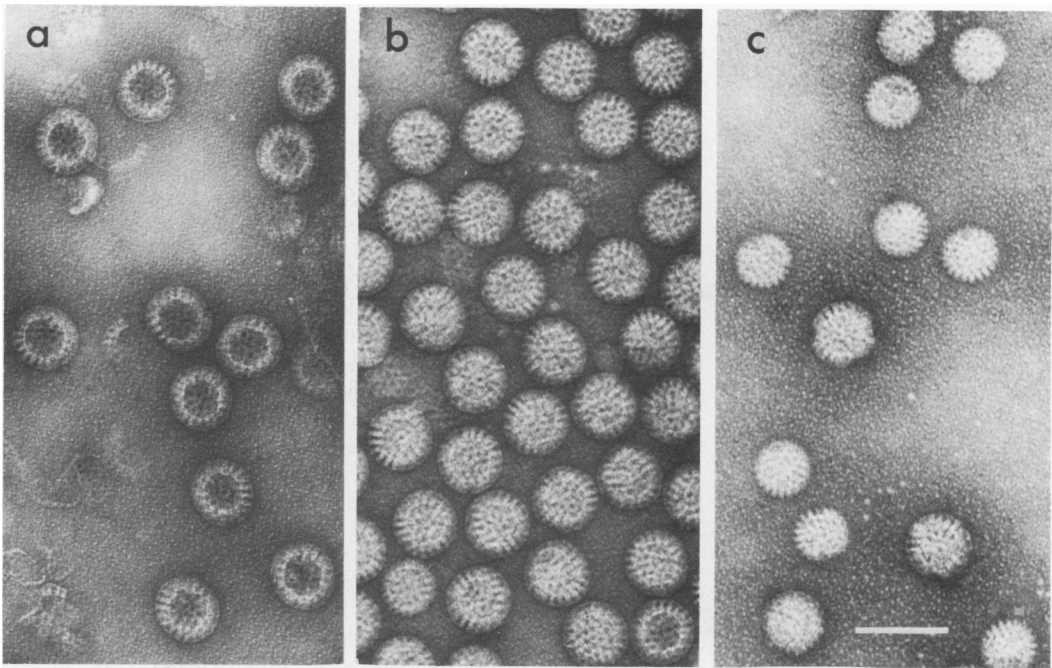


FIG. 1. (a) Particles of calf diarrhea virus from the band at density 1.30 g/ml in a CsCl gradient. (b) Particles of calf diarrhea virus from the band at density 1.36 g/ml. Note the predominance of double-shelled particles. (c) Particles of human diarrhea virus from the band at density 1.37 g/ml. Note the predominance of particles lacking the outer shell. All preparations were negatively stained with one-tenth saturated ammonium molybdate. Magnifications are all $\times 120,000$ and the magnification marker represents 100 nm.

and calves on different diets and from different areas throughout Melbourne (humans) and Victoria (calves), we consider it unlikely for any of the polypeptides we have consistently observed by electrophoretic studies to be a contaminant. So far we have been unable to obtain a preparation of top component free of contaminating polypeptides for viral polypeptide analysis.

For the virus of human origin, electrophoresis of dissociated purified preparations of particles yielded eight consistently occurring polypeptides (Fig. 2). For the virus of calf origin, electrophoresis of similar preparations also yielded 8 polypeptides (Fig. 2), but there appear to be some differences in the relative intensities of the bands. It was noticeable that, with the human diarrhea virus, polypeptide bands no. 5, 7, and 8 and, with the calf diarrhea virus, polypeptide bands no. 5, 7, 8, and 9 were diminished in intensity in preparations from cesium chloride gradient bands at density 1.37 compared with preparations from bands at density 1.36. In view of the electron microscope results this suggests that these polypeptides may be associated with the outer shell.

Two further differences were also observed. Firstly, a polypeptide corresponding to human

diarrhea virus polypeptide band no. 3 has only been resolved occasionally in different calf diarrhea virus preparations, and, secondly, a polypeptide corresponding to calf diarrhea virus polypeptide band no. 9 has not been resolved in preparations of the human diarrhea virus. Further studies are in progress to clarify these differences.

