CHARACTERIZATION OF A VIRUS NEWLY ISOLATED FROM THE SMOKY-BROWN COCKROACH, *PERIPLANETA FULIGINOSA* (SERVILLE)

CHIHARU SUTO

Department of Medical Zoology, Nagoya University School of Medicine, Showa-ku, Nagoya 466

(Director: Prof. Nobuo Kumada)

ABSTRACT

A virus newly isolated from the smoky-brown cockroach, *Periplaneta fuliginosa*, was characterized. The virus showed hexagonal structure with a diameter of 19–21 mμ and had a sedimentation value of approximately 110 S and a buoyant density of 1.41 g/ml. Neither treatment with chloroform nor heating at 56°C destroyed its infectivity. The virus was stable at pH 3.0. The virus consisted of about 31% of deoxyribonucleic acid and 69% protein. The viral DNA *in situ* was suggested to be single-stranded by staining property of the virus with acridine orange as well as by reaction to formaldehyde. Furthermore, it was demonstrated that the viral DNA extracted in low salt buffer exhibited single-stranded nature, whereas in high salt buffer double-stranded. Four virion proteins, VP I (M.W., 48,000), VP II (M.W., 52,000), VP III (M.W., 61,000) and VP IV (M.W., 76,000) were detected by SDS-polyacrylamide gel electrophoresis, and they accounted for 6, 58, 30 and 6% of the total viral protein, respectively. These data indicate that the new isolate belongs to the *Densovirus* of the family *Parvoviridae.*

Key words: Cockroach virus; characterization of cockroach virus; Parvovirus; Densovirus; single-stranded DNA virus

INTRODUCTION

In some Orthopteran insects such as cockroaches and locusts, tumor-like lesions of the alimentary canal have been experimentally induced by nerve severance[1], duct ligation[2], decapitation[3], and anal blockage[4], *etc.* Many factors have been discussed by Harshbarger and Taylor[5] to be causative in the formation of these lesions. Matz *et al.*[6] reported that the lesions in *Locusta migratoria* were transmitted by injecting hemolymph of the "tumorous" locusts, cell free extracts and ribonucleic acid extracts prepared from the "tumorous" lesions of *L. migratoria.* On these evidences Matz[7] suggested that the lesion might be infectious and that a RNA virus might be the agent. Meanwhile, Taylor and Preston[8] threw a doubt on the existence of the infectious materials in tumor-like lesions of Madeira cockroach, *Leucophaea maderae*, because they could not detect any infectious agents from *L. maderae* harboring the lesions.

We have recently found a kind of lesion that occurred spontaneously in more than 80% of our laboratory colonies of the smoky-brown cockroach, *Periplaneta fuliginosa* (Serville), that had been collected in Nagoya district and reared in our laboratory. Histological examinations of the lesion suggested that it was not of neoplasmic nature but a sort of repairing

須藤千春

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process of injury at the hindgut; we regarded it as a kind of ulcer[9]. We also noticed unusually high mortalities among late-instar nymphs and adults that suffered from the ulcer. Symptoms of this fatal disease suggested a paralytic one of viral origin.

In the previous paper, we reported isolation of a viral agent from these diseased cockroaches[10]. The agent had some similar properties known for members of the Parvovirus group and was tentatively designated as "cockroach virus".

This report deals with the more detailed physicochemical properties of the virus.

MATERIALS AND METHODS

Insect: The smoky-brown cockroaches, *P. fuliginosa,* used in this study were derived from the stock cultures which had been collected in Nagoya district or offered by several other laboratories in Japan and had been maintained by feeding on dried mouse food (Nippon Clea, Inc.) in our laboratory[9].

Virus: The virus was originally isolated from the diseased cockroaches and then passed and propagated successively in the cockroaches[10]. The virus materials were stored at −20°C until use.

Virus assay: Late-instar nymphs were anaesthetized with carbon dioxide and each of them was injected with 0.01 ml of the serially diluted virus suspensions into the hemocoele. The inoculated cockroaches were reared in a group of about ten in a mouse cage (30 × 15 × 15 cm) at 25°C. Daily mortalities were recorded and the LD_{50} or LT_{50} were calculated according to Reed and Muench[11] or Finny's graphical method[12].

