STUDIES ON FIJI DISEASE VIRUS WITH SPECIAL REFERENCE TO THE VIRAL NUCLEIC ACID

by

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TABLE OF CONTENTS

Page

Page

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STATEMENT

This thesis has not previously been submitted for an academic award at this or any other University, and is the original work of the author, except where due reference is made in the text.

Masato Ikegami

SUMMARY

1. Spherical particles 55-60 nm in diameter have been purified from leaf galls of sugarcane infected with Fiji disease virus (FDV) by differential centrifugation, treatment with the nonionic detergent Nonidet P40 and sucrose density-gradient centrifugation; they are subviral particles.

2. Immunization of mice with preparations of FDV subviral particles resulted in the production of antibodies to both viral protein and viral RNA. These antibodies were present in both the blood serum and in ascitic fluid produced in response to injection with Krebs 2 Ascites tumour cells.

3. Positive serological reactions were observed in immunodiffusion tests between FDV and antisera to rice dwarf and maize rough dwarf viruses, and between maize wallaby ear virus and antisera to FDV. However, the reactions were shown to result not from the precipitation of viral proteins but of double-stranded (ds) RNA.

4. The specificities of antibodies to ds-polyribonucleotides in sera from animals immunized with three plant viral antigens and one synthetic ds-RNA were studied in immunodiffusion tests with four distinct ds-RNA antigens. Although antibodies in all four antisera reacted with all four antigens tested, the antibodies showed some specificity.

5. Nucleic acid isolated from subviral particles of FDV was identified as ds-RNA by the following properties: (1) Positive orcinol reaction; (2) Negative diphenylamine reaction; (3) Resistance to ribonuclease (RNase) in 1 x SSC (0.15M-sodium chloride and 0.015M-sodium citrate buffer, pH 7) but not in 0.1 x SSC; (4) Susceptibility to RNase in 1 x SSC after thermal denaturation; (5) Sharp thermal denaturation curve with a melting temperature of 76°C in 0.01 x SSC; (6) Buoyant density of 1.60 g/cm^3 in Cs_2SO_4; and (7) No increase in ultraviolet absorption on treatment with formaldehyde at 37°C. On electrophoresis in polyacrylamide gel, FDV-RNA separated into nine RNA segments with a total molecular weight of 15.3×10^6.

6. RNA-dependent RNA polymerase activity was detected in concentrated extracts of gall tissue from FDV-infected leaves but not in similar extracts from healthy leaf tissue. The polymerase activity was correlated with FDV antigen and some polymerase activity was also detected in preparations of FDV subviral particles. Optimal polymerase activity occurred at 35°C, at pH between 8.5 and 9.0, and in the presence of 8 mM $MgCl_2$ and 200 mM NH_4Cl. The polymerase product was single-stranded RNA which was apparently transcribed from FDV ds-RNA.

7. On the basis of data presented in this thesis FDV can be included in the family Reoviridae.

ABBREVIATIONS

The following abbreviations are used in the text.

A	Absorbance (optical density)
ATP	Adenosine-5'-triphosphate
Bisacrylamide	N N'-methylenebisacrylamide
BTV	Bluetongue virus
CTA	Cetyltrimethylammonium bromide
CTP	Cytosine-5'-triphosphate
cpm	Counts/min
CPV	Cytoplasmic polyhedrosis virus
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ds	Double-stranded
EDTA	Ethylenediaminetetra-acetic acid di-sodium salt
FDV	Fiji disease virus
GE buffer	0.1M-glycine, 0.005M-EDTA (pH 8.5)
GM buffer	0.1M-glycine, 0.01M-$MgCl_2$ (pH 8.5)
GMP	Guanosine-5'-phosphate
GTP	Guanosine-5'-triphosphate
g	Acceleration due to gravity
h	Hour
LNYV	Lettuce necrotic yellows virus

Leoning's buffer	40mM-Tris-HCl, 20mM-sodium acetate and 1mM-EDTA (pH 7.8)
M	Molar
min	Minute
mM	Millimolar
mmol	Millimole
MRDV	Maize rough dwarf virus
MWEV	Maize wallaby ear virus
nm	Nanometer (10^{-9}m)
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
Poly [I] : Poly [C]	Poly inosinic : Poly cytidylic acid
POPOP	1,4-di-2-(5-phenyloxazolyl) benzene
PPO	2,5 diphenyloxazole
PTA	Potassium phosphotungstic acid (adjusted to pH 6.8 with KOH)
PTE buffer	0.04M-sodium phosphate, 0.015M-Tris, 0.002M-EDTA (pH 7.6)
RBSDV	Rice black-streaked dwarf virus
RDV	Rice dwarf virus
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
ss	Single-stranded

1 x SSC	0.15M-NaCl, 0.015M-sodium citrate (pH 7.0)
ST buffer	0.1M-NaCl, 0.05M-Tris-HCl, 0.001M-EDTA (pH 6.7)
STE buffer	0.1M-NaCl, 0.05M-Tris-HCl, 0.005M-EDTA (pH 7.5)
STM buffer	0.1M-NaCl, 0.01M-Tris-HCl, 0.01M-$MgCl_2$ (pH 7.3)
TAM buffer	0.05M-Tris, 0.1M-NH_4Cl, 0.09M-2-mercaptoethanol (pH 8.4)
TEMED	N,N,N',N"-tetramethylethylenediamine
TCA	Trichloroacetic acid
Tm	The melting temperature
TMV	Tobacco mosaic virus
TNE buffer	0.1M-Tris-HCl, 0.1M-NaCl, 0.01M-EDTA (pH 7.0)
Tris	Tris(hydroxymethyl)aminomethane
UAc	Uranyl acetate
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
WTV	Wound tumour virus
^oC	Degree Centigrade

CHAPTER I

GENERAL INTRODUCTION

Fiji disease of sugarcane takes its name from the Fiji Islands in the South-West Pacific where it was first observed by Muir (1910) and Lyon (1910). The disease has been recorded in Fiji, New Guinea, Australia, Philippines, Samoa, Madagascar, New Britain and New Hebrides (Hutchinson and Francki, 1973).

Fiji disease causes a severe stunting of all parts of an affected sugarcane plant and galls develop on the lower surfaces of the leaves and sheaths. The galls originate in the phloem tissues of the fibro-vascular bundles. There is at first a proliferation of the cells, and then as the mass enlarges, it extends outward from the leaf (Hutchinson and Francki, 1973).

In Queensland, Mungomery and Bell (1933) observed that nymphs of *Perkinsiella saccharicida* Kirk which had been caged on Fiji diseased cane, transmitted the disease; this was confirmed by North and Barber (1935). In the Philippines, Ocfemia (1934a, 1934b) and Ocfemia and Celino (1939) reported that all nymphal stages and adults of *P.vastatrix* Breddin are able to transmit Fiji disease. It has been established that *P. vitiensis* Kirkaldy can transmit Fiji disease in Fiji and Samoa (Husain *et al.*, 1965). The disease can be transmitted to sugarcane (*Saccharum officinarum*), other *Saccharum* spp., maize (*Zea mays*) and *Sorghum* spp. by these three leafhopper species (Hutchinson *et al.*,1972).

Mungomery and Bell (1933) reported the presence of X-bodies in the cells of the abnormal gall tissues. More recently, polyhedral virus-like particles, about 70 nm in diameter, have been observed in the cells with X-bodies (Giannotti *et al.*, 1968; Teakle and Steindl, 1969; Francki and Grivell, 1972). These virus-like particles are very similar to those of reovirus and other viruses with double-stranded (ds)-RNA genomes such as wound tumour virus (WTV), and rice dwarf virus (RDV) (Teakle and Steindl, 1969; Francki and Grivell,1972). Similar particles have also been observed in *P. saccharicida* which had been caged on Fiji diseased cane (Francki and Grivell, 1972). It was concluded that these particles are probably the causal agent of Fiji disease. Francki and Jackson (1972) detected ds-RNA in gall tissues from Fiji diseased plants but not in tissues of healthy sugarcane leaves by using an antiserum to poly inosinic : poly cytidylic acid (poly [I] : poly [C]) with specificity for ds-polyribonucleotides. These observations have led to the provisional conclusion that Fiji disease is caused by a ds-RNA virus (Hutchinson and Francki, 1973). In this thesis the 70 nm particles associated with Fiji disease will be referred to as Fiji disease virus (FDV) particles.

The aim of the work described in this thesis was to purify and characterize FDV particles and to investigate their serological properties.

CHAPTER II

GENERAL MATERIALS AND METHODS

This chapter contains those materials and methods which were used throughout this study. Other materials and methods are described in the individual chapters of the thesis.

1. Virus-infected plant materials

Infected sugarcane plants (var. NCO-310) grown in the field or an insect-proof glasshouse were used. Galls were excised from leaves of infected plants with razor-blades taking care to include as little surrounding normal tissue as possible. The excised galls were used as starting material. Excised galls were stored at 4°C up to 4 days until required.

The U1 strain of tobacco mosaic virus (TMV) (Siegel and Wildman, 1954) was maintained on tobacco plants (*Nicotiana tabacum* v. White Burley). Upper surfaces of tobacco leaves were inoculated mechanically, and the plants were maintained in a constant temperature room at 25 ± 2°C under fluorescent lights providing continuous illumination of 350 - 450 ft-C.

2. Preparation of nucleic acids from plant tissues

Nucleic acids were extracted from frozen plant material by grinding it in a pestle and mortar with a little acid-washed sand, 3 ml of TNE buffer (0.1M-Tris-HCl, 0.1M-NaCl, 0.01M-EDTA, pH 7.0),

1 ml of sodium dodecyl sulphate (SDS), and 4 ml 90% phenol containing 0.1% 8-hydroxyquinoline for every 1 gm of leaf tissue (Francki and Jackson, 1972). The slurry was transferred to screw-topped bottles and shaken on a mechanical shaker for 45 to 60 min at 25°C. After centrifugation at 3,000 g for 5 min the buffer phase was extracted three more times by shaking with phenol for 5 to 10 min. The nucleic acids were precipitated from the buffer phase with 3 volumes of 95% ethanol and left at -15°C overnight. The precipitate was isolated by centrifugation, washed with 80% ethanol, and suspended in ST buffer (0.1M-NaCl, 0.05M-Tris-HCl, 0.001M-EDTA, pH 6.7). After centrifugation at 3,000 g for 5 min the nucleic acids were again precipitated from the supernatant with ethanol, washed in ethanol, dried with acetone, and suspended in the required buffer. If necessary, polysaccharides in these preparations were removed as described by Ralph and Bellamy (1964). Nucleic acid preparations in 0.025M-Tris-HCl buffer, pH 8.1, containing 0.025M-NaCl were mixed with equal volumes of 2.5M-potassium phosphate buffer (pH 8.0) and 2-methoxyethanol at 4°C. The mixture was centrifuged at 5,000 g for 2 min and the upper phase was added to an equal volume of 0.2M-sodium acetate and half a volume of 1% cetyltrimethylammonium bromide (CTA). After 5 min on ice, the CTA nucleate was isolated by centrifugation at 5,000 g for 5 min and converted to the sodium salt by washing three times with cold 70% ethanol containing 0.1% sodium acetate. The precipitate was further washed

with ethanol and then acetone, dried under vacuum and resuspended in the required buffer.

3. Preparation of viral RNA

Nucleic acid was extracted from purified virus preparations by either of two methods.

(a) Phenol-SDS method

Purified virus in STE buffer (0.1M-NaCl, 0.05M-Tris-HCl, 0.005M-EDTA, pH 7.5) was shaken for 10 min with one-third volume of 4% SDS and an equal volume of 90% phenol containing 0.1% 8-hydroxyquincline. After centrifugation at 3,000 g for 5 min the buffer phase was re-extracted two more times by shaking with phenol for 2-5 min. RNA was precipitated from the buffer phase by adding 3 volumes of cold ethanol and a few drops of 3M-sodium acetate (pH 5.5). The mixture was left at -15°C overnight. The precipitate was recovered by centrifugation, washed 3 times with cold ethanol, once with acetone and then dried under vacuum. The dried precipitate was resuspended in the required buffer and stored at -15°C.

(b) Pronase-SDS method

SSC buffer (0.15M-NaCl, 0.015M-sodium citrate, pH 7.0) containing 0.1 - 0.15% pronase and 0.5% SDS was kept at 37°C for 30 min to digest any contaminating nuclease. It was then used to resuspend virus pellets. The mixture was incubated at 37°C for 20 - 24 h (Murant *et al.*, 1972). After precipitation with ethanol as described above the nucleic acid was stored in 70% ethanol at -15°C until required.

4. Polyacrylamide gel electrophoresis (PAGE)

(a) Recrystallization of acrylamide and bisacrylamide

Acrylamide and bisacrylamide were recrystallised as described by Loening (1967). A 70 gm sample of acrylamide was dissolved in 1 l of chloroform at 50°C and the solution was filtered hot, without suction. Crystals from the filtrate formed at -15°C were recovered by filtration in a chilled filter funnel, washed briefly with cold chloroform, and dried. Bisacrylamide was dissolved in acetone (approximately 10 g/l at 40 - 50°C) and filtered hot. The solution was slowly cooled to -15°C; the crystals were recovered and washed with cold acetone by filtration.

(b) PAGE

A stock solution containing 15% recrystallised acrylamide and 0.3% recrystallised bisacrylamide in distilled water was stored at 4°C in darkness until use (Reddy and Black, 1973b). To prepare 5% polyacrylamide gels, 10 ml of stock acrylamide solution, 6 ml of 5 x PTE buffer (1 x PTE buffer; 0.04M-sodium phosphate, 0.015M-Tris and 0.002M-EDTA, pH 7.6) and deionized water were first mixed and degassed under vacuum for 20 sec. Then 0.04 ml of TEMED (N,N,N',N"-tetramethylethylenediamine) and 0.4 ml of freshly prepared 10% aqueous ammonium persulphate solution were added (Loening, 1967). After mixing, the solution was rapidly pipetted into vertical perspex tubes (0.6 cm internal diameter x 14 cm) to a depth of 11 cm. The lower end of

each tube rested on a dialysis membrane. Distilled water was floated over the gels with a glass capillary to ensure a plane gel surface. Tubes were left at room temperature for at least 2 h before use. PTE buffer was used for electrophoresis (Reddy and Black, 1973b). A 1M-sodium phosphate buffer was stored at -15°C. Stock solutions of 1M-Tris and 0.2M-EDTA were prepared separately and stored at -15°C. PTE buffer was prepared in distilled water just before use. RNA samples containing 10-15 µg were suspended in 15-20 µl of PTE buffer containing 7.5% sucrose. Bromophenol blue (0.2%, 10 µl) was used as a tracking dye. Electrophoresis was carried out at 6°C. Gel columns were pre-run for 1 h at 4mA per gel. After the RNA samples were loaded, the current was set at 2.5mA per gel and after 20 min at 4mA per gel. Electrophoresis was continued for a further 30 or 45 h. PTE buffer in each reservoir (300 ml) was changed every 5-6 h.

After electrophoresis, the gels were rinsed in 0.4M-acetate buffer (0.4M-sodium acetate, 0.4M-acetic acid, pH 4.7) for at least 15 min and then stained with 0.1% toluidine blue O in 0.4M-acetate buffer for 30 min (Reddy and Black, 1973b). Destaining was accomplished with several changes of distilled water. The stained gels were scanned in a Joyce-Loebl Chromoscan at 620 nm.

The 2.4% polyacrylamide gels (8 x 0.6 cm) were prepared using 2.4% acrylamide, 0.019% bisacrylamide, and 25 µl TEMED in a buffer

containing 40 mM-Tris, 20 mM-sodium acetate and 1 mM-EDTA (pH 8.5) (Loening, 1968). Polymerization was catalyzed with 5 mg ammonium persulphate. Electrophoresis buffer consisted of 40 mM-Tris-HCl, 20 mM-sodium acetate and 1 mM-EDTA, pH 7.8 (Loening, 1968). Electrophoresis was carried out for 2 h at 4 mA per gel.

5. Serological techniques

All serological tests were carried out in glass petri dishes 10 cm in diameter containing 12 ml of 0.75% agar in 0.01M-phosphate buffer, pH 7.6, with 0.02% sodium azide as preservative (Francki and Habili, 1972). Holes, 3 mm in diameter and 3.5 mm apart, were cut, and each was charged with 10 µl of serum or antigen. The plates were observed for precipitin lines and photographed if required after 7 days incubation at 25°C in a moist atmosphere. Sera or antigens were titrated in 2-fold dilution series diluted with 0.14M-NaCl.

6. Heat-denaturation of ds-RNA

Heat-denatured ds-RNA was obtained by heating the RNA samples in either 0.1 x SSC or 0.01 x SSC in stoppered test tubes at 100°C for 10 min and cooling rapidly by plunging into an ice-ethanol bath.

7. Spectrophotometry

Solutions of virus and nucleic acid were examined in a Unicam SP800 or an SP1800 spectrophotometer. Concentrations were estimated using assumed extinction coefficients ($E_{1\ cm}^{0.1\%}$) at 260 nm of 25 for single-stranded (ss)-RNA and 16.7 for ds-RNA (Ito and Joklick, 1972).

8. Precautions against ribonuclease (RNase)

All buffers and solutions coming in contact with RNA were autoclaved at 15 psi for 15 - 20 min. Glassware was either autoclaved or heated at 120°C for at least 2 h. Plastic tubing was washed with 0.1M-NaOH and then rinsed in autoclaved distilled water.

9. Instruments for centrifugation

High-speed centrifugations were carried out in either a Beckman Model L or a Model L2-65 ultracentrifuge. For low speed centrifugations, a MSE Magnum with a swing-out rotor, a MSE-18, a Sorvall RC2-B or a RC3 centrifuge was used.

10. Sources of chemicals

The sources of some chemicals and biochemicals used are listed in Table 1. All other chemicals were those commonly available reagent grade preparations. Antisera to poly [I] : poly [C], TMV and potato virus X were gifts from Dr. R.I.B. Francki (Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, South Australia). Antisera to rice dwarf virus (RDV) and maize rough dwarf virus (MRDV) were supplied by Dr. I. Kimura (Institute for Plant Virus Research, Japan) and Dr. E. Luisoni (Laboratorio di Fitovirologia Applicata del Consiglio Nazionale delle Ricerche, Italy). RNA isolated from bacteriophage Ø6 by phenol-SDS extraction was supplied by Dr. J. van Etten (Department of Plant

Pathology, University of Nebraska, U.S.A.) and that isolated from reovirus type 3, Dearing strain, by the phenol-SDS procedure by Dr. A.R. Bellamy (Department of Cell Biology, University of Auckland, New Zealand). ^{32}P-GTP was a gift from Dr. R.H. Symons (Department of Biochemistry, University of Adelaide, South Australia). Tumerogenic mice were supplied by Professor E. Martin (School of Biological Sciences, Flinders University, South Australia).

TABLE 1: The commercial sources of some chemicals and biochemicals used and referred to in the text.

Chemical	Source
Acrylamide	BDH Chemicals Ltd., England
Adjuvant, Freund's complete	Becton, Dickinson & Co., U.S.A.
Actinomycin D (Dactinomycin; pure substance)	Merck, Sharpe & Dohme (Aust.)Pty.Ltd.
ATP	Sigma Chemical Co., St. Louis,Missouri.
Bisacrylamide	BDH Chemicals Ltd., England.
CTP	Sigma Chemical Co., St. Louis, Missouri.
DNase	Sigma Chemical Co., St. Louis, Missouri.
Poly [I] : Poly [C]	Nutritional Biochemicals Corp., Cleveland, Ohio.
GTP	Sigma Chemical Co., St. Louis, Missouri.
NCS	Amersham/Searle, Illinois
Pronase (type VI, fungal protease)	Sigma Chemical Co., St. Louis, Missouri.
Pyruvate kinase	Sigma Chemical Co., St. Louis, Missouri.
Phospho(enol)pyruvate (Trisodium salt)	Sigma Chemical Co., St. Louis, Missouri.
RNase	Sigma Chemical Co., St. Louis, Missouri.
TEMED	Sigma Chemical Co., St. Louis, Missouri.
Tris; Sigma A 7-9	Sigma Chemical Co., St. Louis, Missouri.
UTP	Sigma Chemical Co., St. Louis, Missouri.
UTP-^{14}C (CFA 364)	The Radiochemical Centre, Amersham.
Nonidet P40	Shell Co., U.S.A.
POPOP (Scintillation Grade)	Ajax Chemicals Ltd., Sydney, Melbourne.
PPO (Scintillation Grade)	Koch-Light Laboratories Ltd., Colnbrook, Bucks. England.

CHAPTER III

STUDIES ON PURIFICATION OF FDV

INTRODUCTION

Francki and Jackson (1972) were able to detect ds-RNA in FDV-induced gall tissues but not in tissues of healthy sugarcane plants using antiserum to poly [I] : poly [C]. Since they were unable to detect ds-RNA prior to phenol extraction, it was concluded that the ds-RNA was encapsidated in the virus particles. In order to further characterize FDV particles, it was necessary to develop a suitable method for purifying the virus. In this chapter, the development of a procedure for the purification of FDV is described.

METHODS

1. Assay of FDV

The amount of FDV in tissue extracts was assumed to be proportional to the amount of ds-RNA present. The ds-RNA was assayed by immunodiffusion against an antiserum to poly [I] : poly [C].

The tissue fraction or extract to be assayed was deproteinized by the phenol-SDS method as described in Chapter II. Two-fold dilutions of this preparation were used for immunodiffusion tests (Chapter II) and the reciprocal of the dilution end-point was taken as the relative concentration of ds-RNA and hence FDV.

2. Preparation of celite filters

Celite filters were prepared as described by McLean and Francki (1967). Half to one gm of celite (Hyflo Super-Cel) was mixed with 10 ml of distilled water. This mixture was poured over 2 Whatman No. 1 filter papers, 2 cm in diameter, in a Millipore apparatus. Such pads were used for the filtration of approximately 10 ml of plant extract.

3. Electron microscopy

(a) Negative and positive staining

Virus preparations were stained with either 2% potassium phosphotungstate, pH 6.8, (PTA) or 2% uranyl acetate (UAc). A drop of virus preparation was mixed in the staining solution on a piece of plastic film (parafilm). Copper grids covered with carbon-stabilized Formvar membranes were touched onto the surface of the virus-stain solution. After 30 seconds (negative staining) or 30 min (positive staining) excess solution was removed with filter paper. The specimens were then examined in a Philips 100 or a Siemens Elmniskop I electron microscope. Particles were measured from the micrographs, with a stereoscopic microscope fitted with a micrometer eyepiece.

(b) Freeze-drying

Virus preparations were suspended in a 40 µl of distilled water. The suspension in a 100 µl pipette was mounted on carbon-coated grids

which had been ionized. Excess suspension was drained away from the grid with a needle so that a thin layer of suspension was formed on the grid. As soon as drying started from the edge of the grids, the grids were dropped into liquid Freon 22 and frozen quickly to -155°C, followed by dropping them in liquid nitrogen (-196°C). The grids were then transferred within 2 seconds to the specimen stage of a Balzer's freeze-etching apparatus previously cooled down to -150°C. The bell jar was immediately evacuated. When the pressure in the bell jar reached 1×10^{-4} Torr, the cooling device of the specimen stage was switched off. After pumping down for 45 - 60 min, shadowing by platinum was done at an angle of 45°. At the time of shadowing the pressure in the bell jar was $5\text{-}8 \times 10^{-6}$ Torr and the temperature of the specimen stage was about 20°C (room temperature).

EXPERIMENTAL

1. Location of ds-RNA antigen in FDV-infected sugarcane plants

The following experiment was carried out to determine from which type of tissue highest yields ds-RNA antigen could be obtained. Ds-RNA antigen was extracted from similar weights of galls, leaf tissue without galls and roots of FDV-infected plants and its concentration in each tissue was compared. Galls were the only tissue in which ds-RNA antigen was detected (Table 2). These results confirm electron microscopic observations indicating that FDV particles were confined to gall tissue (Dr. R.I.B. Francki, personal communication). Therefore, in all succeeding experiments excised galls only were used as starting material for FDV purification.

TABLE 2: Localization of ds-RNA antigen in FDV-infected plants

Source of tissue from infected plants	Antigen dilution end-point[a]	
	Experiment 1[b]	Experiment 2[c]
Galls	8	8
Leaf tissue without galls	0	0
Roots	0	0

a) Titres are expressed as reciprocals of highest dilutions producing a visible precipitin line in gel-diffusion tests.

b) Antigen obtained from 4 gm of each tissue was finally suspended in 370 µl of ST buffer.

c) Antigen obtained from 3.5 gm of each was finally suspended in 350 µl of ST buffer.

2. Extraction of virus from infected tissue

(a) Effect of extraction buffer

The choice of buffer influences the efficiency of extraction, the stability of the virus and its aggregation (Brakke, 1969). Preliminary experiments showed that significant losses of ds-RNA antigen into low-speed pellets occurred when GM buffer (0.1M-glycine, 0.01M-$MgCl_2$, pH 8.5) was used for extraction as recommended for the purification of WTV (Black, 1965). Brakke (1969) reported that EDTA (ethylenediaminetetra-acetic acid di-sodium salt) can reduce the amount of virus aggregation. Therefore, an experiment was carried out to compare the effectiveness of 0.1M-glycine buffer (pH 8.5) and GE buffer (0.1M-glycine, 0.005M-EDTA, pH 8.5) as extraction media. Two similar samples of gall tissue were ground in glycine buffer and GE buffer respectively. Extracts were strained through a double layer of cheesecloth and centrifuged at 1,500 g for 5 min. Each supernatant was then adjusted to pH 7.2 with 0.1N-NaOH and centrifuged at 5,000 g for 10 min.