The molecular weights of the polypeptides of both diarrhea viruses are listed in Table 1. The molecular weights quoted represent the average obtained from a series of different experiments.

Determination of the nucleic acid type of the calf diarrhea virus. Data obtained from the orcinol and diphenylamine reactions with the calf diarrhea virus nucleic acid and the standards are presented in Table 2. The results show that the calf diarrhea virus nucleic acid reacted with orcinol rather than diphenylamine, thus indicating a ribonucleic acid genome.

Thermal denaturation characteristics of the nucleic acid of the calf diarrhea virus. Figure 3 shows the melting curve obtained when deproteinized nucleic acid of the calf diarrhea virus was heated to 98 C in $0.1\times$ SSC. A 45% increase in relative absorbance was observed. This sharp increase is of a similar magnitude to

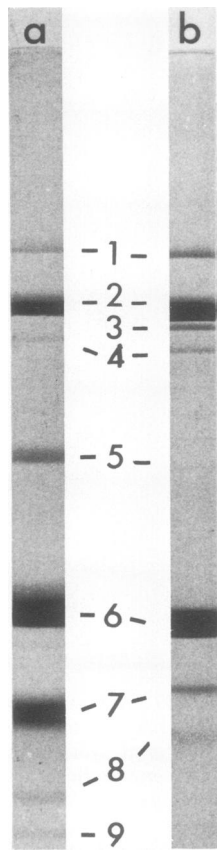


FIG. 2. Electrophoresis of polypeptides of diarrhea viruses of (a) calf origin (from the band at density 1.36 in a CsCl gradient) and (b) human origin (from the band at density 1.37 in a CsCl gradient) on 8.75% polyacrylamide gels. Migration was from top to bottom.

that observed for reovirus and orbivirus nucleic acids and indicates that the calf diarrhea virus nucleic acid is double-stranded in nature. The measured T_m was 78 C, which is close to the value of 81 C obtained by Welch and Thompson (31). Both these values are somewhat lower than that obtained for reovirus ($T_m = 84$ C) in $0.1 \times$ SSC by Bellamy et al. (2).

Electrophoresis of the nucleic acid of the calf diarrhea virus and molecular weight determination. Unlike the reovirus and orbivirus genomes, the calf diarrhea virus genome could not be resolved on 7.5% polyacrylamide gels unless the nucleic acid preparation was deproteinized before electrophoresis. Using this technique, 11 bands were resolved compared with the 10 bands for reovirus shown in Fig. 4. The bands do not appear to follow the charac-

teristic grouping of the reovirus genome bands into three distinct size classes.

The calculated molecular weights (see Materials and Methods for method of calculation) of the calf diarrhea virus genome bands are listed in Table 3, together with those of the reovirus genome bands. To determine if any of the bands obtained for either virus genome contained more than one nucleic acid component, molar ratios were calculated by the following method. Densitometer tracings were made of the polyacrylamide gels after fractionation of the reovirus and calf diarrhea virus genomes (Fig. 5). The resolution obtained by scanning was not as fine as that observed on the stained gels. Eight peaks were obtained for the calf diarrhea virus genome and seven for the reovirus genome. For each virus, the area under each peak was cut out of the homogeneous chart paper and weighed, providing an estimate of the amount of nucleic acid represented by the peak. The molar ratios were then calculated from the relative molarities (Table 3). These results show that the reovirus genome is composed of 10 segments, which agrees with previous reports (11, 25), and that the calf diarrhea virus genome is composed of 11 segments. The genome segments represented by each peak are shown in Table 3, and the segments represented by each band resolved on polyacrylamide gels are shown in Fig. 4. In both these figures, the numbers do not represent a peak for peak or band for band comparison between the two virus genomes.