Chloroform sensitivity: The crude virus suspension was thoroughly mixed with 1/10 volume of chloroform in a stoppered tube. This mixture was kept at 25°C for 15 min and centrifuged to separate aqueous phase, which was assayed for infectivity after serial dilution.

Temperature stability: The serially diluted virus suspensions were incubated at 56°C for 20 min and their infectivities were assayed.

pH stability: One volume of the virus suspension was serially diluted with nine volume of 0.1 M citrate buffer solution at pH 3.0 in a stoppered tube and incubated for 3 h at 5°C.

Purification of the virus: A mass of the diseased cockroaches weighing 100 g was homogenized in 900 ml of phosphate buffered saline (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.2) containing streptomycin (100 μg/ml) and penicillin (500 u/ml). The homogenate was squeezed to remove large fragments and centrifuged twice at 10,000 x*g* for 20 min at 4°C. The supernatant (Sup-1) was shaken with an equal volume of chloroform and *n*-buthanol mixture (v/v, 1:1), and the emulsion was centrifuged at 10,000 x*g* for 20 min to separate the aqueous phase. The aqueous phase was ultracentrifuged at 105,000 x*g* for 90 min at 4°C in a Spinco L or IEC B-60 ultracentrifuge. The pellets obtained were resuspended in about 10 ml of PBS by Teflon homogenizer, and the suspension was centrifuged at 10,000 x*g* for 20 min. Then the suspension was layered on the top of 10–30% linear sucrose gradient in PBS and centrifuged at 25,000 rpm for 90 min at 4°C in a Spinco SW 27 rotar. Fractions containing the virus bands were pooled and dialyzed against PBS with two or more changes. The dialyzed virus suspension was adjusted to give a density of 1.39 g/ml by adding solid CsCl and centrifuged at 39,000 rpm for 18 h in a Spinco SW 50 rotar. The resultant gradient was fractionated by puncturing the bottom of the tube, and optical densities of the fractions were measured at 260 mμ.

Electron microscopy: The purified virus preparations were negatively stained with 1%

potassium phosphotungstate (pH 7.0), and the grids were examined in a Hitachi HU-11D electron microscope.

Chemical analysis of the virus: Protein determination was made by the method of Lowry *et al.*[13] with bovine serum albumin as standard. DNA determination was made by the diphenylamine reaction modified by Burton[14], using salmon sperm DNA as standard. To test the presence of RNA the orcinol reaction of Mejbaum[15] was employed.

Analytical ultracentrifugation: Sedimentation velocity of the virions was measured in a Spinco model E analytical ultracentrifuge equipped with schlieren optics. Virus preparation in PBS was centrifuged at 12°C and 37,020 rpm using AnD rotar. Photographs were taken at 4 min intervals.

Acridine orange staining: Droplets containing the purified virus were allowed to dry on coverslips at room temperature and fixed in Carnoy's fluid for 5 min. After dehydration in ethyl alcohol series, the coverslips were rinsed in distilled water and placed in McIlvaine's citric acid-disodium phosphate buffer at pH 4.0 for 10 min before staining in 0.01% acridine orange as described by Mayor and Hill[16]. To test nuclease sensitivity, Carnoy-fixed virus preparations were incubated for 30 min at 37°C in 0.05% ribonuclease-A in McIlvaine's buffer at pH 4.0 or deoxyribonuclease-1 in 0.01 M phosphate buffer containing 0.003 M $MgSO_4$ at pH 7.2. As reference, tobacco mosaic virus (single-stranded RNA), Chilo iridescent virus (double-stranded DNA), and reovirus (double-stranded RNA) were stained with the same method.

Reaction with formaldehyde: The purified virus preparations were tested for reactivity with 1.8% formaldehyde[17]. The virus suspension was mixed with 10% neutralized formalin to give a final concentration of 1.8% formaldehyde. The mixture was incubated in a stoppered tube at 37°C for required length of time and the absorption spectrum was determined. The viral nucleic acids extracted in appropriate buffers were made to react with formaldehyde in the same manner.