Concentration of ds-RNA antigen in the resulting supernatants and pellets were assayed. The results summarized in Table 3 show that significant losses of ds-RNA antigen into the low-speed pellet occurred when glycine buffer was used, but addition of EDTA to the extraction buffer reduced these losses.

TABLE 3: Effect of EDTA in extraction buffer on the sedimentation of ds-RNA antigen in extracts of FDV-induced galls subjected to low-speed centrifugation.

Extraction buffer	Dilution end-point of antigen[a] in	
	Supernatant after 5000 g for 10 min	Pellet after 5000 g for 10 min
0.1M-glycine, pH 8.5	32	16
0.1M-glycine, 5mM-EDTA, pH 8.5 (GE)	>32	1

a) Titres are expressed as reciprocals of the highest dilutions at which the preparations produced a precipitin line in gel-diffusion tests against antiserum to poly [I] : poly [C].

(b) Effect of pH

An experiment was carried out to determine the optimum pH for isolating material containing ds-RNA. Gall tissue was extracted in GE buffer. The pulp was strained through a double layer of cheesecloth and centrifuged at 1,500 g for 5 min. The supernatants were adjusted to pH 7.0, pH 7.5, pH 8.0 and pH 8.5 with 0.1N-NaOH and centrifuged at 5,000 g for 10 min. The concentration of ds-RNA antigen in the supernatant and pellet of each treatment was assayed. The results summarized in Table 4 show that significant losses of ds-RNA antigen into the pellet occurred above pH 8.0 following low-speed centrifugation.

3. Clarification of extracts

During the next stage of a purification procedure, it is desirable to remove as much plant material as possible from the extract. Organic solvents are often used as clarifying agents. They coagulate host proteins, disrupt ribosomes and dissolve lipids. The use of organic solvents as clarifying agents, such as chloroform (Suzuki, 1969; Wetter *et al.*, 1969), carbon tetrachloride (Kitagawa and Shikata, 1969; Black and Knight, 1970; Milne *et al.*, 1973), ether and Freon 113 (Streissle and Granados, 1968; Milne *et al.*, 1973) resulted in serious losses of ds-RNA antigen and did not remove host materials efficiently (Dr. R.I.B. Francki, personal communication). Therefore, the use of these methods was not considered.

TABLE 4: Effect of pH on the sedimentation of ds-RNA antigen in extracts of FDV-induced galls subjected to low-speed centrifugation.

pH of extract	Dilution end-point of antigen[a] in	
	Supernatant after 5000 g for 10 min	Pellet after 5000 g for 10 min
7.0	8-16	1
7.5	16	1
8.0	8	8
8.5	8	8

a) Titres are expressed as reciprocals of the highest dilutions at which the preparations produced a precipitin line in gel-diffusion tests against antiserum to poly [I] : poly [C].

Filtration has the advantage that the extract can be passed through a pad of celite, which removes much of the green materials (Francki, 1972). This adsorbant has been useful in the purification of several unstable viruses such as lettuce necrotic yellows virus (LNYV) and maize rough dwarf virus (MRDV) (McLean and Francki,1967; Wetter *et al.*, 1969). An experiment was carried out to test the effectiveness of this procedure for the clarification of extracts containing FDV. Extracts in GE buffer adjusted to pH 7.5 were subjected to low-speed centrifugation and divided into two samples. One sample was filtrated through a pad of celite and the concentration of ds-RNA in the filtrate was compared with that of ds-RNA in the unfiltered sample. The results are shcwn in Table 5. Approximately 50% of ds-RNA antigen in the unfiltered sample was lost during filtration and the filtrate retained much green material. Celite filtration was considere unsuitable. In subsequent experiments attempts were made to concentrate ds-RNA antigen from the supernatant after the second low-speed centrifugation. No attempt was made to further clarify the extracts before concentration.

4. Concentration of ds-RNA antigen from extracts

(a) Precipitation with polyethylene glycol (PEG)

Following the work of Hebert (1963) on the use of PEG for the precipitation of four plant viruses, this substance has been effectively employed to selectively concentrate a number of plant, animal

TABLE 5: Clarification of FDV extracts by filtration through a celite pad.

Extract assayed	Dilution end-point of ds-RNA antigen[a)]	
	Experiment 1	Experiment 2
Extract	16	32
Filtrate	8	8-16

a) The titres are expressed as the reciprocals of the highest dilutions at which the preparations produced a precipitin line in gel-diffusion tests against antiserum to poly [I] : poly [C].

and bacterial viruses (Venekamp and Misch, 1964 a, b; Leberman,1966; Van Kammen, 1967; Kanarek and Tribe, 1967; Clark, 1968; Yamamoto *et al.*, 1970; McSharry and Benzinger, 1970; Friedmann and Haas,1970). Recently PEG has also been used to concentrate WTV and RDV (Reddy and Lesnaw, 1971; Reddy and Black, 1973a; Kimura, 1973).

Experiments were carried out to determine the optimum conditions for the precipitation of ds-RNA antigen. Gall tissue was extracted in GE buffer (pH 8.5) and the extract was adjusted to pH 7.5 with 0.1N-NaOH. After measuring the volume of the extract, NaCl was added to a final concentration of 0.3M with varying amounts of PEG (Reddy and Lesnaw, 1971). After standing for 2.5 h at 4°C, the mixture was centrifuged at 10,000 g for 7 min. The concentrations of ds-RNA antigen in the supernatant and pellet were determined. For complete precipitation of ds-RNA antigen a concentration of 10% PEG was required (Table 6). However, this concentration of PEG also precipitated much of the green material with ds-RNA antigen which could not be removed by differential centrifugation.

(b) Concentration by high-speed centrifugation

An experiment was carried out to determine the optimum conditions for sedimentation of ds-RNA antigen. The extract in GE buffer adjusted to pH 7.5 was clarified at low-speed centrifugation and the supernatant was centrifuged at 165,000 g for 10, 20 and 30 min. Concentration of ds-RNA antigen in the resultant supernatant and

TABLE 6: Concentration of ds-RNA antigen by PEG precipitation

Concentration of PEG[a]	Centrifugation at 10,000 g for 7 min	Dilution end-point of ds-RNA antigen[b]			
		Experiment 1[c]	Experiment 2[c]	Experiment 3[c]	Experiment 4[d]
4	Supernatant	4	-	-	-
	Pellet	0	-	-	-
6	Supernatant	4	-	-	-
	Pellet	0	-	-	-
8	Supernatant	-[e]	1	-	-
	Pellet	-	1	-	-
10	Supernatant	-	0	1	0
	Pellet	-	2	8-16	8

a) Concentration of NaCl was 0.3M.

b) The titres are expressed as the reciprocals of the highest dilutions at which the preparations produced a precipitin line in gel-diffusion tests against antiserum to poly [I] : poly [C].

c) PEG 4000 was used.

d) PEG 6000 was used.

e) Not done.

pellet after each treatment was assayed. The results summarized in Table 7 show that centrifugation for 20 - 30 min was adequate to sediment ds-RNA antigen. Therefore, to ensure complete sedimentation centrifugation for 30 min was used routinely. When virus preparations were layered over 2 ml of 10% sucrose cushions, centrifugation was increased to 40 min.

5. Comparison of resuspension buffers

In the next stage of the purification procedure, it was necessary to resuspend the concentrated ds-RNA antigen.

Data summarized in Table 8 indicate that ds-RNA antigen sedimented during low-speed centrifugation at pH above 7 when Mg^{2+} was added to the resuspension buffer as recommended for the purification of WTV (Black and Knight, 1970) and MRDV (Wetter *et al.*, 1969). Significant losses of ds-RNA antigen into the low-speed pellet were also observed when SSC buffer (pH 7.5) (recommended by Smith *et al.*, 1969, for the purification of reovirus) was used. But addition of EDTA to the resuspending buffer minimized these losses. Best results were obtained when the pellet was taken up in STE buffer (0.1M-NaCl, 0.05M-Tris-HCl, 0.005M-EDTA, pH 7.5), dispersed in a glass homogenizer and left at $0^{o}C$ for at least 30 min.

6. Solubilization of contaminating membranous material by Nonidet P40

Nonionic detergents are commonly used in enzymology, biology and virology for selective solubilization of cellular membranes (Glassman, 1950; Bottomley, 1970; Nozu and Yamaura, 1971; Van Oosten, 1972).

TABLE 7: Concentration of ds-RNA antigen by high-speed centrifugation

Centrifugation at 165,000 g for	Dilution end-point of ds-RNA antigen[a] in	
	Supernatant	Pellet
10 (min)	4	2
20	0	4-8
30	0	8

a) The titres are expressed as the reciprocals of the highest dilutions at which the preparations produced a precipitin line in gel-diffusion tests against antiserum to poly [I] : poly [C].

TABLE 8: Effect of resuspension buffers on the sedimentation of ds-RNA antigen in extracts of FDV-induced galls[a)]

Experiment Number	Resuspension buffer	Dilution end-point of antigen[b)] in pellet after	
		low-speed centrifugation (5,000 g for 10 min)	high-speed centrifugation (165,000 g for 30 min)
1	0.1M-glycine,0.01M-$MgCl_2$, pH 7.5 (GM)	>32	2
2	0.15M-NaCl,0.015M-Na citrate,pH 7.5 (SSC)	16	32
3	0.1M-NaCl,0.05M-Tris-HCl, 0.001M-EDTA, pH 7.5 (ST)	4	16
4	0.1M-NaCl,0.05M-Tris-HCl, 0.005M-EDTA, pH 7.5 (STE)	8	128

a) Excised leaf galls were extracted with 3 volumes (w/v) of GE buffer and the pH of the extract was adjusted to pH 7.5 with 0.1N-NaOH. The extract was clarified by low-speed centrifugation and the supernatant was subjected to high-speed centrifugation. The pellets were suspended in the buffers specified, treated with Nonidet P40 and again subjected to centrifugation at 5,000 g for 10 min. The supernatant was then centrifuged at 165,000 g for 30 min.

b) Reciprocals of the highest dilutions at which the preparations produced a precipitin line in gel-diffusion tests against antiserum to poly [I] : poly [C].

Electron microscopic examination disclosed that most of the contaminating material in concentrated preparations of ds-RNA antigen containing material from gall tissues consisted of membrane fragments. Therefore, the nonionic detergent Nonidet P40 was used to solubilize this membranous material. Galls were extracted in GE buffer (pH 8.5) and the pH was adjusted to 7.5 with 0.1N-NaOH. The extract was clarified by centrifugation at 5,000 g for 10 min and the supernatant was centrifuged at 165,000 for 30 min. The pellet was suspended in STE buffer, treated with 1% Nonidet P40, stirred for 10 min at 4°C and again subjected to high-speed centrifugation. The results summarized in Table 9 show that most of the ds-RNA antigen was detected in the pellet after high-speed centrifugation. The pellet was resuspended in STE buffer and centrifuged at 5,000 g for 10 min. The supernatant containing the FDV antigen was usually pale green. Thus, Nonidet P40 was considered satisfactory for solublizing contaminating host membranous material.

7. Sucrose density-gradient centrifugation for further purification

Sucrose density-gradient centrifugation is the most commonly used method in the final stages of purification of plant viruses (Brakke, 1960) and has been used in the purification of WTV, RDV, MRDV and RBSDV (rice black-streaked dwarf virus) (Black, 1965;

TABLE 9: Effect of Nonidet P40 on the sedimentation of the ds-RNA antigen in extracts of FDV-induced galls.

Experiment Number	Dilution end-point of ds-RNA antigen[d] in	
	Supernatant after 165,000 g for 30 min	Pellet after 165,000 g for 30 min
1[a]	0	8
2[b]	0	>32
3[b]	0	>32
4[c]	8	128

a) Excised leaf galls were extracted in GE buffer. The extract was centrifuged at 5,000 g for 10 min and the supernatant was subjected to high-speed centrifugation.

b) Galls were extracted in GE buffer. The extract was centrifuged at 5,000 g for 10 min and the supernatant was mixed with 1% Nonidet P40 and stirred for 10 min at 4°C. The mixture was subjected to high-speed centrifugation.

c) Experiment was carried out as described in the text.

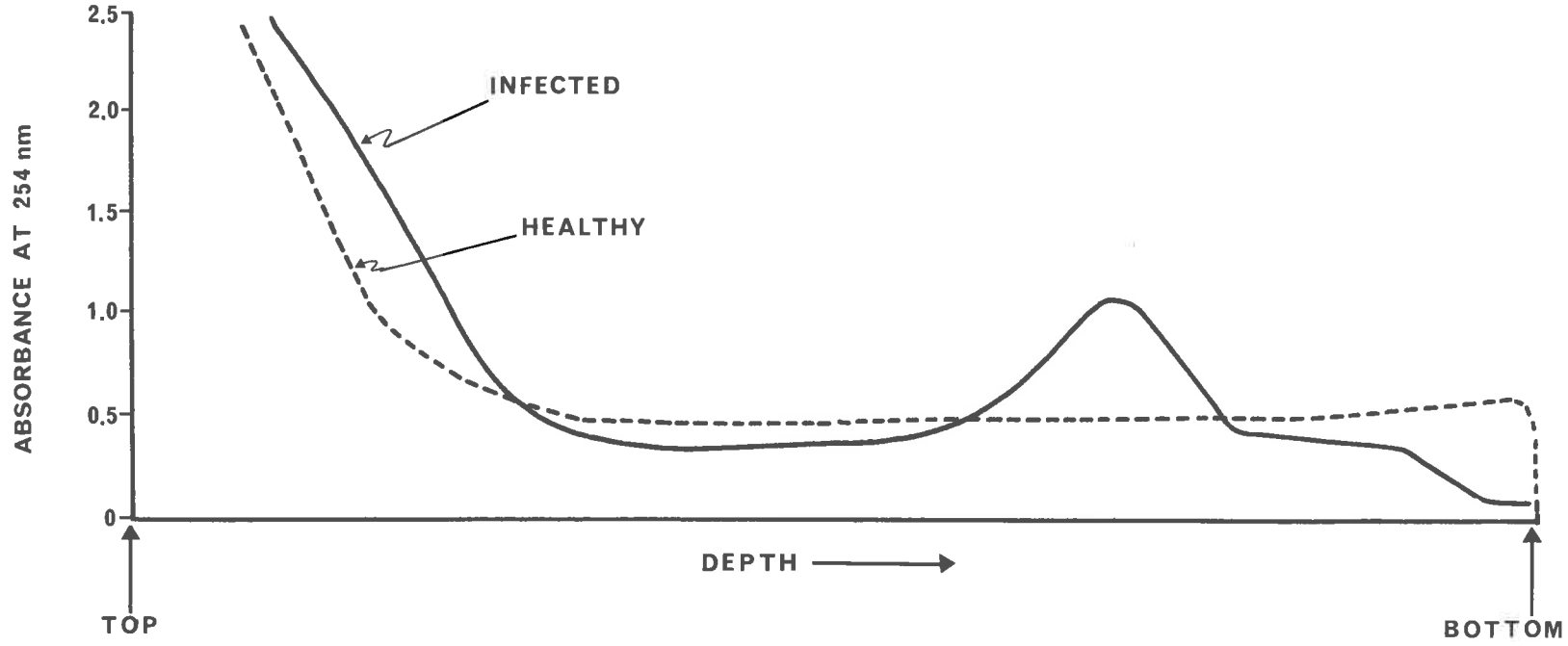
d) The titres are expressed as reciprocals of the highest dilutions at which the preparations produced a precipitin line in gel-diffusion tests against antiserum to poly [I] : poly [C].

Kimura and Kodama, 1968; Wetter *et al.*, 1969; Kitagawa and Shikata, 1969). Samples of the Nonidet P40 treated and re-concentrated ds-RNA antigen preparations (0.5 ml) were layered on 30 to 60% (W/V) linear sucrose density-gradients (prepared in STE buffer) in 5 ml tubes and centrifuged at 40,000 rpm for 60 min in a Spinco SW50.1 rotor. The tube contents were scanned and fractionated with an ISCO model D density-gradient fractionator and densitometer assembly. Typical density-gradient scans of preparations from gall tissue and healthy sugarcane leaves are presented in Fig. 1. A peak about one quarter of the way from the bottom of the tube can be observed in the tube containing material from diseased leaves. Material producing this FDV-specific peak was recovered, dialysed against STE buffer overnight and concentrated by centrifugation at 165,000 g for 30 min. The pellet (final FDV preparation) was resuspended in either distilled water or buffer depending on what the preparation was to be used for. The pellet was usually whitish and contained ds-RNA as determined serologically.

8. Final method adopted for the purification of virus-like particles from FDV-infected plants

Fig. 2 shows the final method adopted for the purification of virus-like particles. Preparations from about 10 gm of isolated galls finally suspended in 1 ml of distilled water had an optical density at 260 nm between 0.15 - 0.25. This method was used in all subsequent experiments.

Fig. 1. Sucrose density-gradient profiles of material purified by differential centrifugation from leaf galls of FDV-infected sugarcane leaves (———), and healthy leaves (---).


ABSORBANCE AT 254 nm
2.5
2.0
1.5
1.0
0.5
0
INFECTED
HEALTHY
DEPTH
TOP
BOTTOM

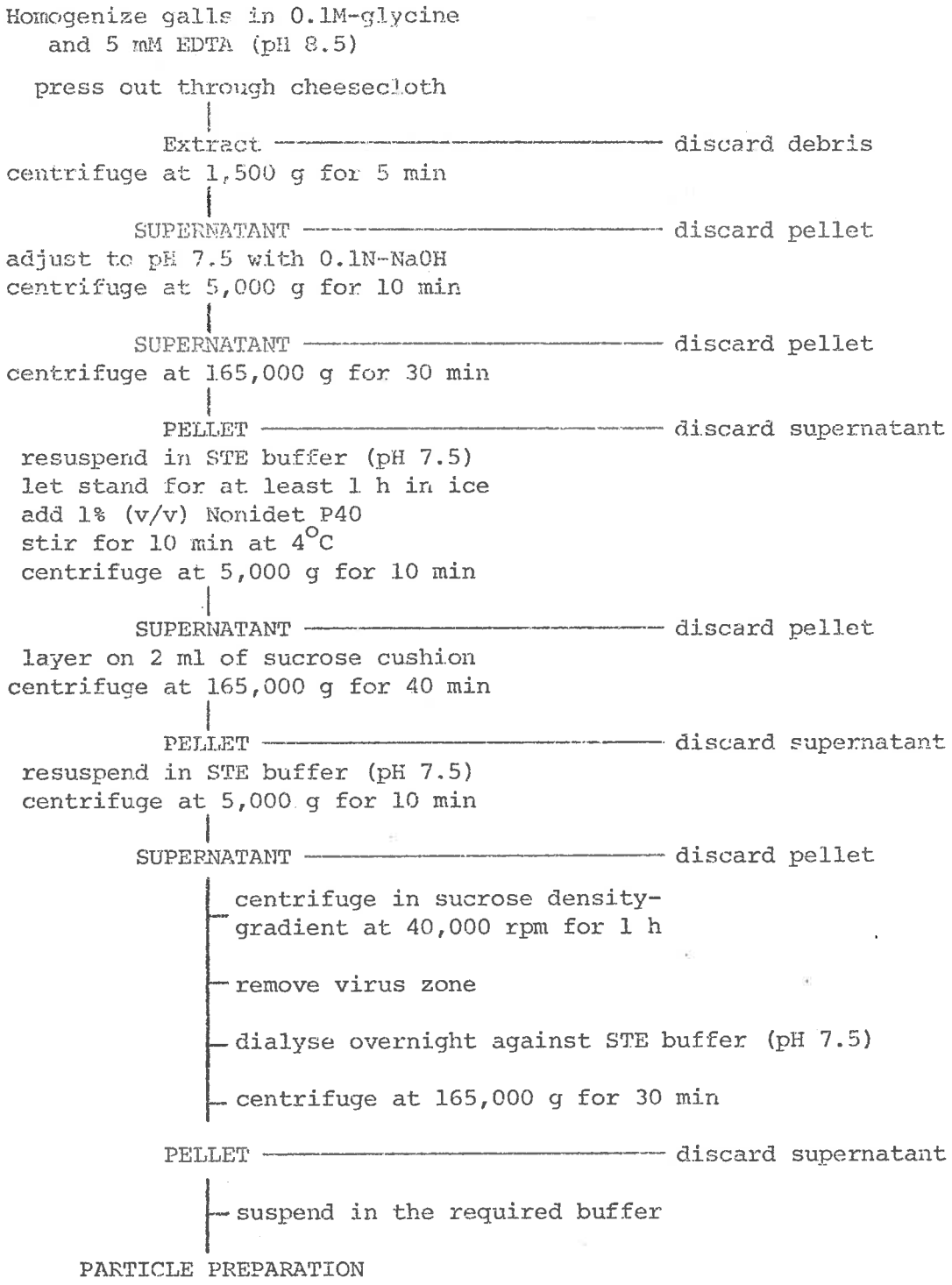


Fig. 2. Flow diagram for the purification of virus-like particles from leaf galls of FDV-infected sugarcane leaves.

9. Some properties and purity of virus-like particle preparations

(a) Electron microscopic observation

Electron microscopic examination of the virus-like particle preparations revealed the presence of numerous roughly spherical particles with a mean diameter of 55-60 nm (Figs. 3 and 6). In preparations stained in UAc, they were 50 nm in diameter (Figs. 4 and 6). Twelve "projections" seem to be present on the surface of the particles purified from FDV-induced galls (Fig. 4C-H). These "projections" were approximately 10.8 nm long and 20.5 nm wide. Each "projection" appears to consist of 5 subunits approximately 7.5 nm in diameter (Fig. 5A). These "projections" were not observed when preparations were stained in PTA (Fig. 3). Numerous strands displaying helical structure were also observed in preparations stained in UAc (Fig. 4A-B), although the numbers of these strands varied from preparation to preparation. Positive staining in UAc of these strands (Dr. T. Hatta, personal communication) indicates the presence of nucleic acid.

(b) Ultraviolet (UV) absorption

Purified virus-like particle preparations had UV absorption spectra characteristics of nucleoprotein (Fig. 7) with a maximum at about 260 nm, a minimum at 245 nm and 260 : 280 nm and 260 : 245 nm ratios of 1.38 and 1.11 respectively.

Fig. 3. Electron micrograph of virus-like particles purified from excised galls of FDV-infected sugarcane leaves and stained in PTA.

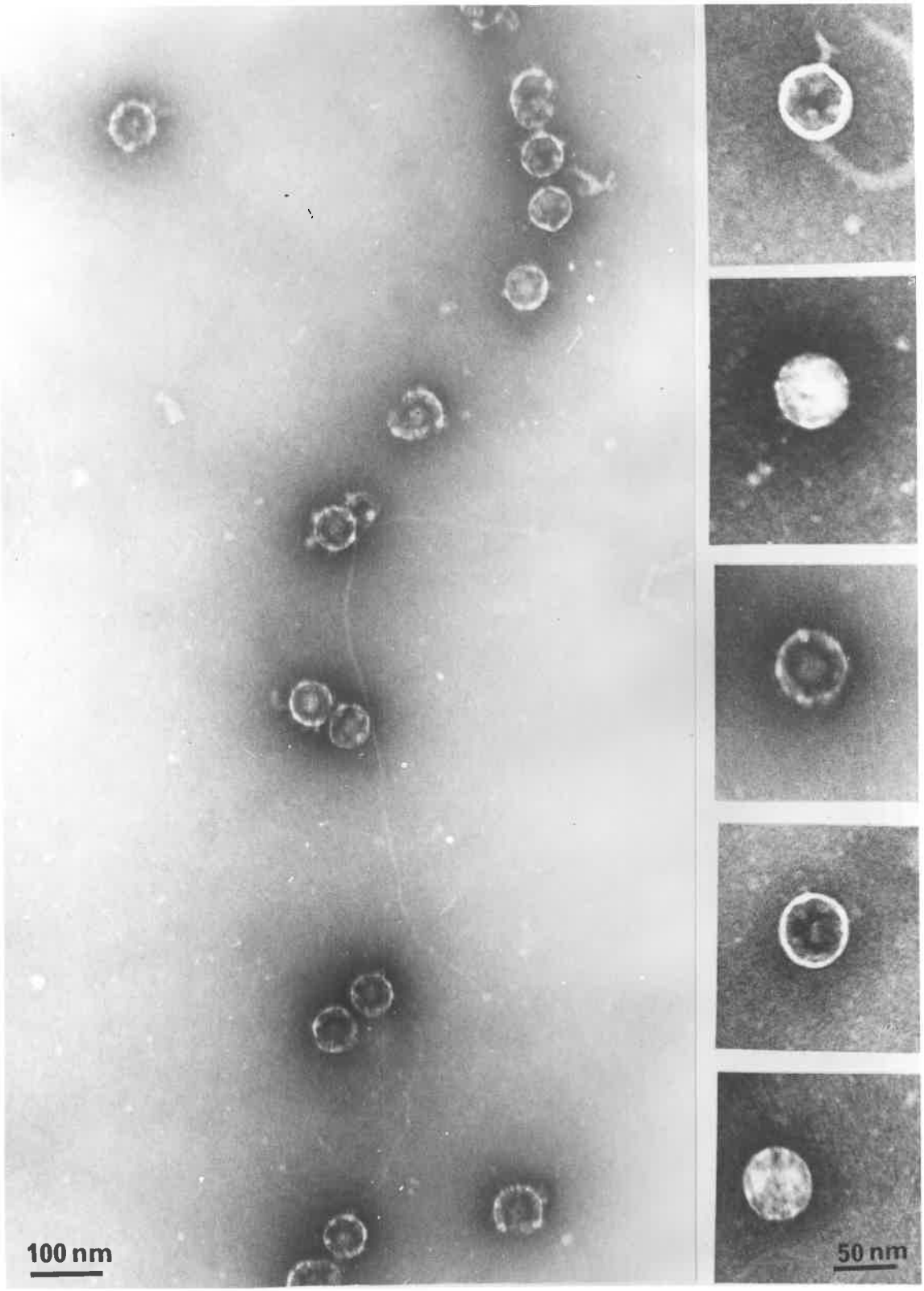

100 nm
50 nm

Fig. 4. Electron micrograph of virus-like particles and strands purified from excised galls of FDV-infected sugarcane leaves. (A, B) Virus-like particles and strands negatively stained in UAc; (C-E) 'Projections' on the surface of virus-like particles negatively stained in UAc; (F-H) Virus-like particles positively stained in UAc.