DISCUSSION

The human and calf diarrhea viruses have been classified as reovirus-like (9, 13, 30) or orbivirus-like (3, 30) on the basis of morphology and morphogenesis; however, neither virus

TABLE 1. Molecular weights of the polypeptides of the calf and human diarrhea viruses

Polypeptide no.	Mol wt of diarrhea viruses	
	Human	Calf
1	127,000	131,000
2	103,000	103,000
3	97,000	97,000 ^a
4	88,000	92,000
5	58,000	58,000
6	32,000	32,000
7	26,000	22,000
8	21,000	16,500
9		14,500

^aThis polypeptide was only detected occasionally.

TABLE 2. Colorimetric determination of calf diarrhea virus nucleic acid using the orcinol and diphenylamine reagents^a

Nucleic acid	Diphenylamine index (OD ₆₀₀ /OD ₂₆₀)	Orcinol index (OD ₆₇₀ /OD ₂₆₀)
Salmon sperm DNA (ca. 100 µg)	0.285/0.920 = 0.310	0.130/1.500 = 0.090
Bakers' yeast RNA (ca. 100 µg)	0.004/1.700 = 0.002	0.285/1.580 = 0.180
Calf diarrhea virus	0.001/1.050 = 0.001	0.125/0.798 = 0.160

^a OD, Optical density.

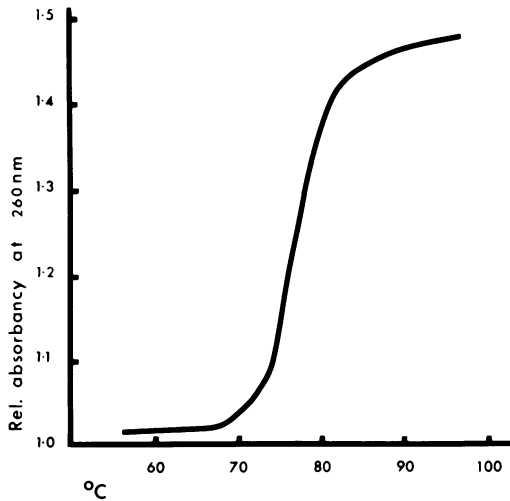


FIG. 3. Thermal denaturation curve of calf diarrhea virus nucleic acid.

shows any serological relationship to either reovirus types 1, 2, and 3 or to any of 20 orbiviruses (13). On the other hand both viruses have been found to be serologically related to each other and to epizootic diarrhea of infant mice virus by immunofluorescence, immunoelectron microscopy, and complement fixation studies (10, 13). Immunofluorescence studies have also shown that the calf diarrhea virus and the simian agent SA-11 are serologically related to each other (Rodger and Holmes, unpublished data). To these morphological and serological findings we now add the results of biophysical and biochemical studies on the human and calf diarrhea viruses.

Polyacrylamide gel fractionation of the diarrhea viruses indicates eight polypeptides, or possibly nine in the case of the calf diarrhea virus. For both the reoviruses and bluetongue virus, however, seven polypeptides have been detected by the same method of analysis of purified virus preparations (16, 26, 28). The fractionation patterns of polypeptides obtained

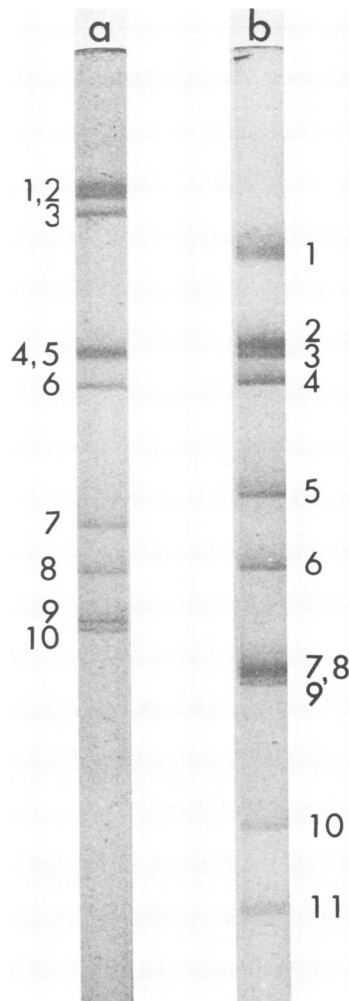


FIG. 4. Electrophoresis of the RNA genomes of (a) reovirus type 3 and (b) calf diarrhea virus on 7.5% polyacrylamide gels. Migration was from top to bottom.

for the diarrhea viruses more closely resembled each other than they did those of the reoviruses or bluetongue virus.