Extraction of DNA: DNA was extracted from the purified virus in 1 mM phosphate buffer (PB) or in 0.1 M NaCl–PB (pH 7.2) by gently shaking with an equal volume of saturated phenol in the buffer at 55°C for 45 min[18]. The suspension was spun at 1,000 xg for 10 min at 4°C to separate phase, from which aqueous phase was removed. Two further phenol extractions were performed at 45°C, followed by two *n*-buthanol-chloroform extractions and three ether extractions at room temperature. Then the DNAs were dialyzed into PB or 0.1 M NaCl–PB, respectively, at 5°C overnight.

Polyacrylamide gel electrophoresis of the viral proteins: The virus preparations were solubilized in 0.01 M phosphate buffer (pH 7.2) containing 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol at 95°C for 5 min. A sample of about 100 μl was layered onto 5 to 10% polyacrylamide gels and then subjected to electrophoresis at 6 mA per gel until bromophenol blue tracking dye reached the end of the gel. The gels were stained overnight with 0.2% Coomassie brilliant blue R250 in methanol-acetic acid-water (50:7:50 by volume) and destained in acetic acid-methanol-water (7.5:5:7.5) or 7.5% acetic acid. The destained gels were scanned with a densitometer at 550 mμ.

Molecular weights of the virus polypeptides were estimated from their migration rates relative to those of the standard proteins (BDH's molecular weight markers; M. W. range 14,300–71,500; Product No. 44223 2U, Poole, England).

RESULTS

Symptoms

When the isolated virus was injected into the cockroaches, they became incoordinated in their movement showing paralysis of the hindlegs, swelling of the abdomens, enlargement of the fat body, and change in the fat body color from brownish white to milk-white. These symptoms were all recognized at later stage of the infection, which were followed by rapid death of the insects. A linear relationship was found between the mean lethal time and the log of the inoculum concentration (Fig. 1).

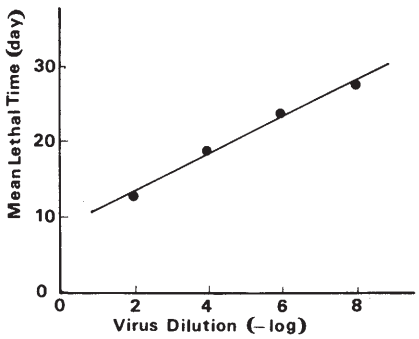

Fig. 1. Relationship between the mean lethal time and the log of the inoculum concentration.

Effect of chloroform, heating and low pH on the viral infectivity

Insensitivities of the virus to lipid solvents, heating at 56° C and acid are in general qualitative criteria for the classification of viruses. Table 1 shows that the virus isolated from the cockroach is not inactivated by any of these factors.

Table 1. Effect of chloroform-, acid-, and heat treatments on stability of the virus.

Virus dilution	LT_{50} (Day)			
	Control (pH 7.2)	Chloroform (*ca.* 25°C, 15 min)	Acid (pH 3.0)	Heat (56°C, 20 min)
2×10^{-2}	14.3	18.0	14.0	15.7
2×10^{-3}	17.0	19.0	16.0	19.8
2×10^{-4}	20.0	19.3	21.2	20.5
2×10^{-5}	23.8	23.8	23.3	24.5
2×10^{-6}	23.5	24.0	25.5	26.0
2×10^{-7}	26.8	27.3	28.0	29.0
$-\log LD_{50}$	6.7	6.1	5.9	5.8

One-hundredth ml of Sup-1 treated with chloroform, acid and heat was inoculated into the hemocoele of mature nymphs of *P. fuliginosa*. The LD_{50}s were calculated from mortalities by the 25th day after inoculation.

Purification of the virus

The crude virus material was partially purified by differential centrifugation, treated with chloroform-buthanol mixture, and then centrifuged through a 10–30% sucrose gradient. Sedimentation profile in the sucrose gradient exhibited a main band(A) with a small shoulder at fraction 11(B) and top component(C) (Fig. 2). Fractions from the main band and the shoulder were pooled and dialyzed against PBS, and then adjusted to give a density of 1.39 g/ml by adding CsCl. Subsequent isopicnic centrifugation at 39,000 rpm for 18 h at 4°C in a Spinco SW 50 rotar yielded a major band(A) with a buoyant density of 1.41 g/ml and a minor band(B) of 1.44 g/ml (Fig. 3). The resolution of the two bands in CsCl was sufficient enough to obtain the materials separately by puncturing the tube at the appropriate positions. All the experiments described below were performed using the purified virus recovered from the major band.