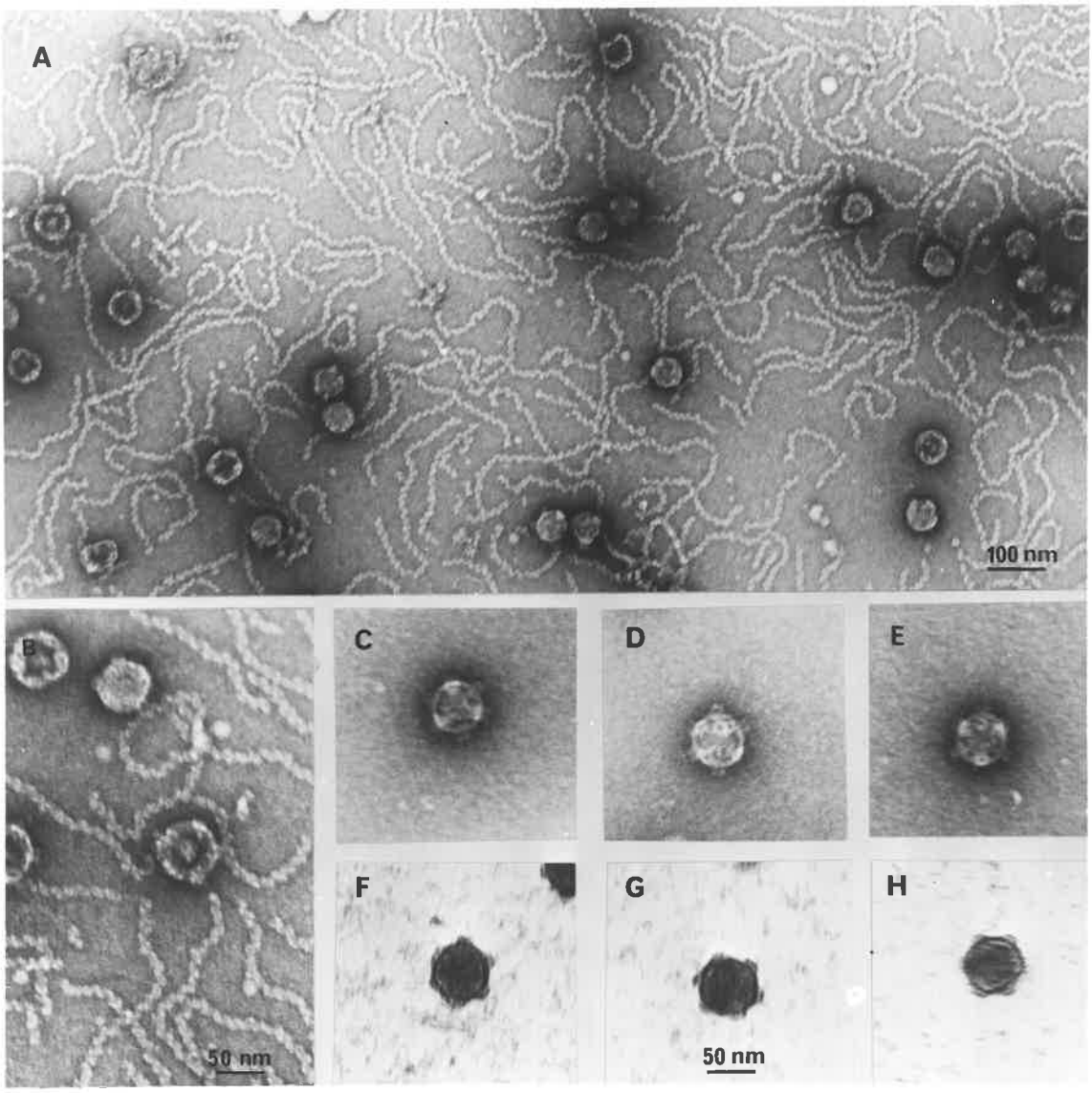

A
100 nm
B
C
D
E
F
G
H
50 nm
50 nm

Fig. 5. Electron micrograph of virus-like particles purified from excised galls of FDV-infected sugarcane leaves. Virus-like particle preparations were freeze-dried and shadowed at 45°. Note the 'projections' on the surface of the virus-like particles. In (A) arrows indicate subunits of 'projection'.

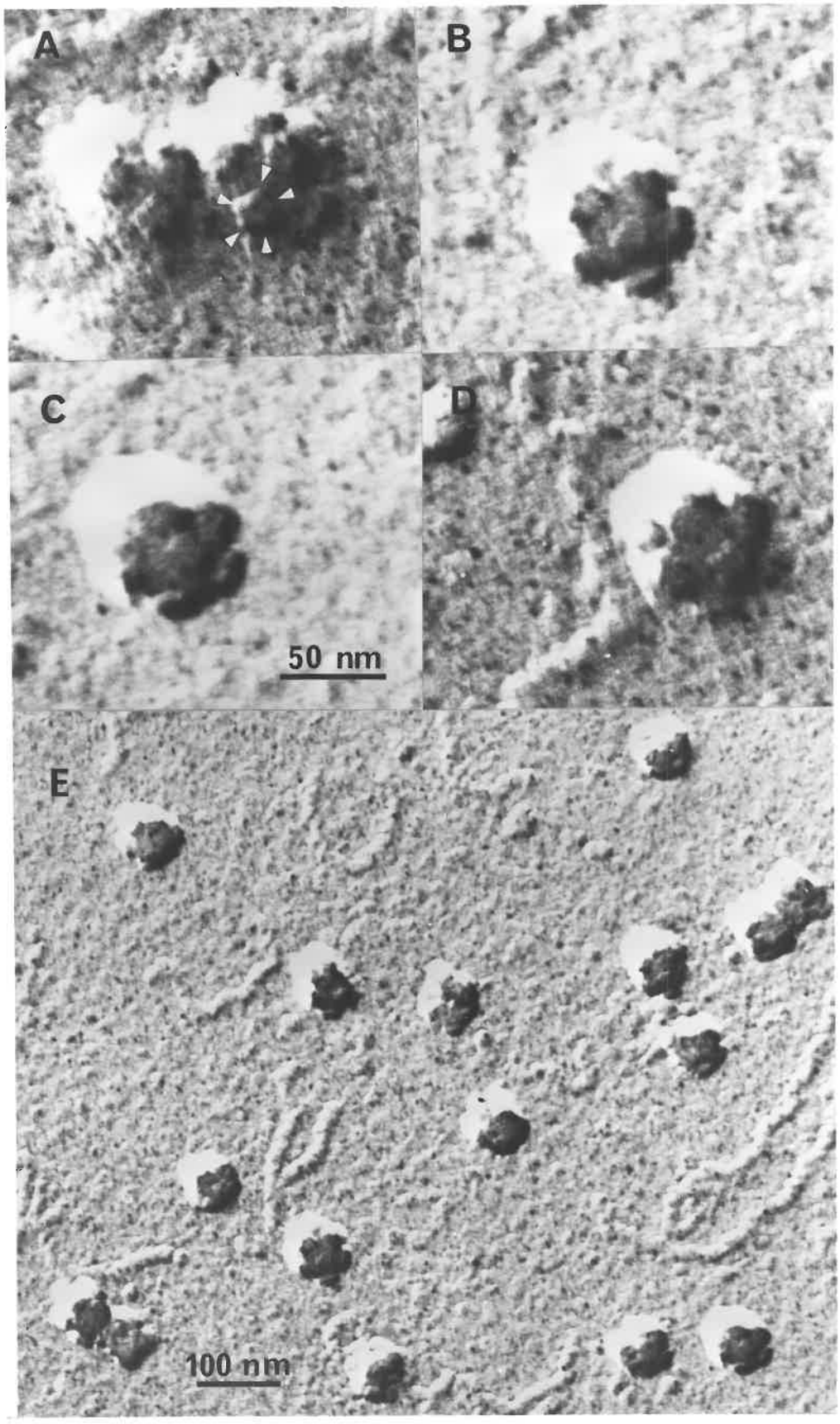

A
B
C
D
E
50 nm
100 nm

Fig. 6. Histograms showing the size distribution of virus-like particles negatively stained in PTA (A) and in UAc (B).

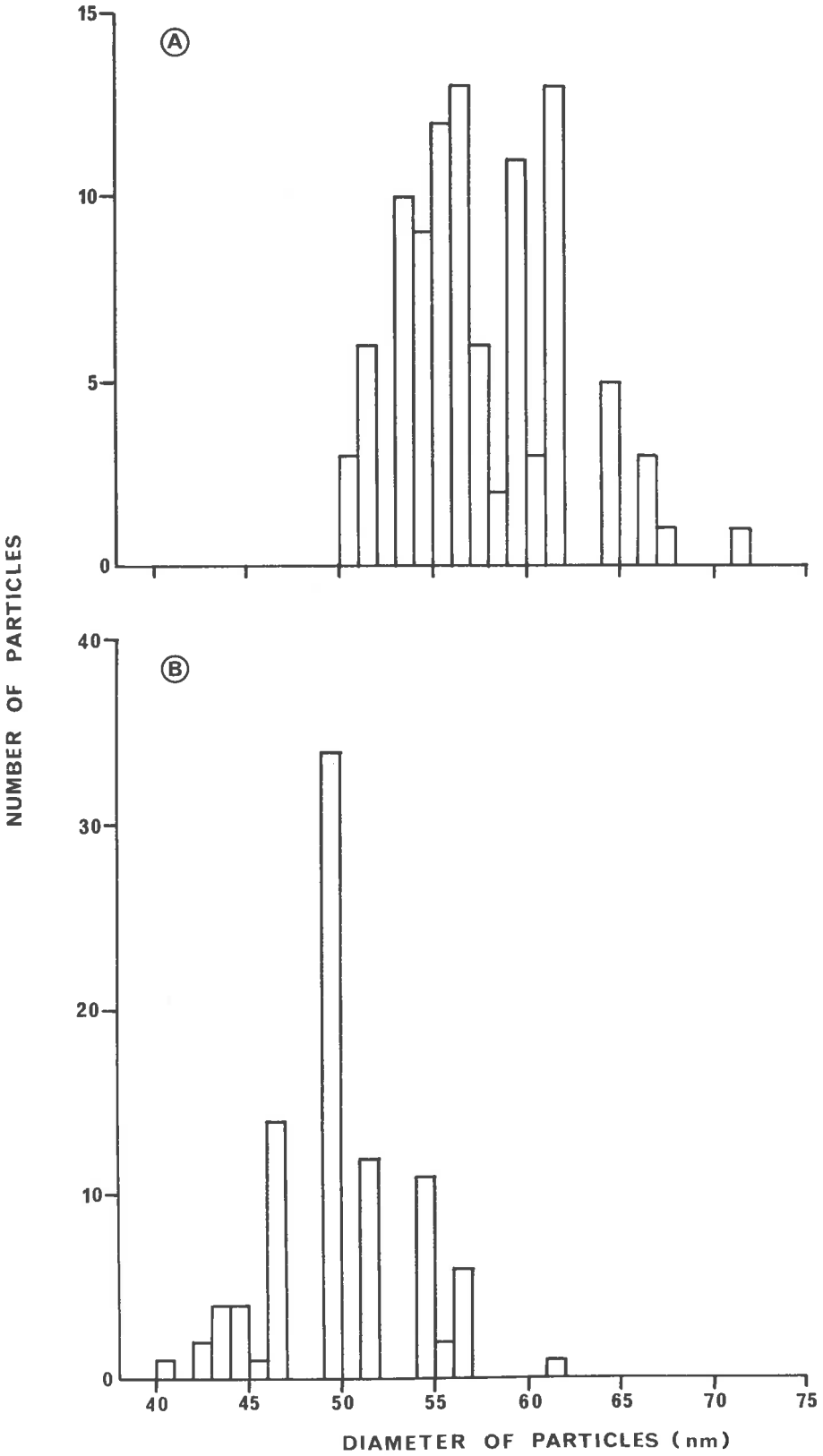
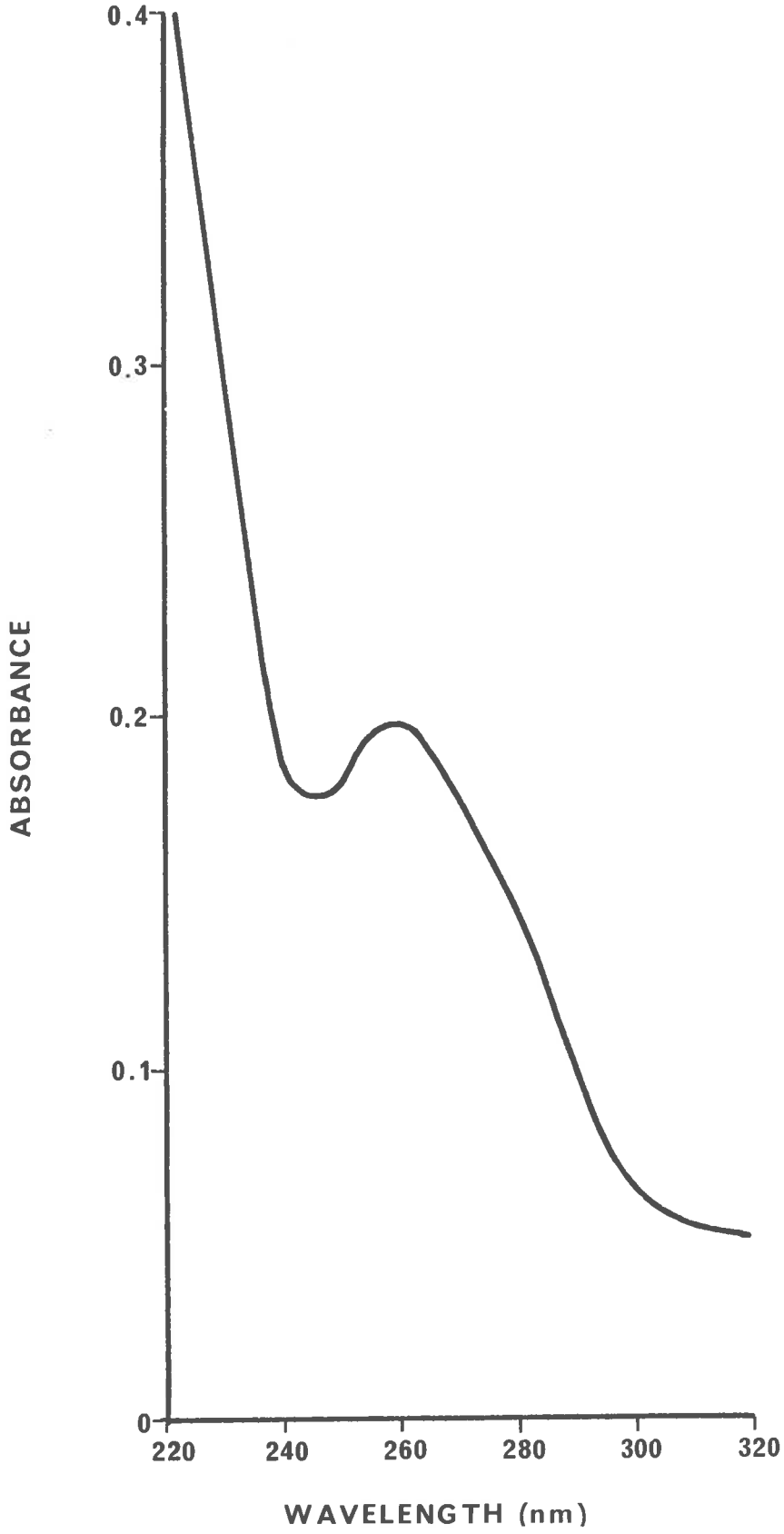

Ⓐ
Ⓑ
NUMBER OF PARTICLES
DIAMETER OF PARTICLES (nm)
0
5
10
15
20
30
40
45
50
55
60
65
70
75

Fig. 7. UV spectrum of a typical preparation of nucleoprotein from excised galls of FDV-infected sugarcane leaves.


0.4
0.3
0.2
0.1
0
ABSORBANCE
220
240
260
280
300
320
WAVELENGTH (nm)

(c) Testing of FDV-specific antiserum

Antiserum against FDV was prepared as described in Chapter IV. In gel-diffusion tests, the titres of blood serum and ascitic fluid were up to 128 and 64 respectively when titrated against FDV protein (Table 11). Antigens prepared from healthy leaves (see Materials and Methods section under Chapter IV) invariably failed to produce any reactions when tested against the antisera (Fig. 8).

10. Attempts to stabilize unpurified FDV particles with formaldehyde

Virus-like particles, 55-60 nm in diameter, were purified from FDV-infected plants. However, these particles seem to be somewhat degraded (see Conclusions section). Attempts were made to purify intact particles by treatment of gall extracts with formaldehyde. Gall tissue extracts in GE buffer adjusted to pH 7.5 were strained through a double layer of cheesecloth and centrifuged at 1,500 g for 5 min. Formaldehyde was added to the supernatant to 1.3% and stirred for 20 min at 4°C (Bar-Joseph *et al.*, 1972). The mixture was centrifuged at 5,000 g for 10 min and then subjected to high-speed centrifugation. The concentration of ds-RNA antigen in the supernatant and pellet was assayed. The results summarized in Table 10 show that most of the ds-RNA antigen was lost into the supernatant during high-speed centrifugation. Thus, the fixation of unpurified FDV particles with formaldehyde was unsuccessful.

Table 10. Treatment of unpurified FDV particles with formaldehyde

Fixation with	Dilution end-point of ds-RNA antigen[a] in	
	Supernatant after 165,000 g for 30 min	Pellet after 165,000 g for 30 min
None	0	16
Formaldehyde	16	4-6

a) The titres are expressed as the reciprocals of the highest dilutions at which the preparations produced a precipitin line in gel-diffusion tests against antiserum to poly [I] : poly [C].

CONCLUSIONS

Studies reported in this chapter demonstrate that:

1. Virus-like particles were purified from galls by differential centrifugation, treatment with Nonidet P40 and sucrose density-gradient centrifugation. EDTA in the extraction and suspension buffers was necessary to prevent particles from sedimenting during low-speed centrifugation. Nonidet P40 was effective in solubilizing contaminating host membranes. Contaminating host material could not be detected in purified preparation of virus-like particles by electron microscopic observations and serological tests.

2. The particles purified by this method were 50 nm and 55-60 nm in diameter after UAc and PTA staining conditions respectively. Particles observed in crude leaf-dip preparations from FDV-induced galls (Teakle and Steindl, 1969; Hutchinson and Francki, 1973) and those observed in thin section of infected cells (Teakle and Steindl, 1969; Francki and Grivell, 1972) measured about 70 nm in diameter. Thus it would appear that the preparations of purified virus-like particles were somewhat degraded.

3. Some virus-like particles showed projecting structures. Numerous strands were observed in virus-like particle preparations stained with UAc.

4. Purified virus-like particles are immunogenic and can be used for the preparation of FDV-specific antisera.

CHAPTER IV

SEROLOGY

INTRODUCTION

Francki and Jackson (1972) were able to detect ds-RNA immunochemically in FDV-infected sugarcane leaves but not in healthy tissues. It appeared that FDV was a ds-RNA virus. FDV is similar to MRDV on the basis of particle morphology, host range, virus morphogenesis in the cells, production of neoplasia in phloem tissues, and propagative transmission by planthoppers both belonging to the Delphacidae (Wetter *et al.*, 1969; Lovisolo, 1971a; Hutchinson and Francki, 1973). FDV also resembles maize wallaby ear virus (MWEV) which is a ds-RNA virus and induces similar symptoms on maize to those of FDV (Hughes and Robinson, 1961; Grylls, 1975; Mr. Grylls, personal communication). Therefore, it was of interest to compare FDV, MRDV, MWEV and another ds-RNA virus RDV, serologically. In this chapter, I describe the serological properties of FDV subviral particles and experiments investigating possible serological relationships among these viruses. I also describe some observations on the specificity of antibodies to ds-RNAs.

MATERIALS AND METHODS

1. Preparation of antiserum

At each immunization time, an FDV preparation, purified from 10 gm of leaf galls and emulsified with 2 volumes of Freund's complete adjuvant, was injected intraperitoneally (Marbrook and Matthews,1966)

into two Swiss white mice (Outbred strain), each weighing between 20 and 30 gm. Blood samples were collected by puncturing the retro-orbital plexus with a Pasteur pipette (Francki and Habili, 1972) and by heart puncture under anaesthesia at the conclusion of the experiment. To prevent the blood clotting both tubes and pipettes coming in contact with the blood were heparinized by allowing a drop of heparin solution (2 mg/ml) to dry in them at 100°C. The blood samples were centrifuged at 1,500 g for 20 min, and the plasma samples were stored at -15°C. Both the injection and bleeding schedules are described in Table 11.

When it was required to induce antibody production in ascitic fluid, the mice were given a booster injection of antigen as described above followed immediately by an intraperitoneal injection of about 1 ml of Krebs 2 Ascites tumour cells feshly obtained from a tumerogenic mouse. A similar technique has been used for the preparation of antisera to animal viruses (Gamble and Kinsley, 1963). After about six days the mice developed markedly distended abdomens. Ascitic fluid accumulated rapidly and had to be drained repeatedly. Ascitic fluid and tumour cells were tapped by puncturing the abdomen with an 18 gauge hypodermic needle allowing the fluid to drain into a beaker by applying gentle pressure to the animal's abdomen. The harvested fluid was centrifuged at 2,500 g for 20 min to remove cells. An aliquot of clear supernatant fluid was stored frozen at -15°C

(Matthews, 1967). Normal sera were obtained from rabbits not subjected to any experimental immunization.

2. Preparation of antigens for immunodiffusion tests

In order to study the serological relationships between FDV, MRDV, MWEV and RDV, and specificity of antibodies to ds-RNA, the following preparations were used as antigens:

(a) FDV-infected sap: FDV infected sap was prepared by grinding excised galls in 2.5 times their weight of STE buffer and the brei was centrifuged at 1,500 g for 5 min. The supernatant was recovered and used without further treatment.

(b) MWEV-infected sap: MWEV-infected sap was prepared as in (a) but from MWEV-infected leafhoppers (*Cicadulina bimaculata* Evans).

(c) Crude FDV nucleic acid: Crude FDV nucleic acid was prepared from excised galls as described in Chapter II, and suspended in STE buffer.

(d) Crude MWEV nucleic acid: Crude MWEV nucleic acid was prepared as in (c) but from viruliferous *C. bimaculata* or MWEV-infected maize roots.

(e) Poly [I] : poly [C]: Poly [I] : poly [C] was dissolved in STE buffer at a concentration of 100 µg/ml.

(f) Healthy sap: The healthy sap was prepared as in (a) but from either healthy sugarcane leaf tissue or non-viruliferous *C. bimaculata*.

(g) Crude host nucleic acid: The host nucleic acid was prepared as in (c), but from healthy sugarcane leaf tissue, non-viruliferous *C. bimaculata* or healthy maize roots.