TABLE 3. Molecular weights and molar ratios of the RNA molecules obtained by electrophoretic fractionation of the genomes of calf diarrhea virus and reovirus type 3

Peak no.	Genome segments ^a	Calf diarrhea virus		Peak no.	Genome segments ^a	Reovirus	
		Mol wt	Molar ratio ^b			Mol wt	Molar ratio ^b
1	1	2.2	0.92	1	1, 2	2.45 ^c	1.84
2	2, 3	1.6 ^c	1.68	2	3	2.3	0.60
3	4	1.5	0.92	3	4, 5	1.5 ^c	2.09
4	5	0.96	0.91	4	6	1.3	0.95
5	6	0.75	0.87	5	7	0.88	1.27
6	7, 8, 9	0.51 ^c	3.10	6	8	0.75	0.92
7	10	0.32	1.19	7	9, 10	0.63 ^c	2.40
8	11	0.24	1.39				

^a Determined from molar ratio calculations.

^b Calculated according to Materials and Methods.

^c Represents an average of the molecular weights of bands resolved on polyacrylamide gels.

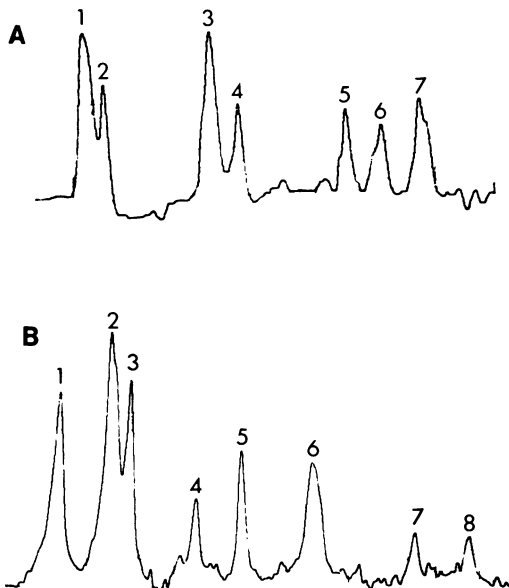


FIG. 5. Densitometer tracings of the fractionated genomes of (A) reovirus type 3 and (B) calf diarrhea virus on polyacrylamide gels.

Analysis of the genome of the calf diarrhea virus showed it to consist of double-stranded RNA. This finding supports that previously reported for this virus (30, 31). By polyacrylamide gel electrophoresis the calf diarrhea virus genome was resolved into 11 segments. Overall, these observations suggest a close similarity between the calf diarrhea virus genome and those of reoviruses and bluetongue virus, although only 10 genomes have been resolved for these viruses (25, 29). Our results also underline a general similarity between the calf diarrhea virus genome and those of wound tumor virus, rice dwarf virus, and cytoplasmic polyhedrosis

virus. However, the double-stranded RNA genomes of wound tumor virus and rice dwarf virus have been resolved into 12 segments (11, 24), whereas that of cytoplasmic polyhedrosis virus has been resolved into 10 segments (11).

On the basis of these results, it is clear that the calf diarrhea virus can be placed within the family *Reoviridae*, but most suitably in a genus separate from the reoviruses and the orbiviruses. Morphological, serological, and preliminary biochemical studies indicate that the human diarrhea virus, epizootic diarrhea of infant mice virus and SA-11 virus can most probably be included along with the calf diarrhea virus in this newly proposed duovirus (6) or rotavirus (10) genus.

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