Centrifugation of the purified virus in an analytical centrifuge gave a sedimentation value of 110 S. Chemical analysis indicated that the purified virus contained DNA and protein with a ratio of 31:69. Fig. 4 shows the electron micrograph by negative staining of the purified virus preparation. The viruses are seen as naked and hexagonal particles. Most of them appeared to be full, but a few empty particles penetrated by the negative stain were also observed. Smaller particles with a diameter of about 10 mμ were intermingled. These smaller particles seemed to be polyferritin granules as observed frequently in purified parvovirus preparations[19,20,21]. Distribution of the virus sizes ranged from 17 to 25 mμ. However, particles with a diameter of 19 to 21 mμ were most frequently observed (Fig. 5).

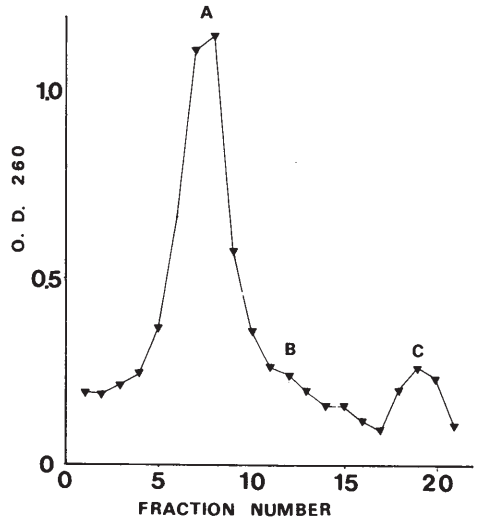

Fig. 2. Sedimentation profile of the virus in a sucrose gradient. A crude virus suspension was shaken with an equal volume of chloroform-buthanol mixture (1:1). Aqueous phase separated by high speed centrifugation was concentrated by ultracentrifugation at 105,000 xg for 90 min. The concentrated suspension was layered on top of 10–30% sucrose gradient and centrifuged at 25,000 rpm for 90 min in a Spinco SW 27 rotar. A, main band; B, shoulder; C, top component.

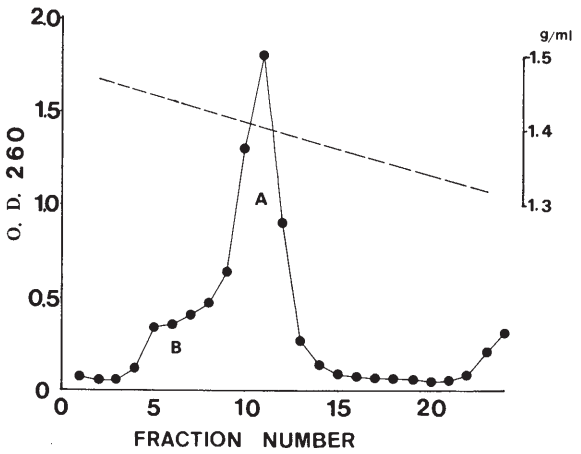

Fig. 3. Isopicnic centrifugation in CsCl of partially purified virus recovered from the sucrose gradient. The virus-CsCl mixture at average density of 1.39 g/ml was centrifuged at 39,000 rpm for 18 h in a Spinco SW 50 rotar and fractions were collected. Left ordinate, optical density at 260 mμ; right ordinate, density of gradient fractions (dashed line).