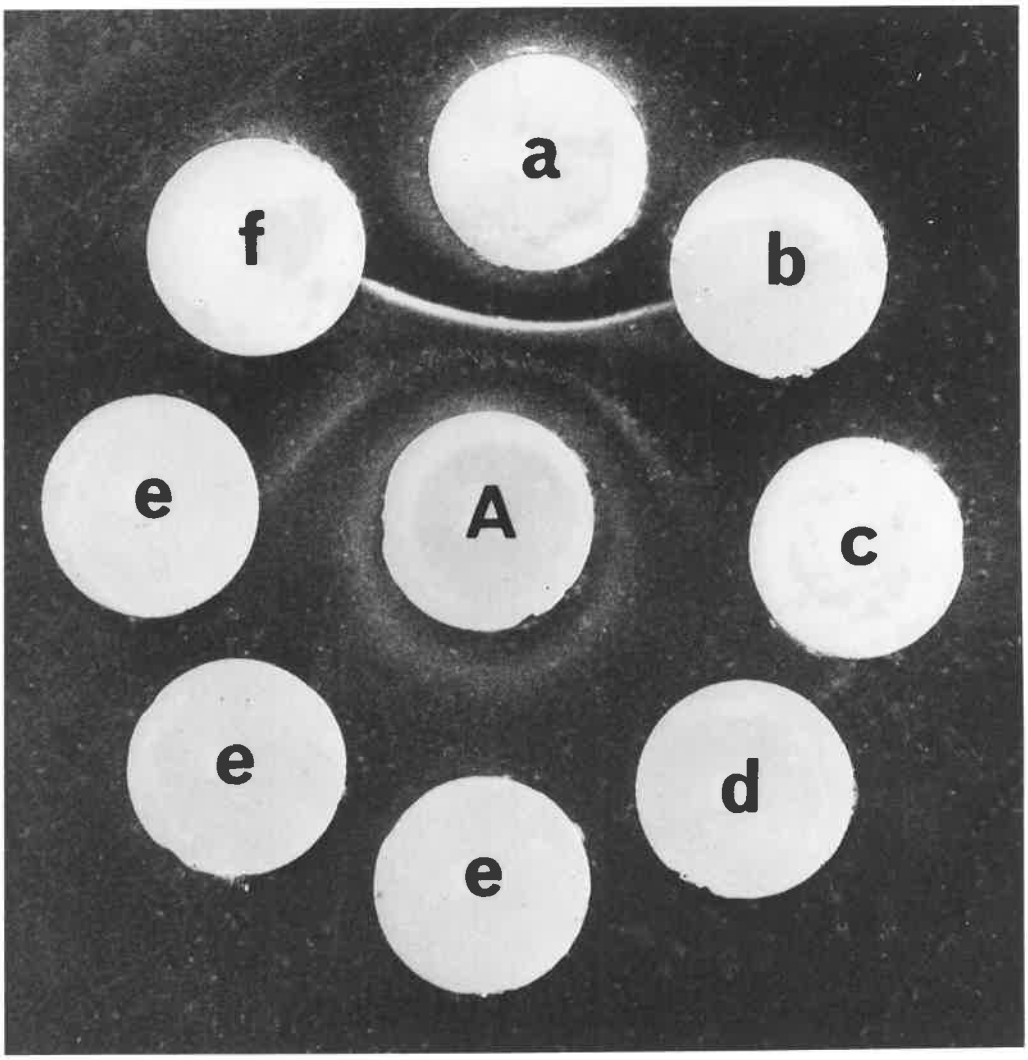
(h) Viral RNA: FDV-RNA was extracted from purified FDV preparations by the phenol-SDS procedure as described in Chapter II. All RNA antigens used in serological tests were suspended in STE buffer at a concentration of 100 µg/ml.

RESULTS

1. Properties of FDV antiserum

Two precipitin lines were obtained when sera of mice immunized with purified FDV preparations were tested against FDV-infected sap; a heavy line was always observed near the antigen well and a fainter one near the antiserum well (Fig. 8). The line nearer the antiserum well was confluent with the single line produced between the antiserum and phenol-extracted FDV antigen (FDV nucleic acid) (Fig. 8). In most, but not all, tests, there was also a weak precipitin line between the anti-FDV serum and poly [I] : poly [C] (Fig. 8). Antigens prepared from healthy sugarcane leaves (both healthy sap and healthy nucleic acid) invariably fialed to produce any reactions when tested with the FDV antisera (Fig. 8). From these experiments it was concluded that antiserum produced in response to injection of FDV preparations was free of host plant antigens but contained antibodies to both FDV protein (producing a precipitin line nearer the antigen well)

Fig. 8. Two-dimensional immunodiffusion of antiserum to preparations of FDV particles (A) and the following antigens: a, FDV-infected sap; b, FDV nucleic acid; c, host nucleic acid; d, healthy sap; f, poly [I] : poly [C]. (Wells marked e remained empty.)


a
f
b
e
A
c
e
d
e

and FDV-RNA (producing a precipitin line nearer the antiserum well).

Details of the immunization schedule and titres of sera from both mice immunized with preparations of FDV are summarized in Table 11. The results show that mouse 2 was very much more immunoresponsive to FDV than mouse 1 and that antisera from both mice invariably contained antibodies to both FDV protein and FDV-RNA. Individual animals are known to vary quite widely in their response to a particular antigen (Sobey, 1954; Matthews, 1970). It is also evident that antibody titres to both FDV protein and RNA were higher in the blood serum than in the ascitic fluid. However, only 50-150 µl of blood serum was obtained from each bleeding whereas yields of as much as 5 ml of ascetic fluid could be obtained by tapping a tumerogenic mouse. In the case of mouse 2 a total of 10.5 ml of ascitic fluid was obtained from the four tappings taken (Table 11). The data summarized in Table 11 also show that both the blood serum and ascitic fluid always had higher antibody titres against FDV protein than against FDV-RNA.

2. Tests for serological relationship between FDV,MRDV,MWEV and RDV

Experiments were carried out to investigate the possibility of a serological relationship between FDV, MRDV, MWEV and RDV. Sap from FDV-diseased tissue produced single precipitin bands when tested with antisera to RDV and MRDV (Fig. 9). However, these bands were confluent with those produced by the antisera and FDV-nucleic acid preparations

TABLE 11. Immunization schedule and antibody production in mice immunized with preparation of FDV particles.

Days after initial immunization	Treatment of mice[a]	Antibody titre[b]							
		Mouse 1				Mouse 2			
		Blood serum against:		Ascitic fluid against:		Blood serum against:		Ascitic fluid against:	
		Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA
0,6 and 13	1st-3rd injection								
19	1st bleeding	4-8	2			32	n.d.[c]		
32	2nd bleeding	n.d.	n.d.			64	32		
39	3rd bleeding	4-8	2-4			128-256	64		
57	4th injection								
67	5th injection 4th bleeding and injection with Ascites Tumor	2-4	2						
73	1st tapping			2	1			32	16
75	2nd tapping			n.d.	n.d.			32	16
77	5th bleeding and 3rd tapping	4	2	2	1			64-128	32
80	6th bleeding and 4th tapping	n.d.	n.d.	n.d.	n.d.	256	64	128	32

a) Bleeding refers to collection of blood for assay of blood serum antibodies and tapping refers to collection of ascitic fluid for antibody assay.

b) Titres are expressed as reciprocals of highest dilutions producing a visible precipitin line in gel-diffusion tests.

c) Not determined.

or poly [I] : poly [C] (Fig. 9). Even after two weeks of incubation, no reactions were observed between the two antisera and similar preparations from healthy leaf tissue (Fig. 9). Failure by Francki and Jackson (1972) to detect any serological reaction between FDV-infected sap and antiserum to poly [I] : poly [C] was probably due to the fact that FDV was not as concentrated in their preparations as in those used for the present studies. No reactions were observed between any of the antigens when tested against two normal sera and antisera to the single-stranded viruses, TMV and potato virus X.

The above observations indicate that the antisera to RDV and MRDV contained antibodies to ds-RNA. This was confirmed by intragel cross-absorption tests (Van Regenmortel, 1967) using poly [I] : poly [C] to absorb antibodies to ds-RNA. From the results summarized in Fig. 10, it can be seen that all antibodies in antisera to RDV and MRDV which reacted with FDV-infected sap could be absorbed with poly [I] : poly [C]. Thus, it is concluded that no serological relationship can be inferred from the above experiments between the viral proteins of FDV and RDV or MRDV.

Sap from MWEV-infected leafhopper tissue produce a single precipitin band when tested against antiserum to FDV (Fig. 11). This band was confluent with those produced by the antisera and MWEV-nucleic acid preparations or poly [I] : poly [C] (Fig. 11). Even after one week of incubation, no reactions were observed between the antiserum

Fig. 9. Two-dimensional immunodiffusion of antisera to RDV and MRDV with various antigen preparations. (A) Antiserum to RDV was placed in the centre well and antigen preparations in the outer wells. Well a, FDV-infected sap; well b, FDV nucleic acid; well c, poly [I]: poly [C]; well d, healthy sap; well e, host nucleic acid. All the antigens were prepared as described in text. (B) Antiserum to MRDV (B) was placed in the centre well and antigen preparations in outer wells as in (A).

Fig. 10. The effect of cross-absorbing antisera to RDV and MRDV with poly [I] : poly [C] on the serological reactions with FDV-infected sap. (A) Antiserum to RDV in a two-fold dilution series was placed in wells marked A and in wells mared Aabs. To the wells marked Aabs, poly [I]; poly [C] had been added 24 h previously. FDV-infected sap was added to wells marked a. (B) Antiserum to MRDV (B) was used but otherwise the arrangement of reactants was similar to that in (A).

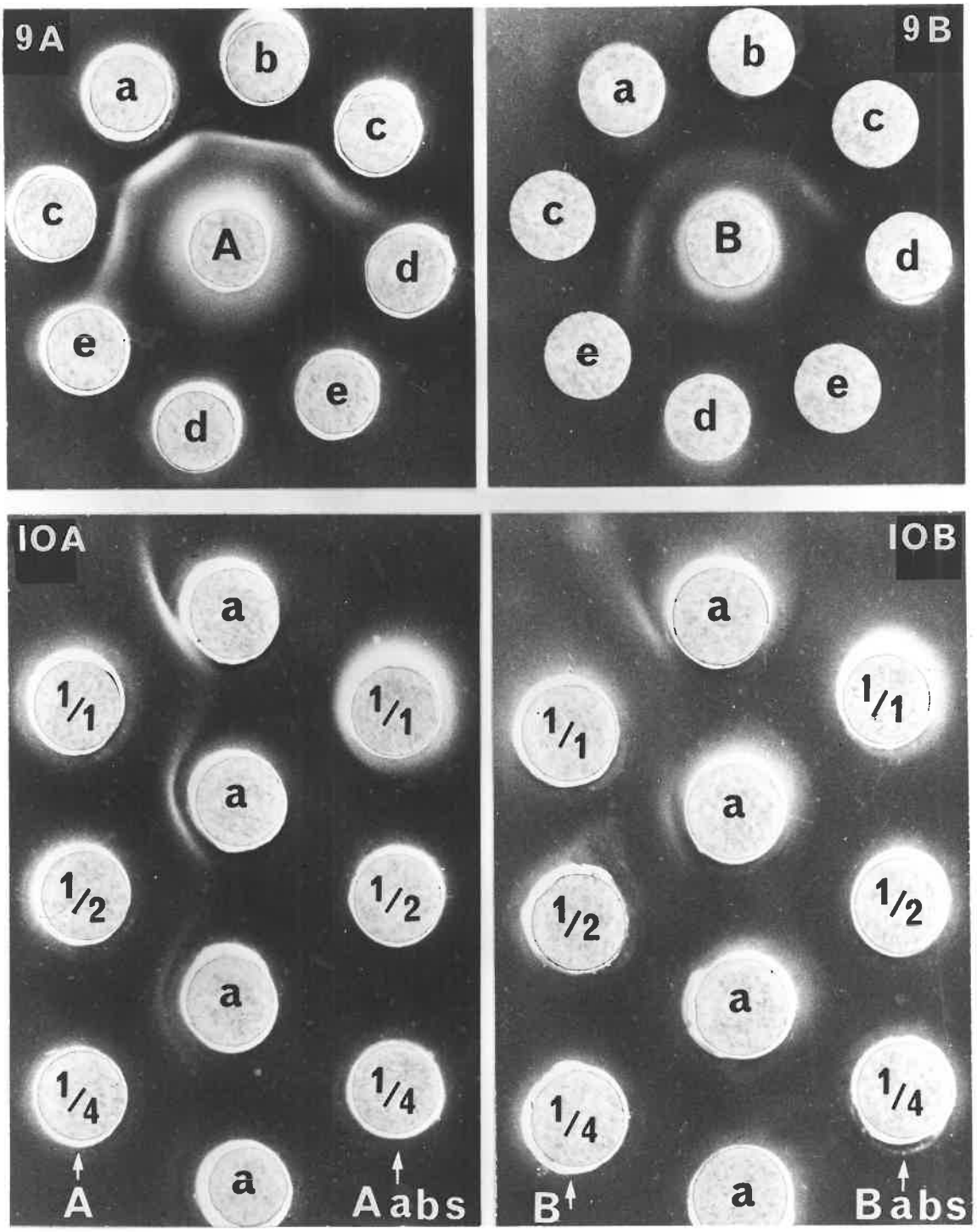
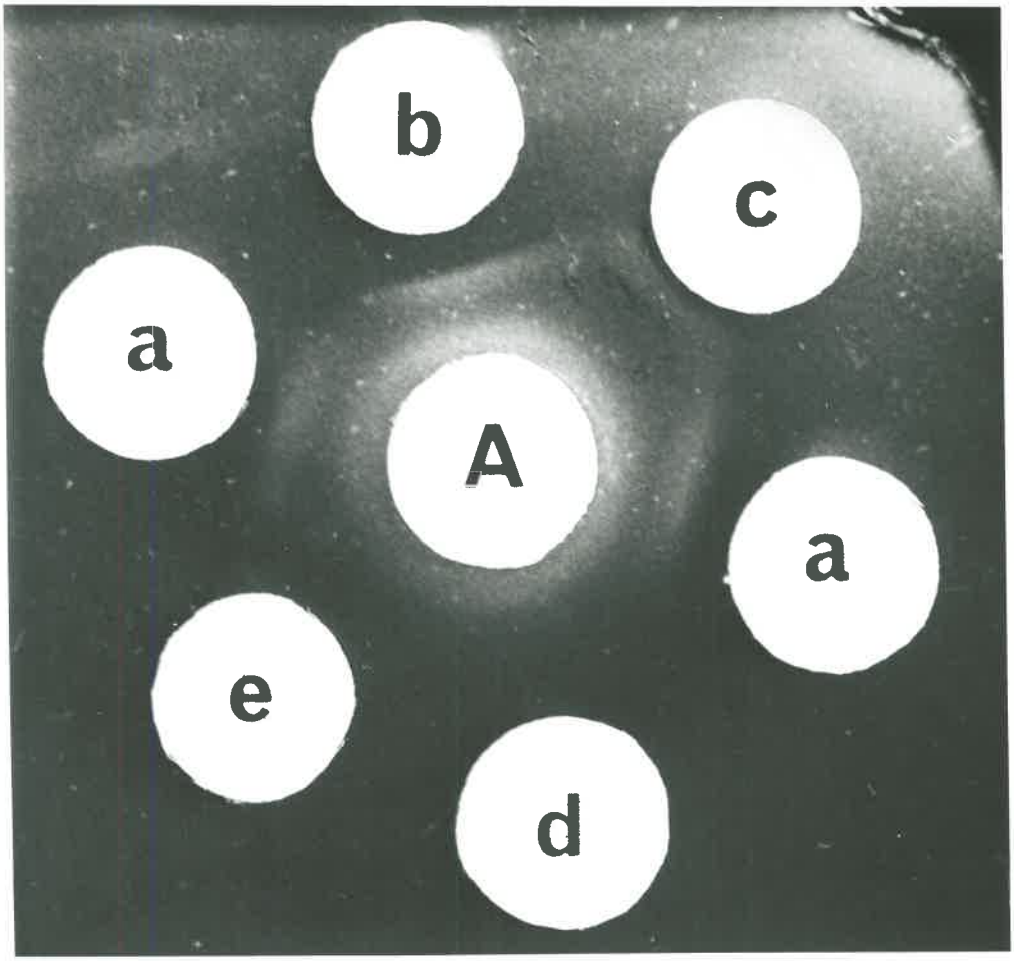

9A
a
b
c
c
A
d
e
e
d
9B
a
b
c
c
B
d
e
d
e
IOA
a
1/1
1/1
a
1/2
1/2
a
1/4
1/4
A
a
Aabs
IOB
a
1/1
1/1
a
1/2
1/2
a
1/4
1/4
B
a
Babs

Fig. 11. Two-dimensional immunodiffusion of antiserum to FDV (A) and the following antigens: a, MWEV-infected sap; b, MWEV nucleic acid; c, poly [I] : poly [C]; d, host nucleic acid; e, healthy sap.


b
c
a
A
a
e
d

and similar preparations from non-viruliferous *C. bimaculata*. From these experiments, it is concluded that antibodies to FDV protein do not recognize protein of MWEV.

3. Antigenic variation among ds-RNAs from viruses and a synthetic polyribonucleotide

Experiments were designed to investigate the specificity of anti-ds-RNA bodies. Data summarized in Table 12 demonstrate that all four antisera used reacted with the four antigens tested confirming previous reporst that antibodies to ds-RNAs react with a wide variety of ds-polyribonucleotides (Stollar, 1973). However, it also appears that there is considerable antigenic variation between the various RNAs. For example, anti-FDV serum had a significantly higher titre against homologous antigen than against any of the heterologous antigens used (Table 12). This antigenic specificity is confirmed by data summarized in Table 13, showing that antibodies in the anti-FDV serum were less efficient at recognizing heterologous antigens. On the other hand, antibodies to poly [I] : poly [C] appear to be far less specific (Table 12 and 13).

Intragel cross-absorption tests demonstrated that whereas all the homologous antibodies in anti poly [I] : poly [C] serum could be absorbed with heterologous antigen such as FDV-RNA (Fig. 12), antibodies to FDV-RNA in anti-FDV serum were not absorbed to any great extent with heterologous antigen such as poly [I] : poly [C] (Fig. 12).

TABLE 12. Reactions between ds-RNAs with antisera to FDV, MRDV, RDV and poly [I] : poly [C]

Antigen Preparation[a]		Titre of antiserum to:[b]			
		FDV	MRDV	RDV	Poly [I] : Poly [C]
FDV-RNA	native	16	2	8	16
	heat-denatured	NR[c]	NR	NR	NR
Reovirus-RNA	native	1	8-16	8	8
	heat denatured	NR	NR	NR	NR
ϕ6-RNA	native	2	4-8	1	16
	heat denatured	NR	NR	NR	NR
Poly [I] : poly [C]	native	1	4	8	16-32
	heat denatured	NR	NR	NR	1

a) All antigen preparations tested were at a concentration of 100 µg/ml suspended in STE buffer.

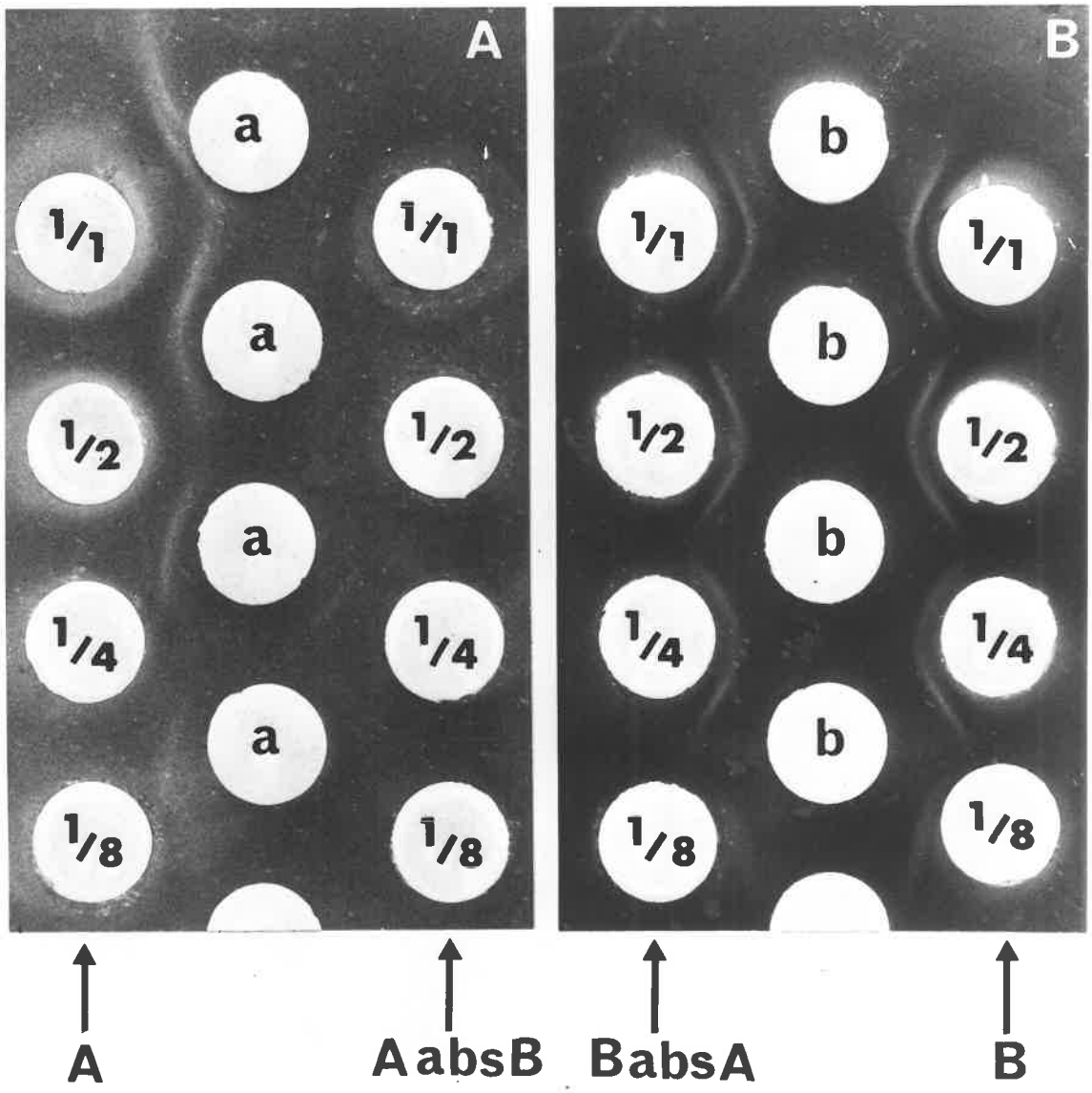
b) Titres are expressed as reciprocals of highest dilution producing a visible precipitin line in gel-diffusion tests.

c) NR indicates no visible reaction with undiluted antigen.

TABLE 13. Minimum amounts of ds-RNA detected by antisera to FDV, MRDV, RDV and Poly [I] : poly [C]

Antiserum to	Minimum amount of ds-RNA detected (µg)			
	FDV-RNA	Reovirus-RNA	ϕ6-RNA	Poly [I]: poly [C]
FDV	0.04	100	100	100
MRDV	0.30	0.30	0.60	0.08
RDV	0.60	0.30	100	0.08
Poly [I] : poly [C]	0.08	0.08	0.60	0.04

Fig. 12. The effect of cross-absorbing antisera to FDV particles and poly [I] : poly [C]. (A) Antiserum to poly [I] : poly [C] in a twofold dilution series was loaded to wells marked A and in those marked AabsB. To the well marked AabsB, FDV nucleic acid was added 24 h previously. Poly [I] : poly [C] was added to wells marked a. (B) Antiserum to FDV particles in a twofold dilution was loaded in wells marked B and BabsA. To the wells marked BabsA, poly [I] : poly [C] was added 24 h previously. FDV nucleic acid was added to wells marked b.


A
a
1/1
1/1
a
1/2
1/2
a
1/4
1/4
a
1/8
1/8
B
b
1/1
1/1
b
1/2
1/2
b
1/4
1/4
b
1/8
1/8
A
AabsB
BabsA
B

Although I was unable to test the anti-MRDV and anti-RDV sera with homologous antigens, data summarized in Tables 12 and 13 indicate that antibodies to ds-RNA in these sera also vary in their ability to recognize different ds-polyribonucleotides. Similarly, ds-RNAs from reovirus and bacteriophage ϕ6 appear to differ antigenically. For example, antibodies in anti-RDV serum appear to be far less efficient at recognizing ϕ6-RNA than either FDV-RNA, reovirus-RNA or poly [I] : poly [C] (Tables 12 and 13). This was confirmed by intra-gel cross-absorption tests showing that antibodies in anti-RDV serum which recognized FDV-RNA could be absorbed with reovirus-RNA but not with ϕ6-RNA (Fig. 13).

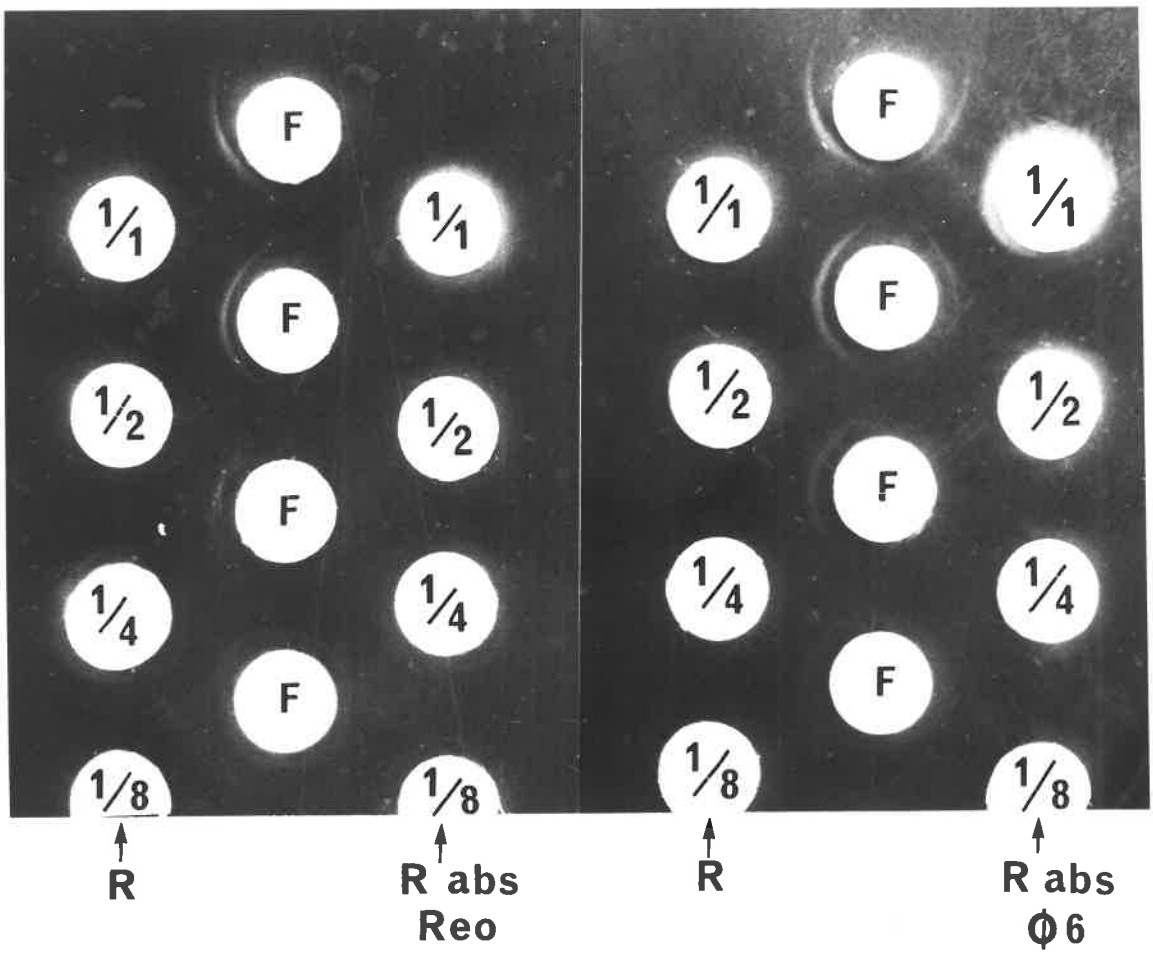
The antigenicity of the polyribonucleotides was destroyed by heat denaturation (Table 12).

CONCLUSIONS

The studies reported in this chapter demonstrate that:

1. Antisera against FDV, MRDV and RDV contained antibodies to ds-RNA as well as to the viral proteins.
2. Antibodies in ascitic fluid could be used, when large volumes of serum are needed.
3. Antibody titres to both FDV protein and ds-RNA were higher in the blood serum than in the ascitic fluid.
4. Both the blood serum and ascitic fluid always had higher antibody titres against FDV protein than against FDV-RNA.

Fig. 13. Two-dimensional immunodiffusion of FDV-RNA (F) with anti-RDV serum before (R) and after cross-absorption with reovirus-RNA (Rabs Reo) and $\phi 6$ bacteriophage-RNA (Rabs $\phi 6$). Each well containing antigen was loaded with ds-RNA at a concentration of 100 µg/ml.


F
1/1
1/1
F
1/2
1/2
F
1/4
1/4
F
1/8
1/8
R
R abs
Reo
F
1/1
1/1
F
1/2
1/2
F
1/4
1/4
F
1/8
1/8
R
R abs
Φ6

5. Antibodies to MRDV and RDV proteins did not recognize the protein of FDV.

6. Antibodies to FDV protein did not react with the protein of MWEV.

7. There appears to be some degree of specificity among antibodies to ds-RNAs.

CHAPTER V

CHARACTERIZATION OF NUCLEIC ACID FROM FDV SUBVIRAL PARTICLES

INTRODUCTION

FDV subviral particles were purified from FDV-infected plants (Chapter III). The purity of these preparations was sufficient to investigate the viral nucleic acid. In this chapter, the isolation and characterization of nucleic acid from FDV subviral particles are described.

MATERIALS AND METHODS

1. TMV purification

TMV was purified from tobacco plants. All procedures were carried out at 4°C. Frozen and systemically infected leaf tissue was extracted in 0.5M-Na_2HPO_4-KH_2PO_4 buffer (pH 7.2) containing 1% 2-mercaptoethanol. The pulp was strained through a double layer of cheesecloth and n-butanol was added to give the final concentration of 8%, while stirring. The mixture was then centrifuged at 10,000 g for 30 min and 4 gm of PEG (Mol. wt. 6000)/100 ml was added to the supernatant. After the PEG had dissolved at 4°C, the mixture was centrifuged at 10,000 g for 15 min. The pellets were resuspended in 0.01M-phosphate buffer (pH 7.2) and clarified at 10,000 g for 15 min. The supernatant was ultracentrifuged at 105,000 g for 60 min and the virus pellet was suspended in 0.01M-phosphate buffer.

After one more cycle each of low-speed and high-speed centrifugation, the final pellet was resuspended in 0.01M-phosphate buffer (Gooding and Hebert, 1967).

2. Orcinol reaction

The stock reagent was prepared by dissolving 0.135 gm of ferric ammonium sulphate and 0.2 gm of orcinol in 5 ml of solution. Before use, 2.5 ml of the stock reagent was added to 41.5 ml of concentrated HCl and diluted to 50 ml with water. The test was run by adding 1 ml of test solution to 3 ml of the dilute reagent, and heating for 20 min in a boiling water bath. The solution was cooled and its colour was observed (Volkin and Cohn, 1954).

3. Diphenylamine reaction

The reagent was prepared by dissolving 1.0 gm of diphenylamine in 98 ml of redistilled glacial acetic acid and then adding 2 ml of concentrated H_2SO_4. The reagent was prepared just before use. One ml of the sample was added to 2.5 ml of the reagent and the mixture was heated for 5 min in a boiling water bath. The sample was cooled and its colour was observed (Volkin and Cohn, 1954).

4. Thermal denaturation

Thermal denaturation profiles of RNA samples were determined in a Unicam SP1800 spectrophotometer equipped with an SP876 series 2 Temperature Programme Controller and a Philips X-Y recorder. The cell block was heated at $0.5^{\circ}C$/min. The RNA was at a concentration

of 10-50 µg/ml and it was contained in a stoppered 1 ml quartz cuvette with 10 mm path length.

5. Digestion with RNase

RNase solution (100 µg/ml) was added to 1 ml of RNA solution in 0.1 x SSC and 1 x SSC to give final concentrations of 0.04 µg/ml and 0.5 µg/ml respectively. After incubation of the mixture at 25°C, the increase in A_{260} was measured at intervals. A_{260} at time zero was that measured before the addition of the enzyme.

6. Isopycnic ultracentrifugation

FDV-RNA (50 µg) or TMV-RNA (20 µg) in 1 ml of 1 x SSC (pH 7.0) was mixed with 4 ml of caesium sulphate so as to produce a solution with density 1.60 gm/cm^3.

Densities of Cs_2SO_4 solutions were calculated using the following formula:

$$\rho^{25^{\circ}C} = 1.0047 + 0.28369m - 0.017428m^2 \quad (0.5 \leq m \leq 3.5)$$

where m is the molarity (Ludlum and Warner, 1965).

These preparations were centrifuged at 35,000 rpm for 88 h in a Spinco SW50.1 rotor at 5°C. Centrifuge tubes were punctured at the bottom and the contents of each tube were collected dropwise into 25 fractions. The density of each fraction was determined gravimetrically as described by Szybalski (1968). A weighed 50 µl-micropipette was first filled with distilled water, weighed, and dried with acetone by suction. It was then refilled with the various

fractions and weighed at 25°C. Between each fraction, the micropipette was washed with distilled water and dried with acetone. The densities were calculated using the following equation (Szybalski, 1968):

$$\rho_o = \frac{\text{net weight of each fraction}}{\text{net weight of water}} \times 0.99704$$

The absorbance at 260 nm of each fraction was determined after dilution of each sample with 0.8 ml of distilled water.

7. Formaldehyde treatment of RNA

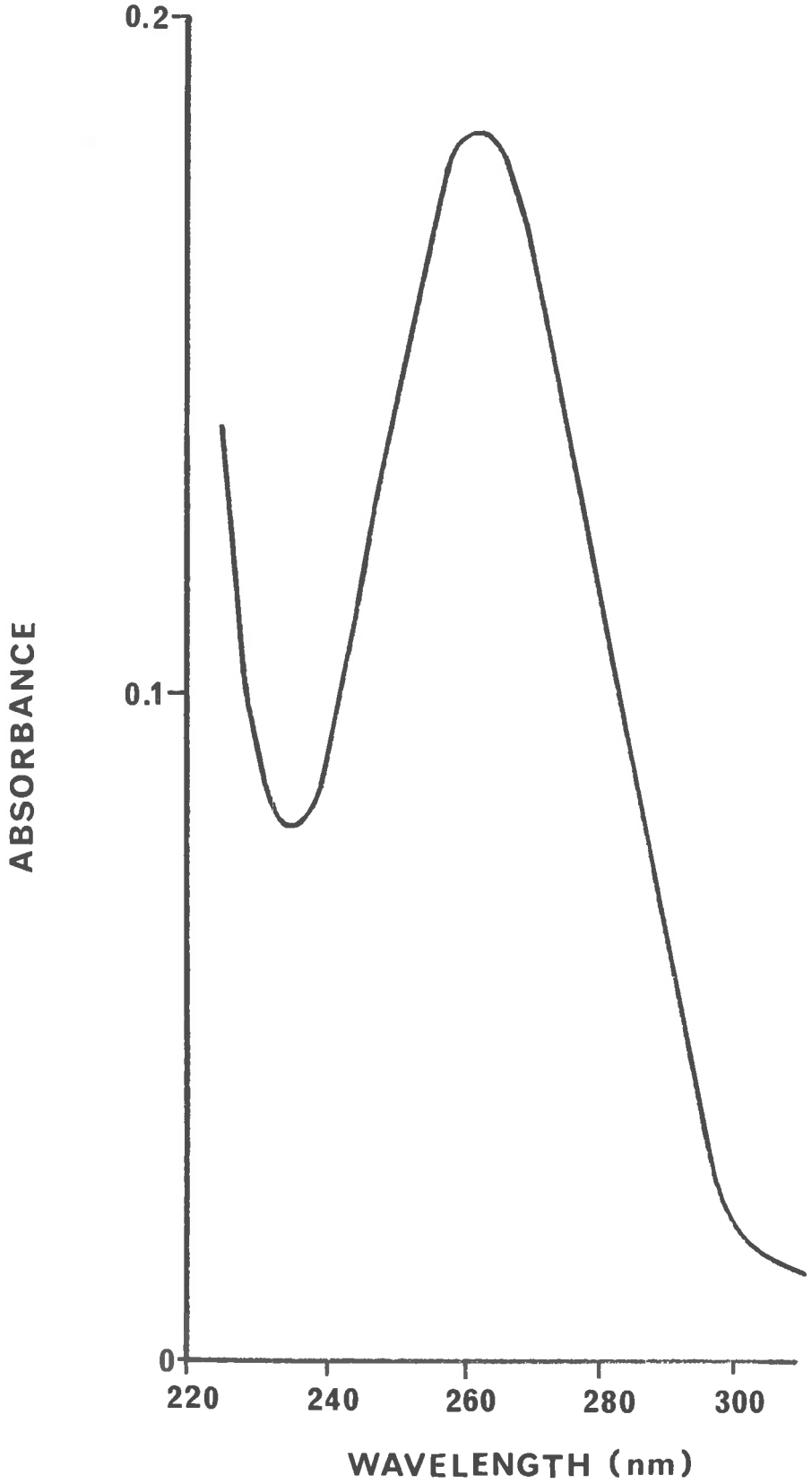
To phenol-SDS prepared RNA in 1 ml of 0.1M-NaCl, was added 50 µl of 39-40% formaldehyde solution to give a final concentration of 1.8% (Miura *et al.*, 1966, 1968). The UV spectra of the mixtures in stoppered cuvettes were examined after 23 h incubation at 37°C in a water bath. The UV absorption at zero time was measured by addition of 50 µl of distilled water instead of formaldehyde solution.

RESULTS

1. Purity of nucleic acids extracted from viruses

Fig. 14 shows a typical UV spectrum of nucleic acid extracted from a purified preparation of FDV subviral particles by the phenol-SDS procedure. A_{260}/A_{230} and A_{260}/A_{280} ratios were about 2.2 and 1.8 respectively. RNA preparations from TMV had UV spectra with A_{260}/A_{230} and A_{260}/A_{280} ratios of about 2.5 and 2.4.

Fig. 14. UV spectrum of a typical preparation of nucleic acid isolated from FDV subviral particles by the phenol-SDS method.


0.2
0.1
0
ABSORBANCE
220
240
260
280
300
WAVELENGTH (nm)

2. Type of nucleic acid extracted from FDV subviral particles

FDV was shown to contain RNA by the following tests: (1) Nucleic acid preparations gave positive orcinol reactions indicating the presence of ribose; (2) the diphylamine reaction for deoxyribose did not induce any colour.

3. Evidence for FDV having ds-RNA

(a) Thermal denaturation

In thermal denaturation experiments, the UV absorption of FDV-RNA increased rapidly around 76°C, when heated in 0.01 x SSC (Fig.15). Maximum increase in A_{260} on heating the RNA in 0.01 x SSC was about 44%. The melting temperature (Tm) was 76°C for FDV-RNA under these conditions.

TMV-RNA showed a temperature-dependent increase in UV absorption over a wide range under the same conditions.

(b) Resistance to RNase

In order to observe denaturation of FDV-RNA by RNase treatment, hyperchromicity at 260 nm was measured (Fig. 16). When 0.04 µg/ml of RNase in 0.1 x SSC was used, the increase in UV absorption was small for both native FDV-RNA and ϕ6-RNA which is known to be double-stranded (Semancik *et al.*, 1973) as compared with TMV-RNA (Fig. 16A). Heat-denatured FDV-RNA showed a hyperchromicity similar to that for TMV-RNA. Furthermore, when suspended in 1 x SSC, both native FDV-RNA and ϕ6-RNA were highly resistant to RNase digestion, unlike heat-denatured FDV-RNA and TMV-RNA (Fig. 16B).

Fig. 15. Thermal denaturation kinetics of FDV-RNA (———) as compared to those of TMV ss-RNA (---). RNAs were isolated by phenol-SDS extraction and were suspended in 0.01 x SSC. Heating was at a rate of 0.50°C/min and reannealing was allowed to take place at a room temperature of 25°C after switching off the heater.

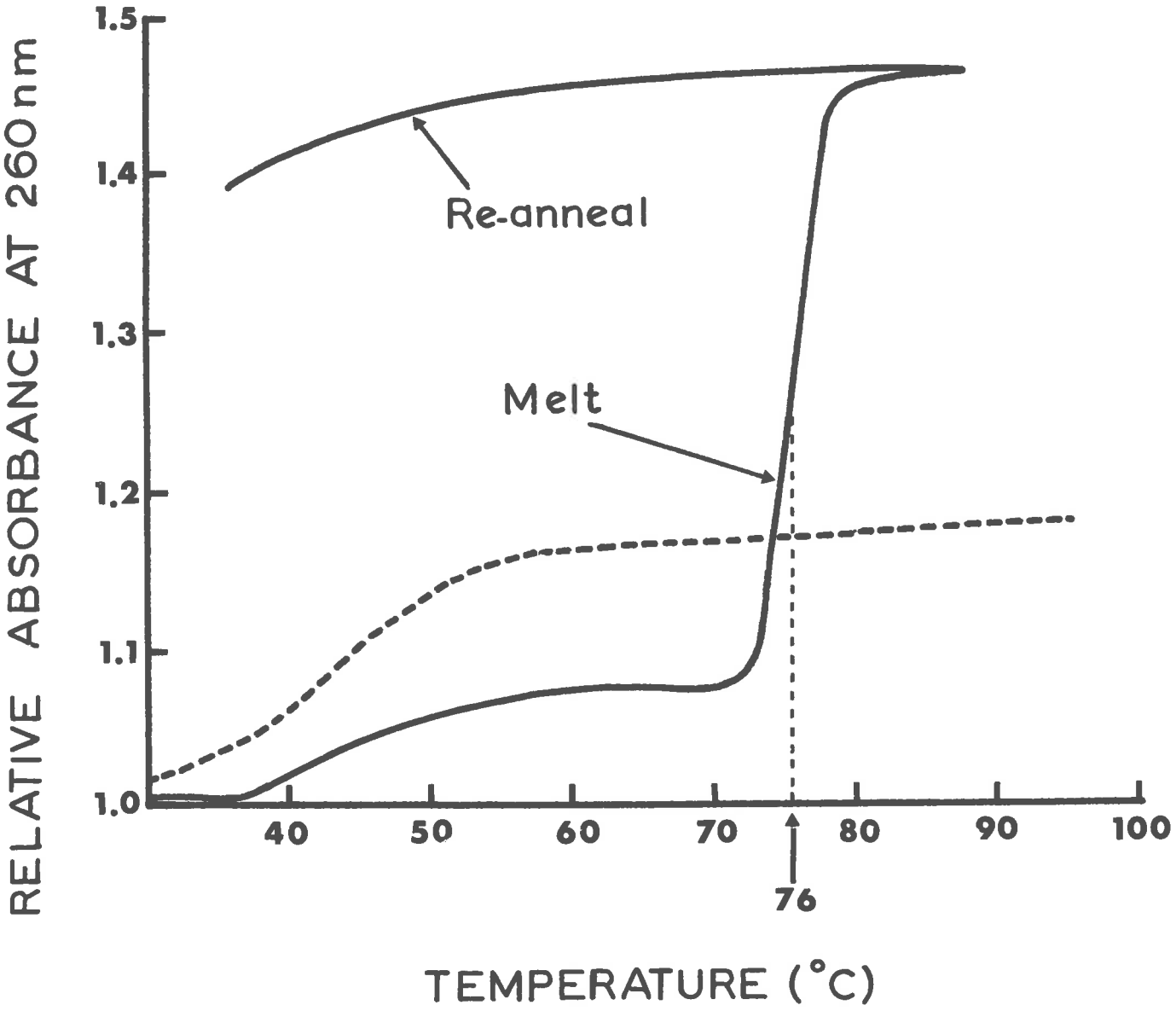
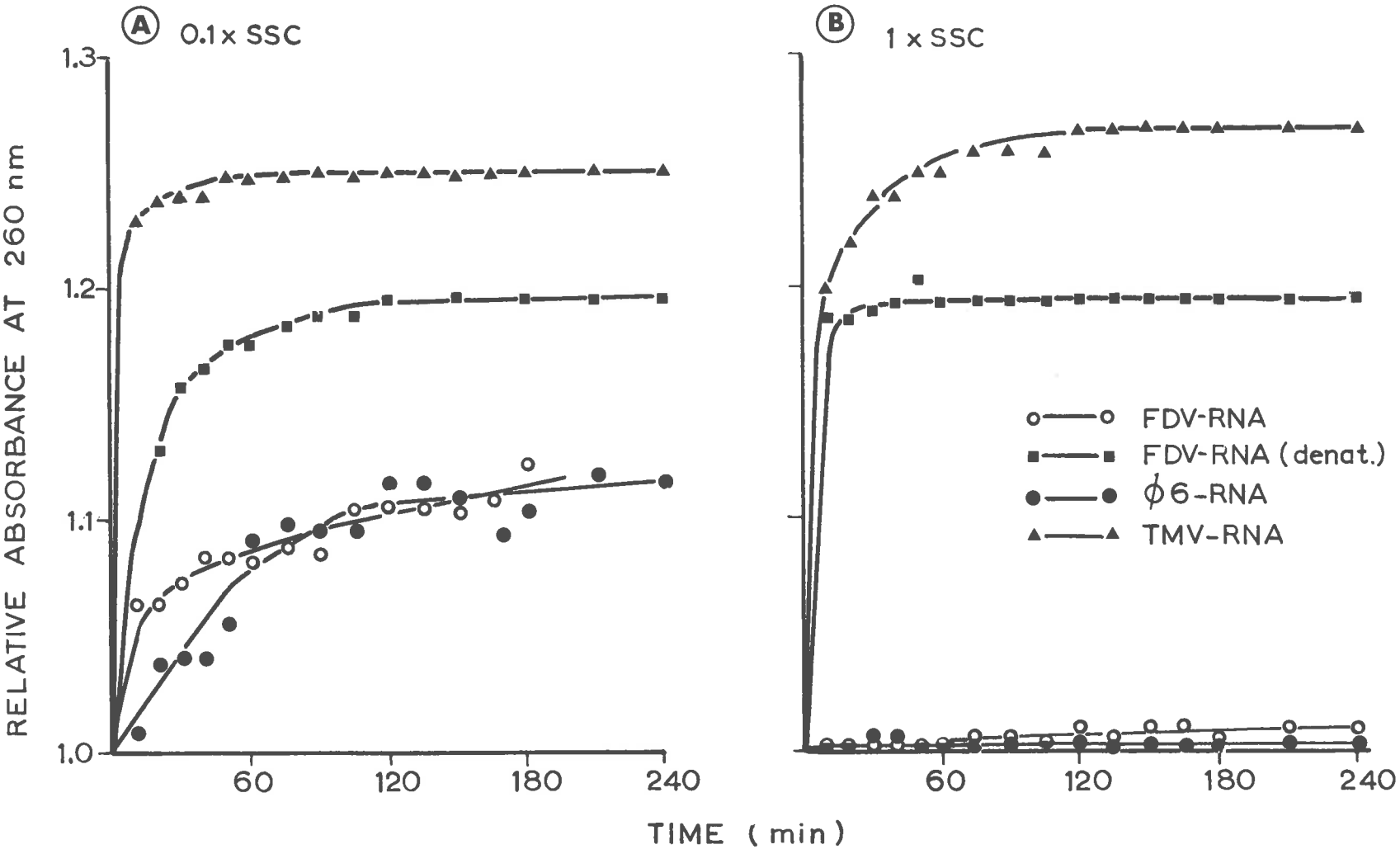

RELATIVE ABSORBANCE AT 260nm
1.5
1.4
1.3
1.2
1.1
1.0
Re-anneal
Melt
40
50
60
70
80
90
100
76
TEMPERATURE (°C)

Fig. 16. Kinetics of digestion of FDV-RNA compared to that of ϕ6 phage ds-RNA and TMV ss-RNA in the presence of RNase.

The nucleic acids were isolated by phenol-SDS extraction and were suspended in either 0.1 x SSC (A) or 1 x SSC (B). FDV-RNA was denatured by heating in 0.1 x SSC for 10 min at 100°C followed by rapid cooling in an ice-ethanol bath.

About 15 µg/ml of each RNA preparation was incubated at 25°C in the presence of 0.04 µg/ml (A) and 0.5 µg/ml (B) of RNase.


A 0.1 x SSC
B 1 x SSC
RELATIVE ABSORBANCE AT 260 nm
1.3
1.2
1.1
1.0
60
120
180
240
TIME (min)
FDV-RNA
FDV-RNA (denat.)
ϕ6-RNA
TMV-RNA

(c) Buoyant density of FDV-RNA in caesium sulphate

The buoyant density of FDV-RNA, as determined by equilibrium density-gradient centrifugation in Cs_2SO_4, was 1.60 gm/cm^3 (Fig. 17). This was easily distinguished from TMV-RNA which banded at a density of 1.66 gm/cm^3 under the same conditions (Fig. 17). The density of FDV-RNA is similar to that reported for reovirus ds-RNA (Shatkin, 1965; Iglewski and Franklin, 1967), ϕ6 ds-RNA (Semancik *et al.*, 1973) and replicative form of poliovirus (Bishop and Koch, 1967).

(d) Nonreactivity with formaldehyde

Fig. 18 shows the effect of formaldehyde on FDV-RNA suspended in 0.1M-NaCl. While TMV-RNA exhibited a marked increase in absorbance at 260 nm (22%) and a shift of maximum absorption to a longer wavelength (4-6 nm), FDV-RNA showed no significant increase or shift in UV absorption maximum and minimum; FDV-RNA is thus like other ds-RNAs (Miura *et al.*, 1966, 1968). After heat-denaturation, FDV-RNA reacted with formaldehyde in the same manner as TMV-RNA.

All these results indicate that FDV-RNA is double-stranded and that heating and rapid cooling produced a single-stranded structure.

It is interesting to note that the relative absorbance of FDV-RNA increased to a greater extent on thermal denaturation (Fig. 15) than digestion of denatured RNA with RNase (Fig. 16), whereas the reverse was observed with TMV-RNA. These data indicate that denatured FDV-RNA undergoes only partial RNase digestion, probably because of rapid partial renaturation.

Fig. 17. Isopycnic density-gradient centrifugation of FDV-RNA as compared to that of TMV ss-RNA in Cs_2SO_4.
Each RNA prepared by pronase-SDS digestion in 1 ml of 1 x SSC was mixed with 4 ml of Cs_2SO_4 to produce a solution of density 1.60 gm/cm^3. The sample was centrifuged at 35,000 rpm for 88 h in a Spinco SW50.1 rotor at 5^oC (absorbancy, 0——0; density ⊗——⊗).