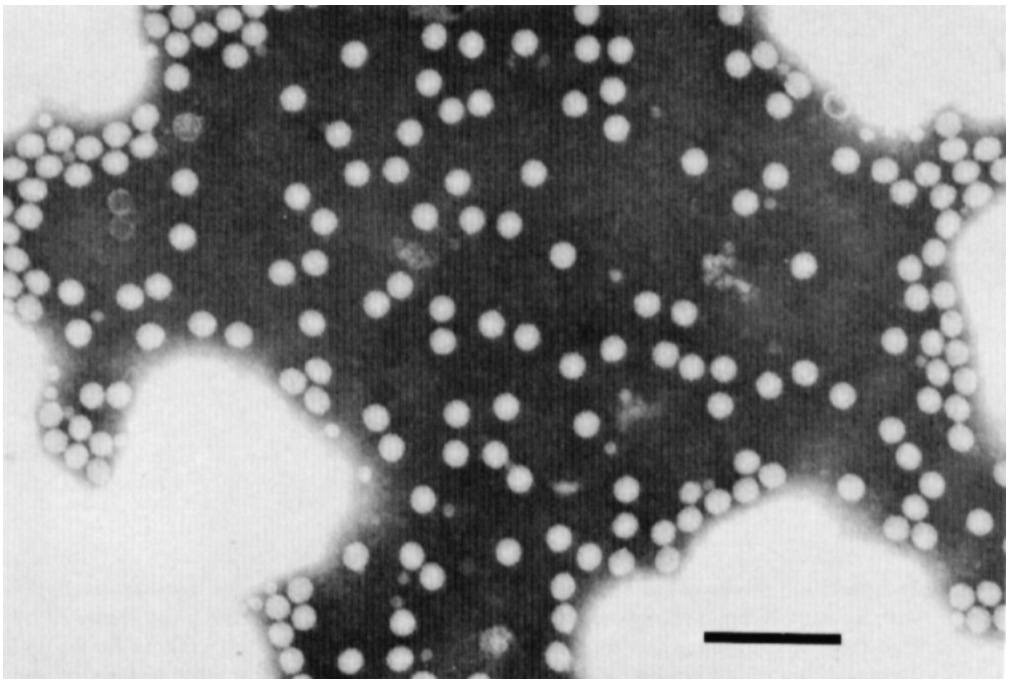

Fig. 4. Electron micrograph of the purified virus obtained from a band at a density of 1.41 g/ml and negatively stained with potassium phosphotungstate. A few empty and the smaller particles among the complete virions are visible. Bar: 100 mμ.

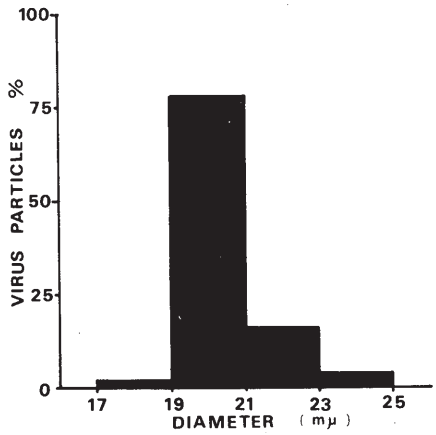

Fig. 5. Size distribution of one hundred virus particles after negative staining.

Analysis of the properties of the viral DNA

Dried droplet preparations of the virus were fixed, stained with acridine orange and observed with fluorescent microscope. The sample gave a brilliant flame-red fluorescence. The development of this red fluorescence of the virus was completely suppressed by prior treatment with DNase, but not affected with RNase (Table 2). Thus the viral DNA appeared to be single-stranded. The nature of DNA was also examined by monitoring the reactions after treatment with formaldehyde.

The absorption spectra of the intact virus before reaction with formaldehyde were typical of those of a nucleoprotein, with a maximum absorption at 263 mμ and minimum at 243 mμ. The A_{260}/A_{280} and A_{max}/A_{min} were 1.43 and 1.48, respectively. As shown in Fig. 6, exposure of the virus to 1.8% formaldehyde caused an increase in absorption and a shift of the absorption maximum to the region of longer wavelength. The increase was about 11% at the maximum and about 6% at 260 mμ. These data also indicate that the viral DNA *in situ* is single-stranded.

The conformation of extracted viral DNA is known to show distinct variations depending on the concentration of salt in the case of single-stranded DNA viruses[18,19,20]. Whether this is the case or not was examined with the extracted viral DNA under low and high salt conditions followed by reaction to formaldehyde.

Table 2. Staining properties of the purified virus with acridine orange.

Virus	Color developed after staining	Nuclease susceptibility	
		DNase	RNase
Cockroach virus	Flame red	+	–
Tobacco mosaic virus	Flame red	–	+
Chilo iridescent virus	Yellowish green	*	*
Reovirus	Yellowish green	*	*

Droplets of the purified virus preparations were fixed in Carnoy's fluid at pH 4.0 and stained with 0.01% acridine orange.
* Not examined.