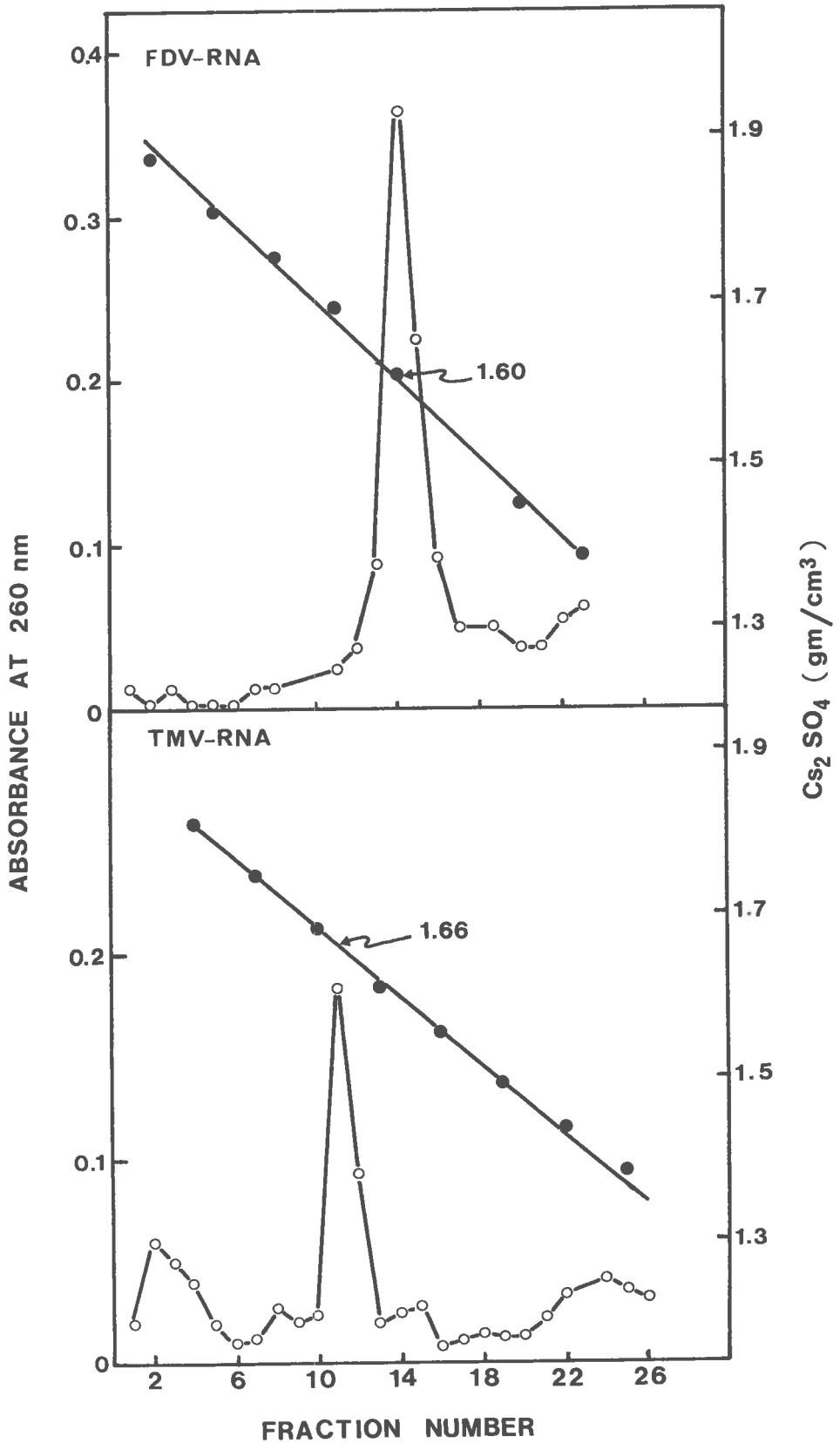
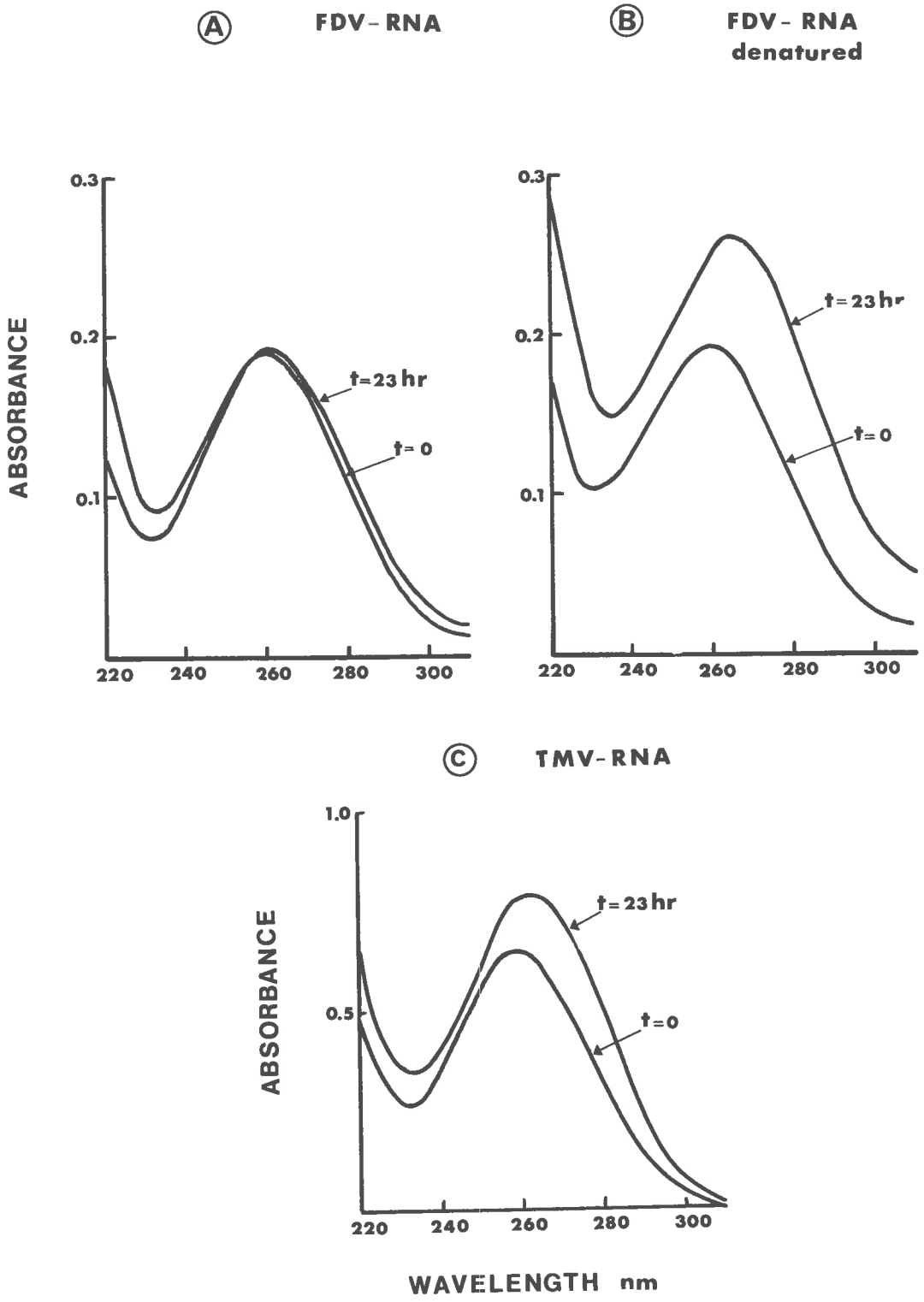

FDV-RNA
TMV-RNA
1.60
1.66
0.4
0.3
0.2
0.1
0
1.9
1.7
1.5
1.3
2
6
10
14
18
22
26
ABSORBANCE AT 260 nm
Cs_2SO_4 (gm/cm^3)
FRACTION NUMBER

Fig. 18. Reaction of native (A) and heat denatured (B) FDV-RNA compared to that of TMV ss-RNA (C) with formaldehyde. The UV spectra of phenol-SDS prepared RNA in 0.1M-NaCl were examined after 23 h incubation at 37°C (t = 23 h) in the presence of 1.8% formaldehyde. The absorbance at zero time (t = 0) is that measured before the addition of formaldehyde.


Ⓐ FDV-RNA
Ⓑ FDV-RNA denatured
ABSORBANCE
0.3
0.2
0.1
t=23 hr
t=0
220
240
260
280
300
Ⓒ TMV-RNA
1.0
0.5
WAVELENGTH nm

4. Molecular weight and segmentation of FDV ds-RNA

Polyacrylamide gel electrophoresis of FDV-RNA preparations for 30 h resulted in the resolution of eight distinct bands (Fig.19). Reovirus-RNA and ϕ6-RNA separated into 9 and 3 bands respectively (Figs. 19 and 21). The electrophoretic profiles of reovirus-RNA and ϕ6-RNA were similar to those obtained by Shatkin *et al.* (1968) and Semancik *et al.* (1973) respectively. The profile of each RNA was reproducible, and specific to the virus. When coelectrophoresed with reovirus-RNA and ϕ6-RNA, it was demonstrated that the molecular weight of the FDV-RNA segments ranged from 2.60 to 1.08 x 10^6 daltons (Figs. 19 and 21).

The intensity of staining of the fastest migrating band suggested that two RNA species of similar molecular weight may have been co-electrophoresing. Although when the time of electrophoresis was increased to 45 h, this band was still single and homogeneous (Fig.20). Since the electrophoretograms were consistently reproducible, estimates of the relative amounts of each RNA class per virion could be made by measuring the areas under each peak from the electrophoretogram (Fig.20). On the assumption that the FDV genome contains a single segment of class 5 RNA, the molar ratio of each band was calculated taking acount of their molecular weights. As shown in Table 14, the fastest migrating band appeared to consist of two components.

Fig. 19. Comparison of RNA molecules extracted from FDV and reovirus.

FDV-RNA isolated by pronase-SDS extraction and reovirus-RNA isolated by the phenol-SDS procedure (sample of about 15 µg in 1 x PTE buffer containing 5% sucrose) were separated by polyacrylamide gel electrophoresis for 30 h as described in Chapter II. The gels were stained in 0.1% toluidine blue 0 and destained in distilled water. Migration is from top to bottom. The molecular weights of the FDV-RNA segments were calculated using reovirus-RNA and ϕ6-RNA segments as markers (see Fig. 21).

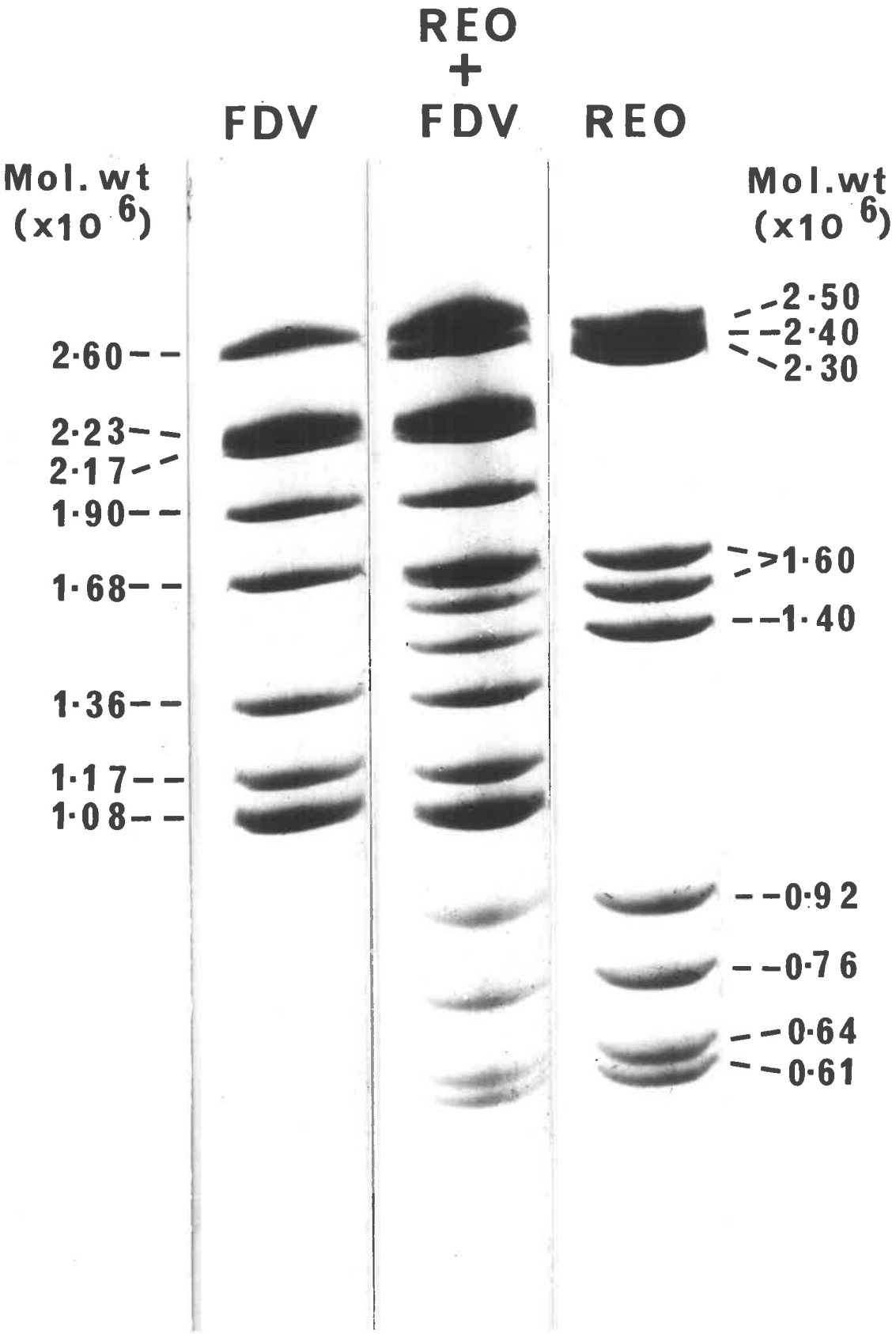

REO
+
FDV
FDV
REO
Mol. wt
(x10 6)
Mol.wt
(x10 6)
2·60
2·23
2·17
1·90
1·68
1·36
1·17
1·08
2·50
2·40
2·30
>1·60
1·40
0·92
0·76
0·64
0·61

Fig. 20. Electrophoretogram of FDV-RNA segments after electrophoresis in PTE buffer for 45 h, staining in 0.1% toluidine blue O, destaining in distilled water and scanning at 620 nm.
Migration is from left to right.

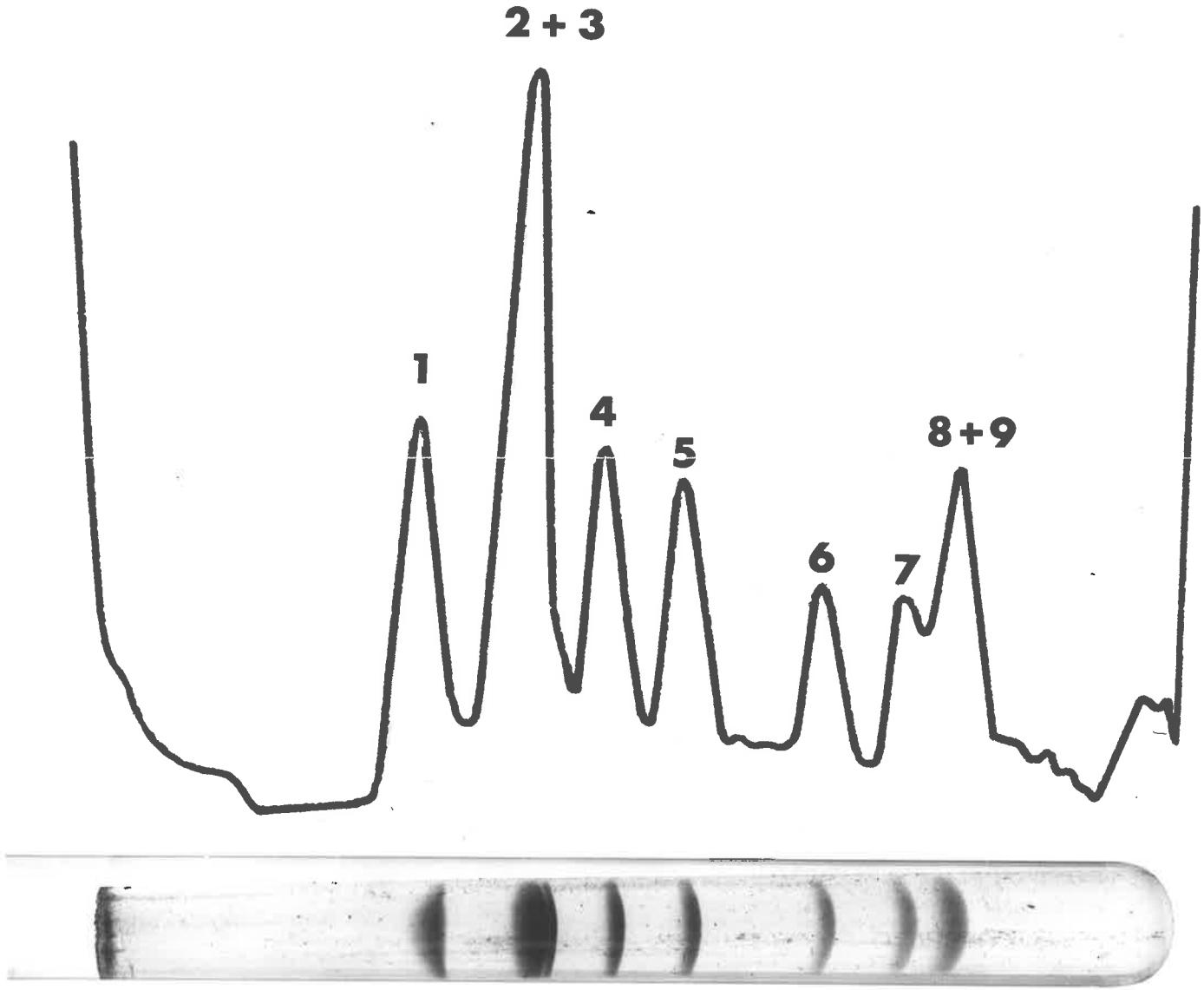

2+3
1
4
5
6
7
8+9

Fig. 21. Relationship between the electrophoretic mobility of RNAs isolated from FDV, reovirus and $\phi 6$ phage.

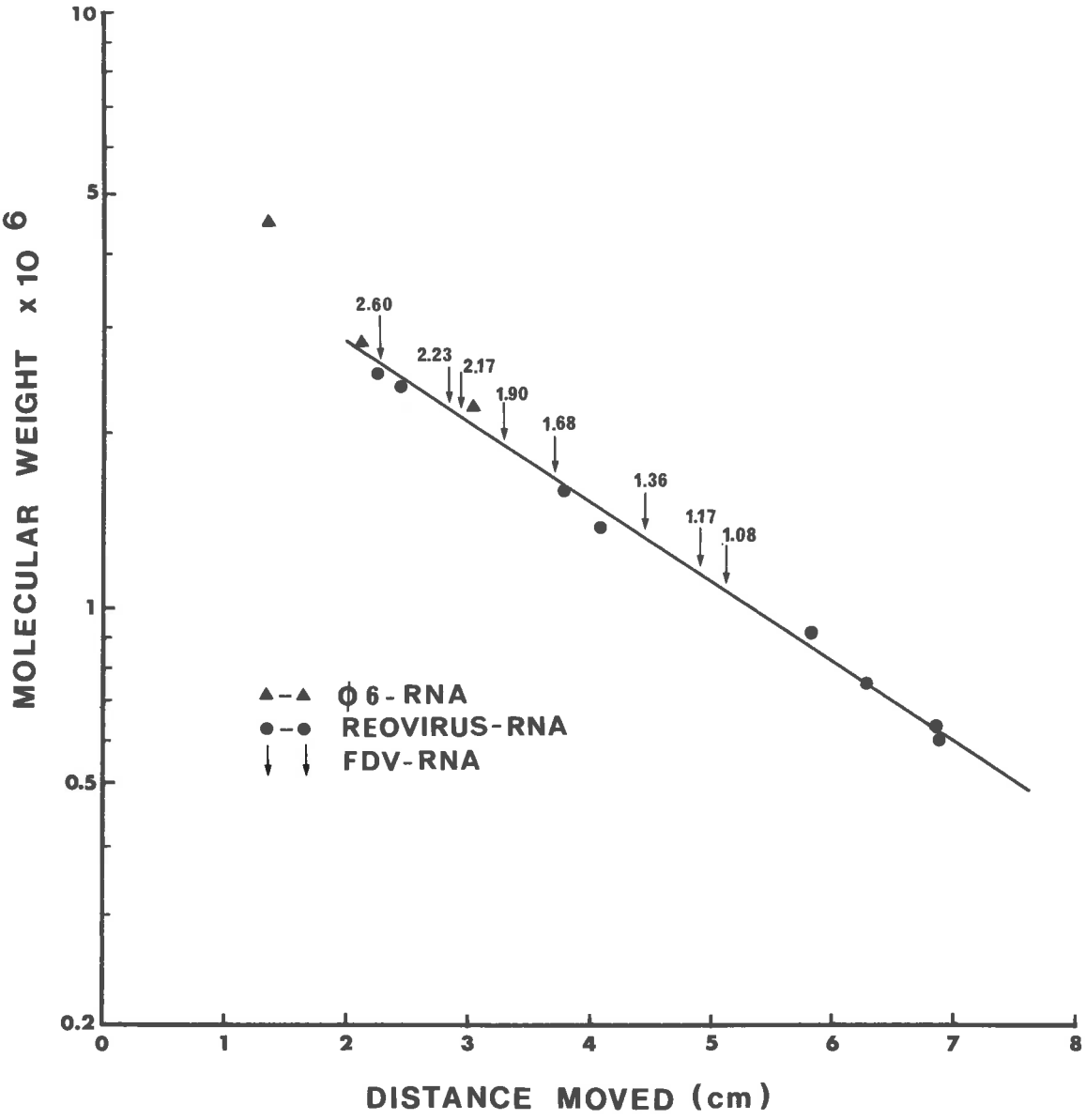

MOLECULAR WEIGHT x 10 6
10
5
1
0.5
0.2
0
1
2
3
4
5
6
7
8
DISTANCE MOVED (cm)
2.60
2.23
2.17
1.90
1.68
1.36
1.17
1.08
▲–▲ ϕ 6 - RNA
●–● REOVIRUS-RNA
FDV-RNA

TABLE 14. Molecular weights and molecular ratios of FDV-RNA segments

Band[a] No.	Molecular weight[b] ($\times 10^6$)	Molar[c] ratio
1	2.60	0.75
2	2.23 }	2.16
3	2.17	
4	1.90	0.96
5	1.68	1.00
6	1.36	0.87
7	1.17	0.98
8	1.08	2.13

a) Separated RNA bands in a gel are numbered from the highest molecular weight band to the lowest one in Fig. 20. Molar ratio is calculated using band No. 5 as standard.

b) Calculated from data in Fig. 21.

c) Calculated from data in Fig. 20.

CONCLUSIONS

Studies carried out in this chapter indicate that:

1. Nucleic acid isolated from FDV subviral particles was identified as ds-RNA by the following properties:

(a) positive orcinol reaction;

(b) negative diphenylamine reaction;

(c) resistance to RNase in 1 x SSC but not in 0.1 x SSC;

(d) susceptibility to RNase in 1 x SSC after heat-denaturation;

(e) sharp thermal denaturation curve with a melting temperature of 76°C in 0.01 x SSC;

(f) buoyant density of 1.60 gm/cm^3 in Cs_2SO_4;

(g) no reaction with formaldehyde.

2. FDV-RNA separated into nine RNA segments with a total molecular weight of 15.3×10^6 daltons.

CHAPTER VI

RNA-DEPENDENT RNA POLYMERASE ASSOCIATED WITH FDV SUBVIRAL PARTICLES

INTRODUCTION

It has been established that RNA-dependent RNA polymerase activity is associated with particles of reovirus (Borsa and Graham, 1968; Shatkin and Sipe, 1968), cytoplasmic polyhedrosis virus (CPV) (Lewandowski *et al.*, 1969), WTV (Black and Knight, 1970), bluetongue virus (BTV) (Verwoerd and Huismans, 1972; Martin and Zweerink, 1972), RDV (Kodama and Suzuki, 1973) and MRDV (Boccardo and Milne, 1975). Fenner *et al.* (1974) have included all these viruses in the family Reoviridae. Since FDV is similar to these viruses on the basis of particle morphology and properties of the viral nucleic acid (Teakle and Steindl, 1969; Francki and Grivell, 1972; Chapters III and IV), I have tested for the presence of an RNA-dependent RNA polymerase associated with FDV subviral particles.

MATERIALS AND METHODS

1. Preparation of enzymatically active extracts

All extracts were prepared at 4°C and kept in an ice bath until assayed. Gall tissue was ground in a pestle and mortar with some acid-washed sand by two methods:

Method 1 - Each gm of fresh tissue was extracted in 2.5 ml of 50 mM-Tris HCl, 100 mM-NH_4Cl and 90 mM-2-mercaptoethanol, pH 8.4 (TAM buffer) (May and Symons, 1971), and strained through cheese-

cloth. The strained liquid was centrifuged for 1 min at 500 g to remove cell debris and the supernatant was centrifuged at 200,000 g for 1 h. The pellet was suspended in 200 µl of TAM buffer unless otherwise stated.

Method 2 - Each gm of tissue was extracted in 2.5 ml of GE buffer and strained through cheesecloth. The extract was centrifuged for 1 min at 500 g and Nonidet P40 was added to the supernatant to a concentration of 1%. After 10 min at 4°C the extract was centrifuged at 5,000 g for 10 min, layered over 2 ml of 10% sucrose in GE buffer and centrifuged at 200,000 g for 1 h. The pellet was suspended in either STE buffer or in TAM buffer.