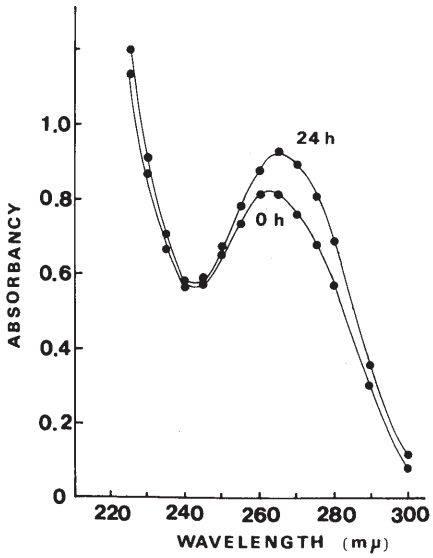

Fig. 6. Effect of formaldehyde on the absorption spectrum of the purified virus. Neutralized formalin was added to a virus suspension in PBS to give a final concentration of 1.8% HCHO. The absorption spectrum was determined immediately and the suspension was incubated at 37°C.

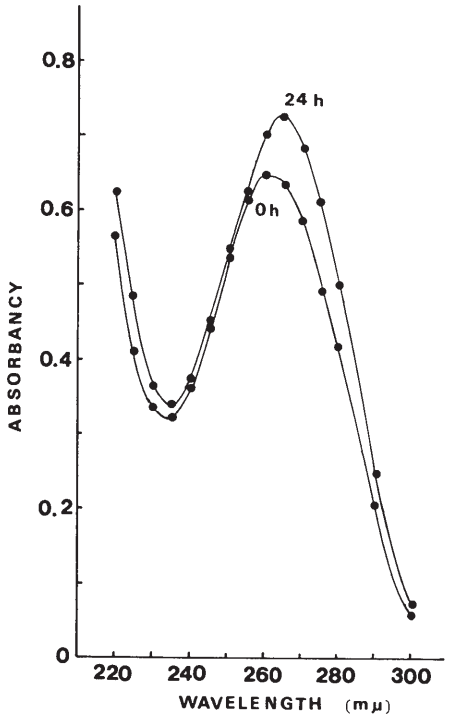

Fig. 7. Effect of formaldehyde on the absorption spectrum of the viral DNA extracted in 1 mM phosphate buffer.

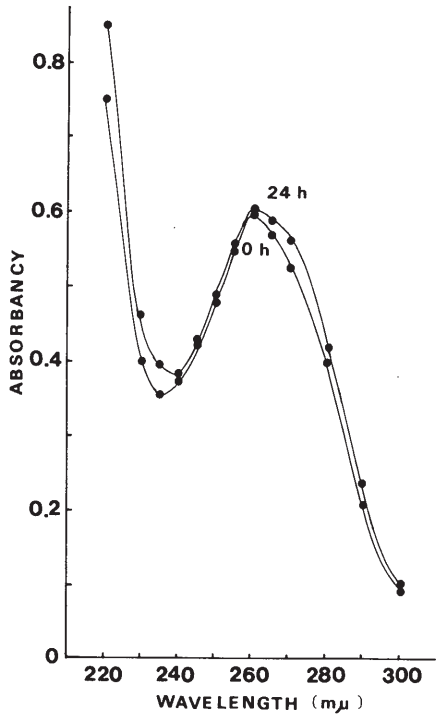

Fig. 8. Effect of formaldehyde on the absorption spectrum of the viral DNA extracted in 0.1 M NaCl–1 mM phosphate buffer.

All preparations of the viral DNA before reaction with formaldehyde had absorption spectra typical of the nucleic acid (Figs. 7, 8). The effect of 1.8% formaldehyde on the viral DNA extracted in 1 mM PB is shown in Fig. 7, which indicates an increase in optical absorption and a shift in the maximum absorption at 260 to 265 mμ. The increase was *ca.* 7% at 260 mμ and *ca.* 13% at 265 mμ. Thus the viral DNA may be single-stranded if extracted with low-salt buffer. In contrast, when the viral DNA extracted in 0.1 M Nacl–1 mM PB, such shift of absorption did not take place and the profile gave a typical one for double-stranded DNA (Fig. 8). These results indicate that the secondary structure of the viral DNA is markedly affected by the environmental salt condition.