2. Assay of RNA-dependent RNA polymerase activity

Enzyme assays were based on the incorporation of labelled ribonucleotide triphosphate into acid-insoluble material. Fifty µl aliquots of preparation to be assayed were added to 200 µl of the standard assay medium. Unless otherwise stated, the standard medium contained:

25 µmoles	Tris-HCl (pH 8.5)
2 µmoles	$MgCl_2$
1 µmole	phospho(enol)pyruvate (PEP)
25 µg (6 units)	pyruvate kinase (PEP kinase)
0.5 µmole	adenosine-5'-triphosphate (ATP)
0.2 µmole	cytosine-5'-triphosphate (CTP)

0.02 µmole	guanosine-5'-triphosphate (GTP)
0.2 µmole	uridine-5'-triphosphate (UTP)
approx. 1 x 10^6 cpm	^{32}P-GTP
4 µg	actinomycin D

Actinomycin D was added to inhibit DNA-dependent RNA polymerase activity. PEP and PEP kinase were added to inhibit the polynucleotide phosphorylase reaction (August and Eqyang, 1967). GTP was used as the labelled substrate to avoid polyriboadenylate polymerase and adenylate-cytidylate pyrophosphorylase activity (August and Eqyang, 1967). All assays were carried out in duplicate, incubated under the conditions specified in the tables and figures presented. The reaction was terminated by chilling the tubes, adding 0.4 ml of 0.08M-sodium pyrophosphate and 0.05 ml of yeast RNA (4 mg/ml), mixing and then adding 0.4 ml of 25% trichloroacetic acid (TCA) (Francki and Randles, 1972). After 30 min, an equal volume of 95% ethanol was added to dissolve chlorophyll, and the remaining TCA-insoluble material was filtered onto glass fibre discs (Type A, 2 cm diameter, Gelman Instrument Co.) in a Millipore apparatus. The discs were washed once with 95% ethanol,and 6 times with 7.5% TCA - 0.02M-sodium pyrophosphate. Residual TCA was removed by washing the glass fibre discs with 5 ml of 95% ethanol. The ethanol washed discs were dried in an oven (100^{o}C for 5-10 min) and then placed in a vial containing 5 ml of scintillation fluid (0.3% PPO and 0.03% POPOP in toluene) (May and

Symons, 1971). Radioactivity was determined in a Packard Liquid Scintillation Spectrometer.

3. RNA-RNA hybridization technique

^{32}P-labelled RNA samples from polymerase active extracts were mixed with samples of test RNA preparations in 0.01M-phosphate buffer, pH 8.2, containing 0.3M-NaCl (Shatkin and Sipe, 1968). The mixtures were heated at 100°C for 10 min and cooled slowly as follows: 90°C to 72°C in 1 h, 72°C for 15.5 h, 72°C to 45°C in 3 h (Schonberg *et al.*, 1971). When cooled, the samples were incubated with 10 µg/ml RNase at 37°C for 30 min. Yeast RNA was added (200 µg/sample) and the mixtures were precipitated with TCA at 4°C. The precipitates were filtered, washed and assayed for radioactivity as described above.

4. Slicing polyacrylamide gels and solubilization of RNA for liquid scintillation counting

The gels were frozen at -15°C and sliced with a multi-bladed apparatus into sections 1.4 mm thick. The individual slices were placed in glass scintillation vials and 0.7 ml of NCS and water in a ratio of 9:1 was added to each vial (Zaitlin and Hariharasubramanian, 1970). After incubation overnight at 45°C, radioactivity was measured as described above.

RESULTS

1. RNA-dependent RNA polymerase activity in crude extracts of FDV-induced galls

In preliminary experiments, significant RNA-dependent RNA polymerase activity could not be detected in purified preparations of FDV subviral particles. However, enzymatic activity was readily detected in crude extracts of leaf galls from FDV-infected sugarcane whereas similar extracts from healthy leaf tissue had no activity (Table 15). After fractionating extracts by differential centrifugation at 500 or 17,000 g, significant polymerase activity was detected in all the fractions (Table 15). Distribution of FDV antigen among these fractions was proportional to that of the enzyme activity (Table 15). Subsequent experiments demonstrated that more than 90% of the polymerase activity was sedimented into pellets after centrifugation at 200,000 g for 1 h (Table 16). Moreover, the proportion of the polymerase activity sedimenting under these conditions was not different if the nonionic detergent, Nonidet P40, was added to the extract to a concentration of 1% (Table 17); a treatment designated to solubilize cytoplasmic membranes.

2. Effect of length of incubation on RNA-dependent RNA polymerase activity

The kinetics of polymerase activity in concentrated extracts of FDV-induced galls are shown in Fig. 22. The rate of incorporation

TABLE 15. Localization of RNA-dependent RNA polymerase activity in crude extracts from FDV-induced galls

Fraction	Polymerase activity (cpm)[a]		Antigen dilution end-point[b]	Ratio Polymerase activity (cpm) / Antigen dilution end-point
	Healthy	Infected		
Extract	0	432	64	7.60
500 g supernatant	-	264	32	10.00
500 g pellet	-	312	32	11.50
1700 g supernatant	-	206	16-32	16.4-8.2
1700 g pellet	-	83	8	17.50

a) Assay mixture, in volume of 0.25 ml, consisted of 0.5 µmole ATP; 0.3 µmole GTP; 0.2 µmole CTP; 25 µmoles Tris (pH 8.5); 3 µmoles $MgCl_2$; 4 µg AMD; 0.25 µCi ^{14}C-UTP and 50 µl of extract from each fraction. Incubation was 28°C for 60 min.

b) Antigen dilution end-point expressed as reciprocals of highest dilutions of antigen producing a visible precipitin line in immunodiffusion tests against ant-FDV serum.

TABLE 16. RNA-dependent RNA activity in supernatant and pellet after high-speed centrifugation of crude extract from PDV-induced galls

Experiment No.	Polymerase activity (cpm) [a] Centrifugation at 200,000 g for 60 min	
	Pellet	Supernatant
1 [b]	1497	143
2 [c]	1359	106

a) Assays done as described in Table 15.

b) Extracts prepared by Method 1 as described in Materials and Methods.

c) Extracts prepared by Method 2 described in Materials and Methods and suspended in TAM buffer.

TABLE 17. Effect of Nonidet P40 on RNA-dependent RNA polymerase activity

Treatment with	Polymerase activity (cpm)[a] in pellet after 200,000 g for 60 min	Antigen dilution end-point	Ratio $\frac{\text{Polymerase activity (cpm)}}{\text{Antigen dilution end-point}}$
None	815	16	51
Nonidet P40	1574	32	49

a) Assays done as described in Materials and Methods. Incubation was at 30°C for 20 min.

b) Antigen dilution end-points are expressed as reciprocals of highest dilutions of antigen producing a visible precipitin line in immunodiffusion tests against anti-FDV serum.

of label from ^{32}P-GTP into a TCA-insoluble product at 30°C increased rapidly during the first 20 min of incubation but did not increase significantly thereafter. Under the same conditions, a similarly prepared extract from healthy sugarcane leaf tissue failed to incorporate detectable amounts of label (Fig. 22).

3. Optimal conditions for RNA-dependent RNA polymerase activity

To maximize the activity of the polymerase, optimal conditions for the reaction were investigated.

(a) pH

The pH of the incubation mixtures was adjusted to the values shown in Fig. 23. Maximum incorporation of ^{32}P-GTP occurred in the pH range 8.5 to 9.0. Below pH 8.5 the rate of incorporation decreased sharply and at pH 7.0 only about 13% of the maximum activity was observed.

(b) Temperature

A series of standard incubation mixtures were prepared as described under Materials and Methods and incubated at various temperatures as indicated in Fig. 24. The optimal temperature for maximum incorporation was 35°C. However, the rate of incorporation was relatively insensitive to small changes within the temperature range 30°C to 40°C.

Fig. 22. Rate of increase of TCA-insoluble ^{32}P-labelled product in the complete assay medium incubated at 30°C containing extract from FDV-induced galls (●——●) and from healthy sugarcane leaf tissue (▲——▲). The extracts were prepared by Method 2 and suspended in TAM buffer as described in Materials and Methods section.

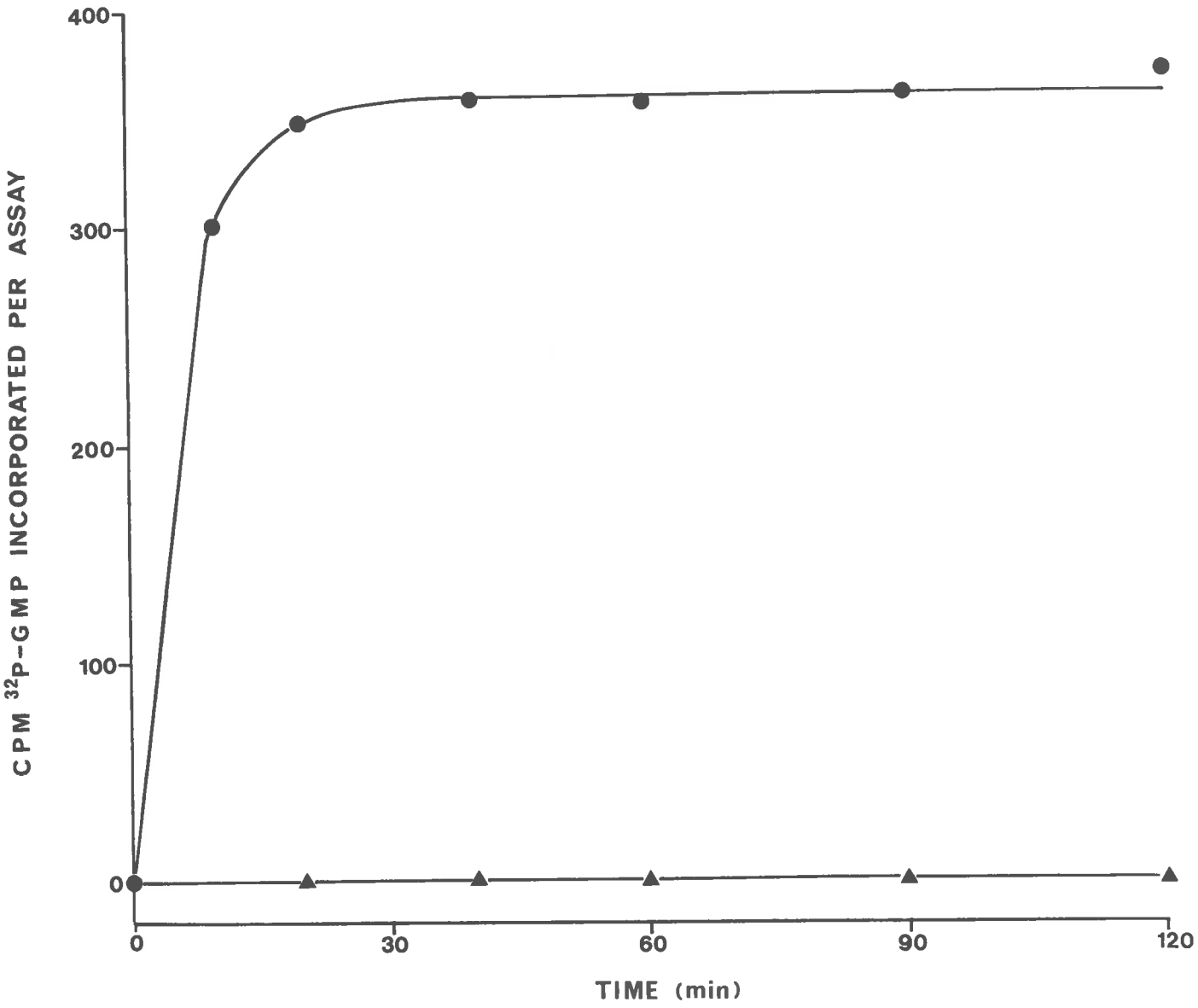

400
300
200
100
0
CPM ^{32}P-GMP INCORPORATED PER ASSAY
0
30
60
90
120
TIME (min)

Fig. 23. Relationship of RNA polymerase activity in extracts from FDV-induced galls and pH. The extracts were prepared by Method J and assayed as described in Materials and Methods section. All samples were incubated at 30°C for 40 min.

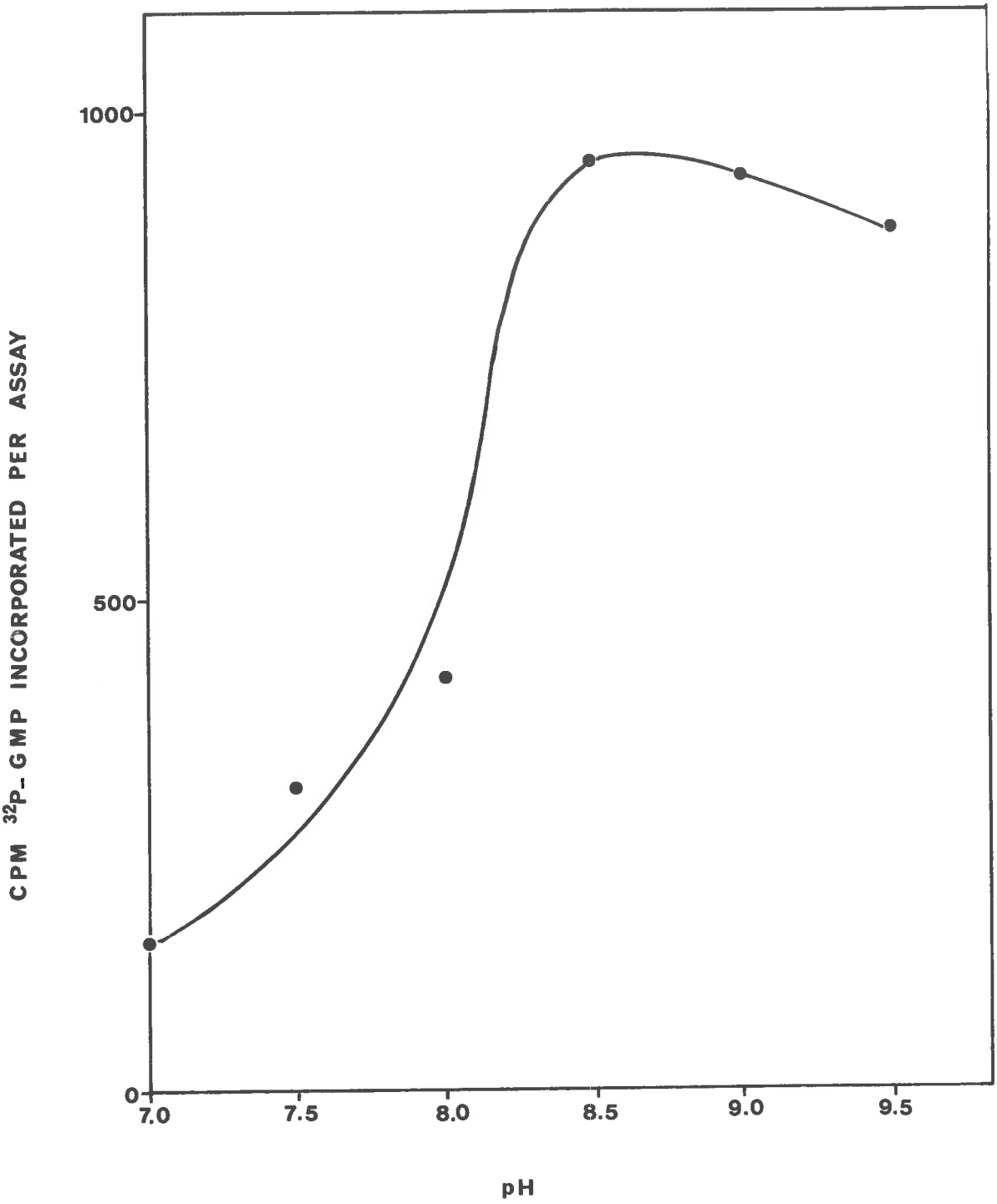
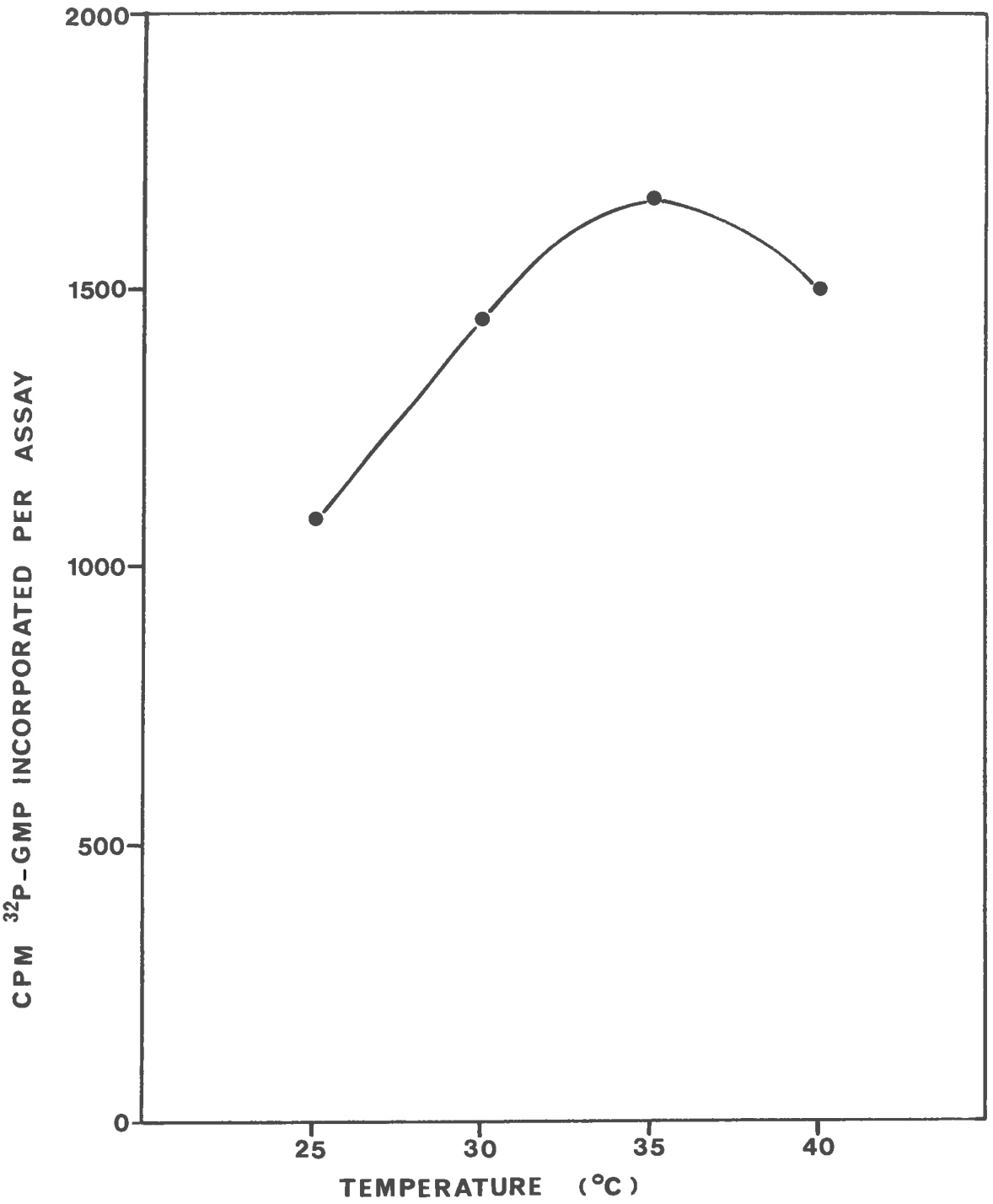

1000
500
0
7.0
7.5
8.0
8.5
9.0
9.5
pH
CPM ^{32}P- GMP INCORPORATED PER ASSAY

Fig. 24. Relationship of RNA polymerase activity in extracts from FDV-induced galls to temperature. Preparations and assay conditions as described in Fig. 23, except that $MgCl_2$ concentration was 4 mM throughout. Incubation was at 30°C for 40 min.


2000
1500
1000
500
0
CPM ^{32}P-GMP INCORPORATED PER ASSAY
25
30
35
40
TEMPERATURE (°C)

(c) Mg^{2+}, NH_4^+ and Na^+

RNA-dependent RNA polymerase activity was dependent on the presence of Mg^{2+} in the assay medium, the optimal concentration being about 8 mM (Fig. 25). The enzyme was also greatly stimulated by NH_4^+ but not by Na^+, the optimum NH_4^+ concentration being about 200 mM (Fig. 26).

4. Characteristics of RNA-dependent RNA polymerase activity

Table 18 summarizes the general characteristics of the polymerase in concentrated extracts of FDV-induced galls. Enzyme activity was dependent on the presence of UTP in the assay medium, and was greatly reduced in the absence of ATP and CTP. Incorporation of ^{32}P-GTP by the polymerase was independent of the addition of PEP and PEP kinase. The insensitivity of the system to DNase and Actinomycin D indicates that the activity of the polymerase was independent of DNA. However, ^{32}P-GTP incorporation was almost completely inhibited by RNase. Addition of EDTA to the assay medium did not affect the enzyme activity (Table 19).

Borsa and Graham (1968) and Shatkin and Sipe (1968) demonstrated that heat treatment or digestion with α-chymotrypsin, respectively, activated the RNA-dependent RNA polymerase of purified reovirus preparations. Similar treatments of concentrated extracts of galls from FDV-infected leaves decreased enzymatic activity slightly, but significantly (Table 20).

Fig. 25. Relationship between RNA polymerase activity in extracts from FDV-induced galls and $MgCl_2$ concentration. The extracts were prepared by Method 1 and assayed at 30°C for 40 min as described in Materials and Methods section.

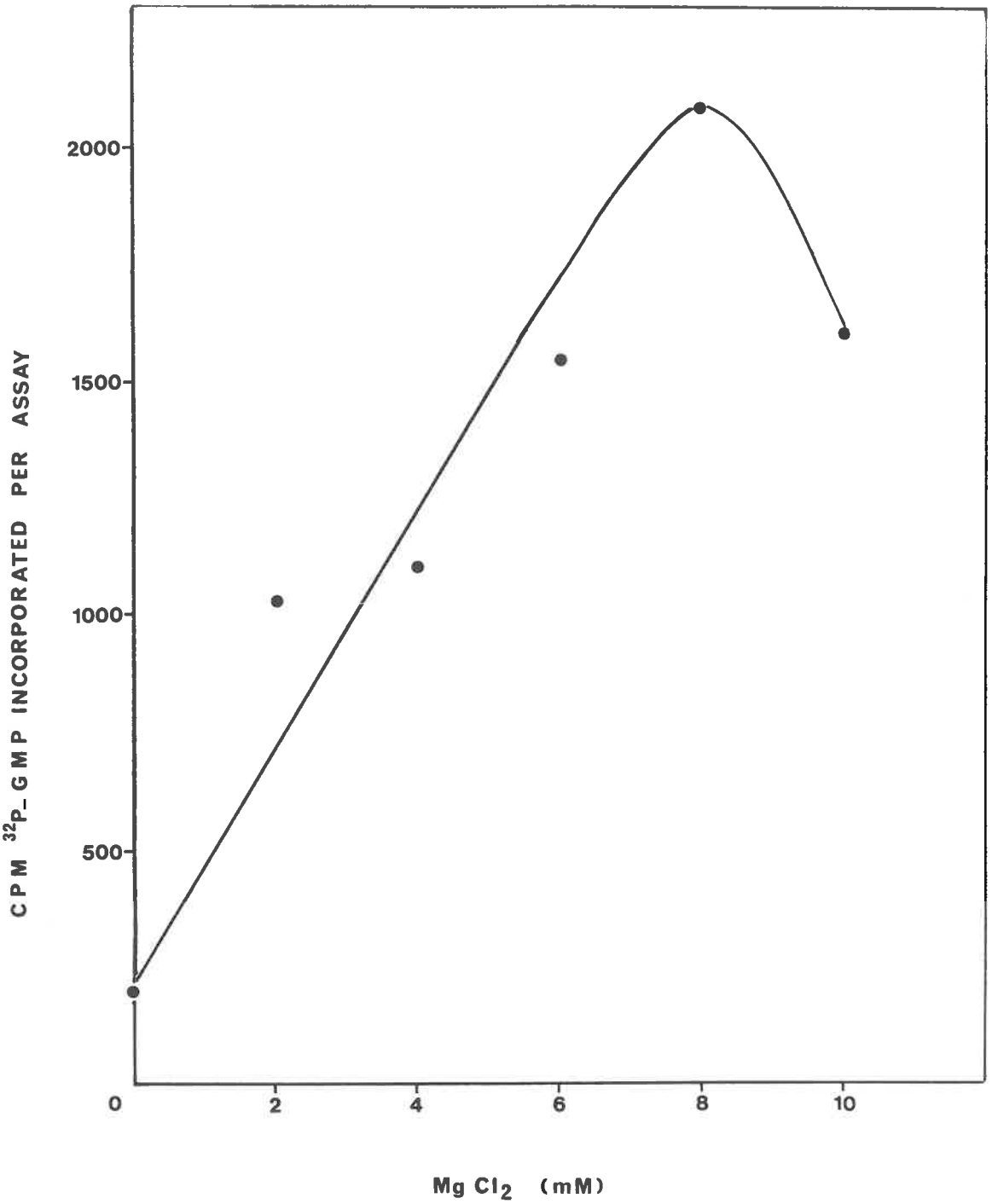

2000
1500
1000
500
0
2
4
6
8
10
CPM ^{32}P-GMP INCORPORATED PER ASSAY
Mg Cl_2 (mM)

Fig. 26. Relationship between RNA polymerase activity in extracts from FDV-induced galls and monovalent cation concentration. The extracts were prepared by Method 1 as described in Methods and Materials section; however, extracts used in Experiments 1 and 2 were suspended in buffer containing no NH_4Cl. Reaction mixtures were incubated at 30°C for 40 min (Experiments 1 and 3) and 60 min (Experiment 2). Radioactivity of the samples in these experiments ranged from 290 to 4,978 cpm per assay.