Structural proteins of the virus

Proteins extracted from the purified virus with SDS were analyzed by electrophoresis on a SDS-polyacrylamide gel followed by staining of the gel with Coomassie brilliant blue. As shown in Fig. 9, four polypeptides were observed on the gel. They were designated as VP I, VP II, VP III, and VP IV in order of their decreasing migration rates; their molecular weights determined by coelectrophoresis with marker proteins were 48,000, 52,000, 61,000 and 76,000, respectively (Fig. 10). These polypeptides accounted for 6, 58, 30 and 6%, respectively, of the total virion protein, when calculated by scanning the individual bands at 550 mμ in a densitometer.

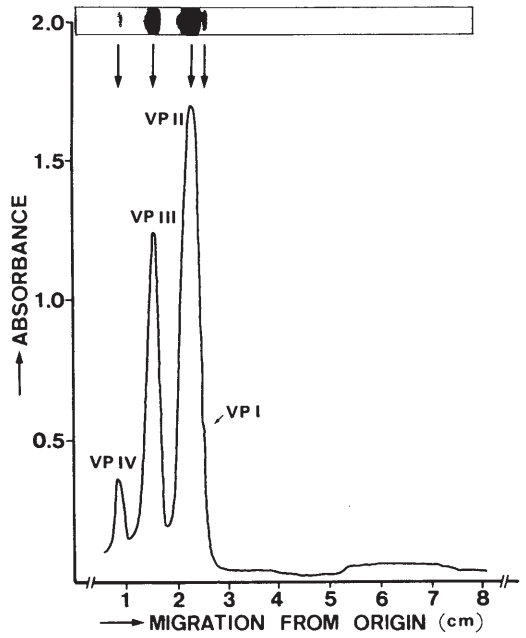

Fig. 9. Densitometer tracing of the virus polypeptides after electrophoresis on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue, destained in 7.5% acetic acid and scanned at 550 mμ. Four polypeptide peaks were identified and named VP I–IV in order of their mobilities.

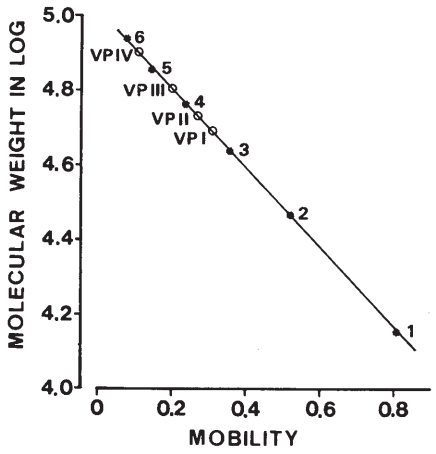

Fig. 10. Estimation of molecular weights of the virus polypeptides by electrophoresis in 10% gels. Marker proteins and the purified virus were treated with SDS and subjected to electrophoresis on parallel gels. After staining, distances migrated by the marker proteins relative to bromophenol blue tracking dye were plotted against logarithms of the molecular weights. The relative distances of migration of the virus polypeptides (VP I–IV) were determined, and their molecular weights were estimated from the plot. Marker proteins: 1, monomer (M.W., 14,300); 2, dimer (28,600); 3, trimer (42,900); 4, tetramer (57,200); 5, pentamer (71,500); 6, hexamer (85,800).

DISCUSSION

The virus highly pathogenic to the smoky-brown cockroach was isolated and analyzed for its physical, chemical and morphological characteristics. The data obtained so far have indicated that the virus is to be classified into parvovirus group[22,23,24]: The virus has a diameter of 19–21 mμ; the virus is hexagonal in shape suggesting its icosahedral symmetry; the virus is resistant to chloroform, acid and heating; the virus contains single-stranded DNA; the virus replicates in the nuclei and forms crystalline arrays in the infected cells, with no helper virus[10].

Density gradient centrifugation in CsCl of the virus revealed two types of the virus: A major peak at 1.41 g/ml and a heavy minor peak at 1.44 g/ml. It is known that parvoviruses have a relatively high density and heterogeneity in isopicnic centrifugation. Hoggan[19] compared the density of nine parvoviruses including densonucleosis virus (DNV), or one of the insect parvoviruses. His results showed that the density values of the major infectious band were in a range of 1.38 to 1.45 g/ml. He also noted that a heavy minor band (1.44–1.47 g/ml) was frequently found in addition to a major infectious band and a number of lighter bands with all the parvoviruses studied. He suggested that this difference in the buoyant densities might be due to the loss of protein layer. But Johnson and his coworkers[25,26] ascribed this difference to a lesser content of minor protein, and Clinton and Hayashi[27] to a proteolytic conversion from the dense to the lighter type. Tijssen *et al.*[28] have recently reported that the difference in densities of DNV might be due to a different protein content and a difference in the balance of forces that determine polypeptide structure of the virion.

In our preliminary analysis, there was no difference of polypeptide patterns between virus particles with a buoyant density of 1.41 g/ml and those with that of 1.44 g/ml. Investigation of these dense particles remains to be done until sufficient amount will be obtained.

Staining reaction with acridine orange and relevant enzyme susceptibility patterns have been used as a simple and direct means for detecting the type and structure of nucleic acid in viruses. Double-stranded nucleic acid exhibits yellowish green fluorescence, while single-stranded one yields flame-red fluorescence[16,29]. The result of the staining reaction and the pretreatment with DNase of the cockroach virus revealed that it contained single-stranded DNA.

Formaldehyde reaction with the virus caused an increase in absorption and a shift of the absorption maximum toward the longer wavelength (Fig. 6). This result suggests that the protein coat of the virus is penetrated by formaldehyde and that the amino groups in the bases of DNA are not occupied by hydrogen bonding between base pairs. Although the increase was not as great as that reported for ϕX 174[17], or minute virus of mice[30], it was almost identical with those in Kilham rat virus[31,32], adeno-associated virus (AAV)[33], and DNV[18].

Reactivities of the extracted DNA with formaldehyde was determined to know whether the single-stranded DNA in the virion was separately encapsidated into "plus" and/or "minus" strands. The results (Figs. 7 and 8) showed that the DNA extracted in low-salt buffer presented properties typical of the single-stranded one as observed in the DNA *in situ*. The DNA in high-salt buffer, however, demonstrated properties of double-stranded one, presumably because the given salt concentration was enough to permit the DNA to form double strands[18]. Thus, the results suggest that the virus contains complementary plus and/or minus single-stranded DNA packaged separately in the virus particles.

The presence of complementary plus and minus single-stranded DNA is not unique, since the AAV's[19] and DNV[20] were also known to contain similar DNA. However, it should be noted that the AAV's are the satellite viruses that depend on adeno viruses for their replication, whereas the cockroach virus and DNV can replicate "autonomously" without requiring the genome of a helper virus for their replication.

Most of the parvoviruses examined so far have three structural proteins in the virions. Tijssen *et al.*[28] recently reported the presence of four polypeptides with M.W.'s of 49,000, 59,000, 69,000 and 98,000 in DNV from the results of different gel concentrations. Tinsley and Longworth[21] reviewed parvoviruses and stated that DNV contained three different polypeptides in 5% polyacrylamide gels, but that four bands were found in 10% gel systems. Results by Kelly *et al.*[18] were compatible with those of Tijssen *et al.* but did not support the results by Tinsley and Longworth. Polyacrylamide gel electrophoresis of the cockroach virus proteins revealed the presence of four polypeptides with molecular weights of 48,000 (VP I), 52,000 (VP II), 61,000 (VP III) and 76,000 (VP IV); and about 60% of the total virus protein was composed of the major polypeptide (VP II).

The resemblance of the protein structure and characteristics of nucleic acid of the cockroach virus with those of DNV is striking and suggests that they may belong to the same group; the *Densovirus* of the family *Parvoviridae*. As the virus was isolated from the cockroach colonies showing high incidence of hindgut ulcer, possibilities of its playing a role in the induction of ulcer or tumor-like lesion should be further investigated.

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