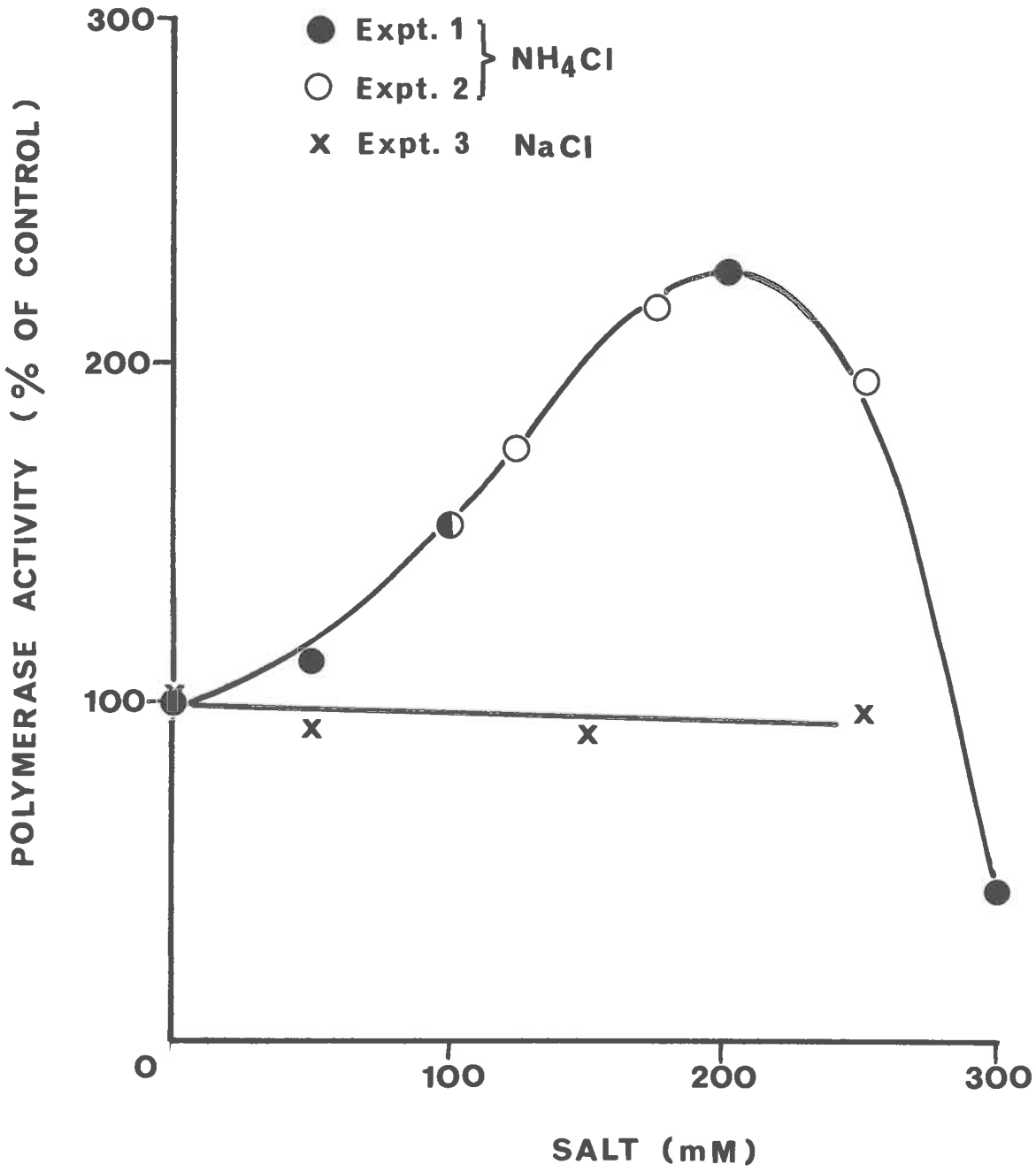

300
200
100
0
Expt. 1
Expt. 2
NH4Cl
X Expt. 3 NaCl
POLYMERASE ACTIVITY (% OF CONTROL)
100
200
300
SALT (mM)

TABLE 13. Properties of the RNA polymerase associated with FDV infection.

Experiment Number	Reaction System	^{32}P-GMP incorporation	
		cpm	% of complete system
1	Complete[a)]	1655	100
	- ATP	111	6.7
	- CTP	479	29
	- UTP	76	4.6
	- ATP, -CTP, -UTP	31	1.9
	- gall extract	0	0.0
2	Complete[a)]	1083	100
	- PEP, -PEP kinase	1772	98
	- Actinomycin D	1561	87
	+ DNase (25 µg/ml)	1716	95
	+ RNase (20 µg/ml)	55	3.1

a) Extracts from FDV-induced galls were prepared by Method 1 and polymerase activity assayed as described in Materials and Methods section. The enzyme activity was assayed at 30°C for 40 min.

TABLE 19. Effect of EDTA on RNA-dependent RNA polymerase activity

Concentration of EDTA (mM)	^{32}P-GMP incorporation	
	cpm[a)]	% of untreated
0	2572	100.0
1	2662	103.8
5	2448	95.2

a) Extracts of FDV-induced galls were prepared by Method 1 and enzyme activity was assayed as described in Materials and Methods. Incubation was at 30°C for 40 min.

TABLE 20. Effects of heat and α-chymotrypsin treatments on RNA-dependent RNA polymerase activity in extracts from FDV-induced gall tissue.

Experiment Number	Treatment of gall extract prior to enzyme assay	^{32}P-GMP incorporation	
		cpm	% of untreated
1	Untreated[a]	932	100
	Heated 40°C for 20 sec[b]	782	84
	Heated 50°C for 20 sec	833	89
	Heated 60°C for 10 sec	829	89
	Heated 60°C for 20 sec	648	70
2	Untreated[a]	1813	100
	+ 50 µg/ml α-chymotrypsin[c]	1433	79
	+ 100 µg/ml "	1450	80
	+ 200 µg/ml "	1437	79
	+ 400 µg/ml "	968	53

a) Extracts of FDV-induced galls were prepared by Method 1 and enzyme activity was assayed as described in Materials and Methods section. Incubation was at 30°C for 40 min.

b) Heat treatment was applied before assaying polymerase activity.

c) α-chymotrypsin was included in the assay medium.

5. Correlation of polymerase activity with FDV antigen

Significant polymerase activity in the extracts of FDV-induced galls was distributed in all fractions after differential centrifugation at 500 g or 1,700 g (Table 15). This, together with the observation that nearly all the polymerase activity in extracts from FDV-induced gall tissue sedimented after centrifugation for 60 min at 200,000 g (Table 16), even after treatment with detergent, indicates that the enzyme must be associated with relatively large macromolecular structures. The sedimentation rate of these structures was compared to that of FDV antigen by subjecting an enzymatically active preparation to centrifugation in a sucrose density-gradient. Each fraction from the gradient was assayed for the presence of FDV antigen and for polymerase activity. Results of the experiment (Fig. 27) indicate a good correlation between the presence of FDV antigen and polymerase activity suggesting that the enzyme is virion associated. In a subsequent experiment the antigen content and polymerase activity of a purified preparation of FDV subviral particles were compared to those of a concentrated gall tissue extract. The results (Table 21) demonstrate that both preparations incorporated ^{32}P-GTP into a TCA-insoluble precipitate and that both contained FDV antigen. However, on the basis of antigen content, the purified subviral particles were only about 10% as active in incorporating ^{32}P-GTP as the concentrated gall extract.

Fig. 27. Correlations of RNA polymerase activity with FDV antigen in extracts from FDV-induced gall tissue. An extract was prepared by Method 2 and suspended in STE buffer as described in Materials and Methods section. A sample (500 µl) of the extract was layered on a 30-60% linear sucrose density-gradient buffered with STE and centrifuged at 40,000 rpm for 30 min in a Spinco SW50 rotor. Fractions were collected by puncturing the bottom of the tube and each fraction was subdivided for assay of polymerase activity in duplicate and FDV antigen. Enzyme activity was assayed at 30°C for 40 min as described in Materials and Methods section except that the assay medium contained 175 mM-NH_4Cl and 90 mM-2-mercaptoethanol. FDV-antigen was assayed by immunodiffusion as described in Chapter II.

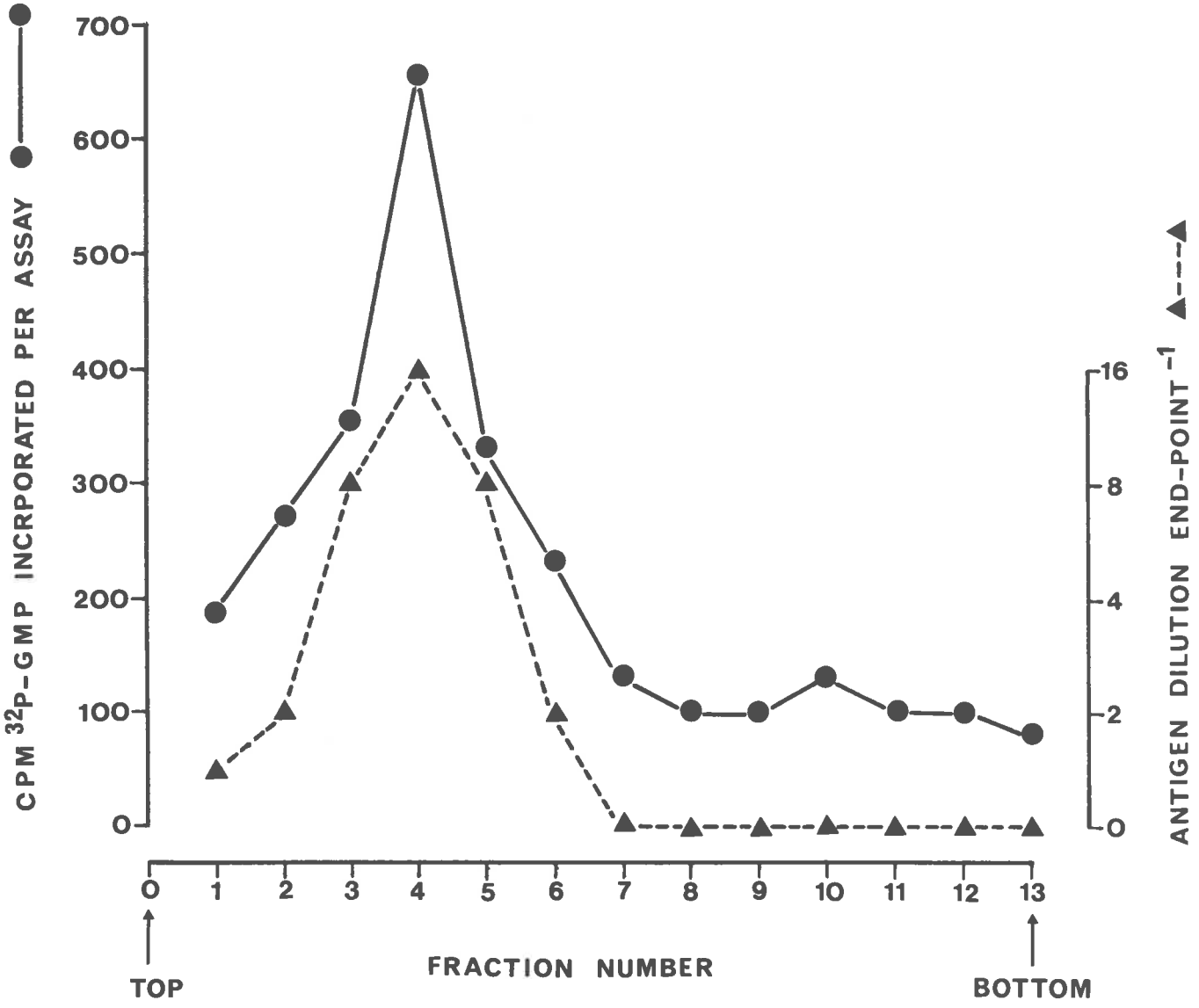

CPM ^{32}P-GMP INCRPORATED PER ASSAY
700
600
500
400
300
200
100
0
ANTIGEN DILUTION END-POINT^{-1}
16
8
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
TOP
FRACTION NUMBER
BOTTOM

TABLE 21. Comparison of the RNA polymerase activity in extracts from FDV-induced gall tissue and purified FDV subviral particles.

Preparation	Polymerase activity (cpm)[a]	Antigen dilution end-point[b]	Ratio Polymerase activity / Antigen dilution end-point
Gall extract[c]			
Undiluted	4109	8	513
Diluted 1/2	2584	4	646
Diluted 1/4	1842	2	921
Diluted 1/8	938	1	938
FDV subviral particles[d]	1228	16	77

a) Assays done as described in Materials and Methods section. Incubation was at 30°C for 40 min.

b) Antigen dilution end-points are expressed as reciprocals of highest dilutions of antigen producing a visible precipitin line in immunodiffusion tests against anti-FDV serum.

c) Extracts prepared by Method 1 described in Materials and Methods section.

d) Preparations purified as described in Chapter III.

6. Properties of the polymerase product

The product of the polymerase appears to be almost entirely an ss-polyribonucleotide under the conditions of incubation used since over 90% of it was readily digested by RNase (Table 22). In a subsequent experiment, the ability of the polymerase product RNA to anneal with FDV-RNA was examined (Table 22). Self-annealing and annealing after the addition of RNA from healthy sugarcane leaf tissue, rendered about half the radioactive product resistant to RNase digestion whereas annealing in the presence of added FDV-RNA increased this to 80%. These observations indicate that most of the ss-RNA product has base sequences complementary to FDV-RNA.

To test if the transcriptase product RNA is released from FDV subviral particles, a concentrated gall extract incubated in the polymerase assay medium with ^{32}P-GTP for 60 min at 30^{o}C was subjected to sucrose density-gradient centrifugation. Nearly all the TCA-insoluble radioactive material sedimented at the same rate as FDV-antigen (Fig.28). Under these incubation conditions, synthesis of the polymerase product would have been completed within the first 15 min of incubation (Fig.22).

A similar preparation incubated in the polymerase assay medium with ^{32}P-GTP for only 20 min was deproteinised by phenol-SDS extraction and subjected to sucrose density-gradient centrifugation. The results summarized in Fig. 29 indicate that most of the product remained at the top of the gradient whereas FDV ds-RNA (assayed by immunodiffusion against poly [I] : poly [C] antiserum) sedimented well into the gradient.

TABLE 22. Annealing of the RNA polymerase product[a] with FDV-RNA

Addition to reaction mixture	Treatment	RNase resistance	
		cpm	% of product added
Nil	Unheated[b]	53	6.3
Nil	Heated and cooled	382	46
RNA from healthy sugarcane leaves (11 µg/assay)	Heated and cooled	451	54
FDV-RNA 3 µg/assay	Heated and cooled	701	84

a) Polymerase product was extracted with phenol-SDS and purified as described in Materials and Methods section. Each reaction mixture contained 839 cpm of the polymerase product.

b) Polymerase product was incubated with 10 µg/ml RNase at 37°C for 30 min without any prior treatment and then precipitated with TCA as described in Materials and Methods section.

c) Polymerase product was heated and cooled slowly as described in Materials and Methods section before RNase treatment and TCA precipitation.

Fig. 28. Cosedimentation of polymerase product RNA and FDV-antigen in sucrose density-gradients. An extract from FDV-induced gall tissue was prepared by Method 2 and suspended in STE buffer and was incubated in the standard polymerase assay medium at 30°C for 60 min as described in Materials and Methods section. The incubated mixture (500 µl) was fractionated by sucrose density-gradient centrifugation as described in Fig. 27. The fractions were assayed for TCA-insoluble radioactivity and FDV-antigen as described in Materials and Methods section.

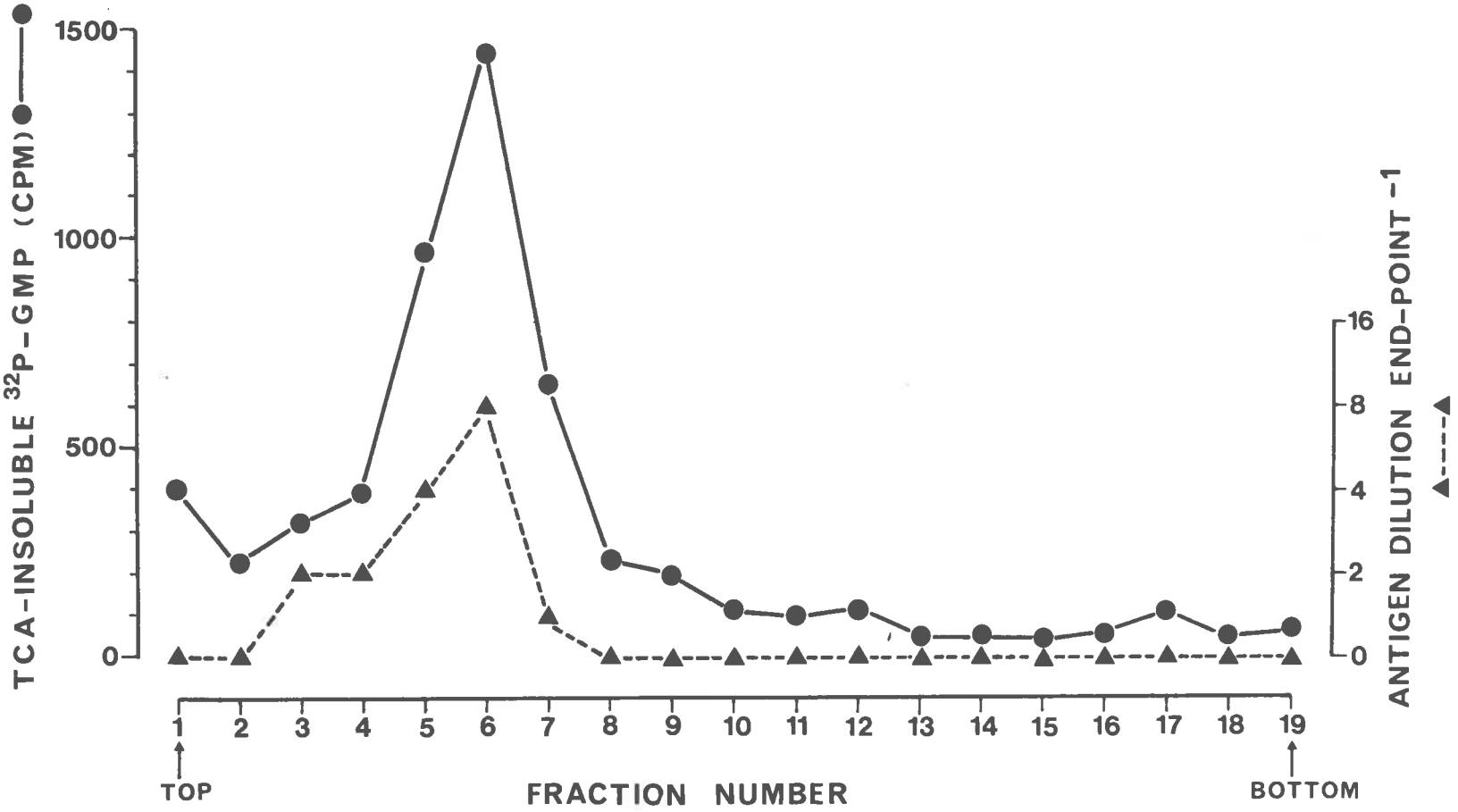
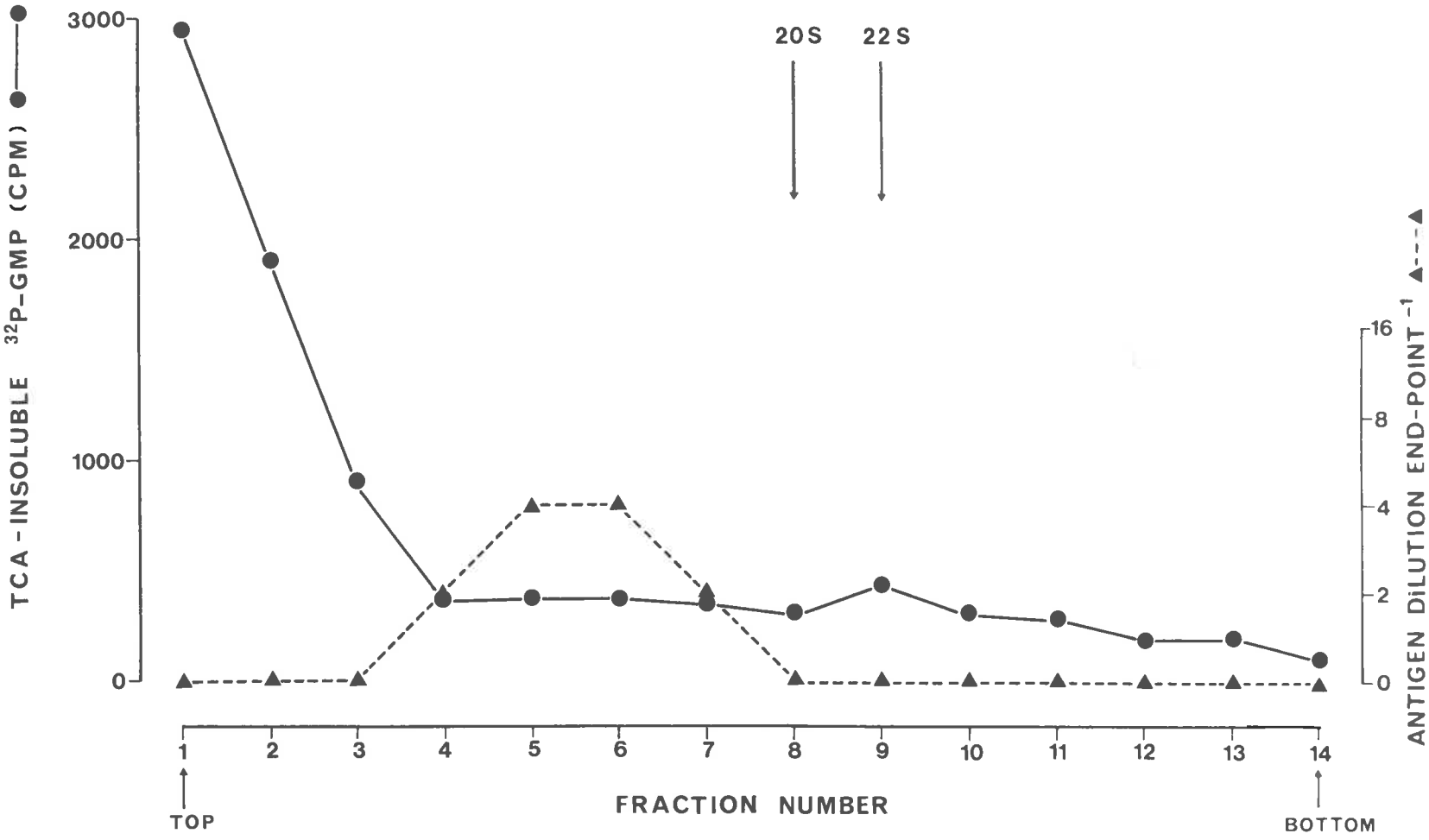

TCA-INSOLUBLE ^{32}P-GMP (CPM)
1500
1000
500
0
ANTIGEN DILUTION END-POINT -1
16
8
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
TOP
FRACTION NUMBER
BOTTOM

Fig. 29. Sedimentation of RNA isolated from an extract of FDV-induced gall tissue incubated with ^{32}P-GTP. The extract was prepared by Method 2, suspended in TAM buffer and incubated at 30°C for 60 min in the standard polymerase assay medium as described in Materials and Methods section. The incubated mixture was deproteinised by the phenol-SDS procedure and subjected to centrifugation in a 10-30% linear sucrose density-gradient in SSC buffer for 4.5 h. The gradients were fractionated as described in Fig. 27, and each fraction was assayed for TCA-insoluble radioactivity and ds-RNA antigen as described in Materials and Methods section. Arrows indicate the position to which marker cucumber mosaic virus RNAs sedimented in a sister tube.


TCA-INSOLUBLE ^{32}P-GMP (CPM)
3000
2000
1000
0
20S
22S
ANTIGEN DILUTION END-POINT^{-1}
16
8
4
2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
TOP
FRACTION NUMBER
BOTTOM

In order to determine the molecular size of the product, a preparation of phenol-SDS-extracted RNA-dependent RNA polymerase product was subjected to PAGE (Fig. 30). Some of the labelled material migrated ahead of the 18S leaf r-RNA, some between 18S and 4S t-RNA and some ahead of the 4S t-RNA. The radioactivity at the top of the gel was probably due to aggregation of the product.

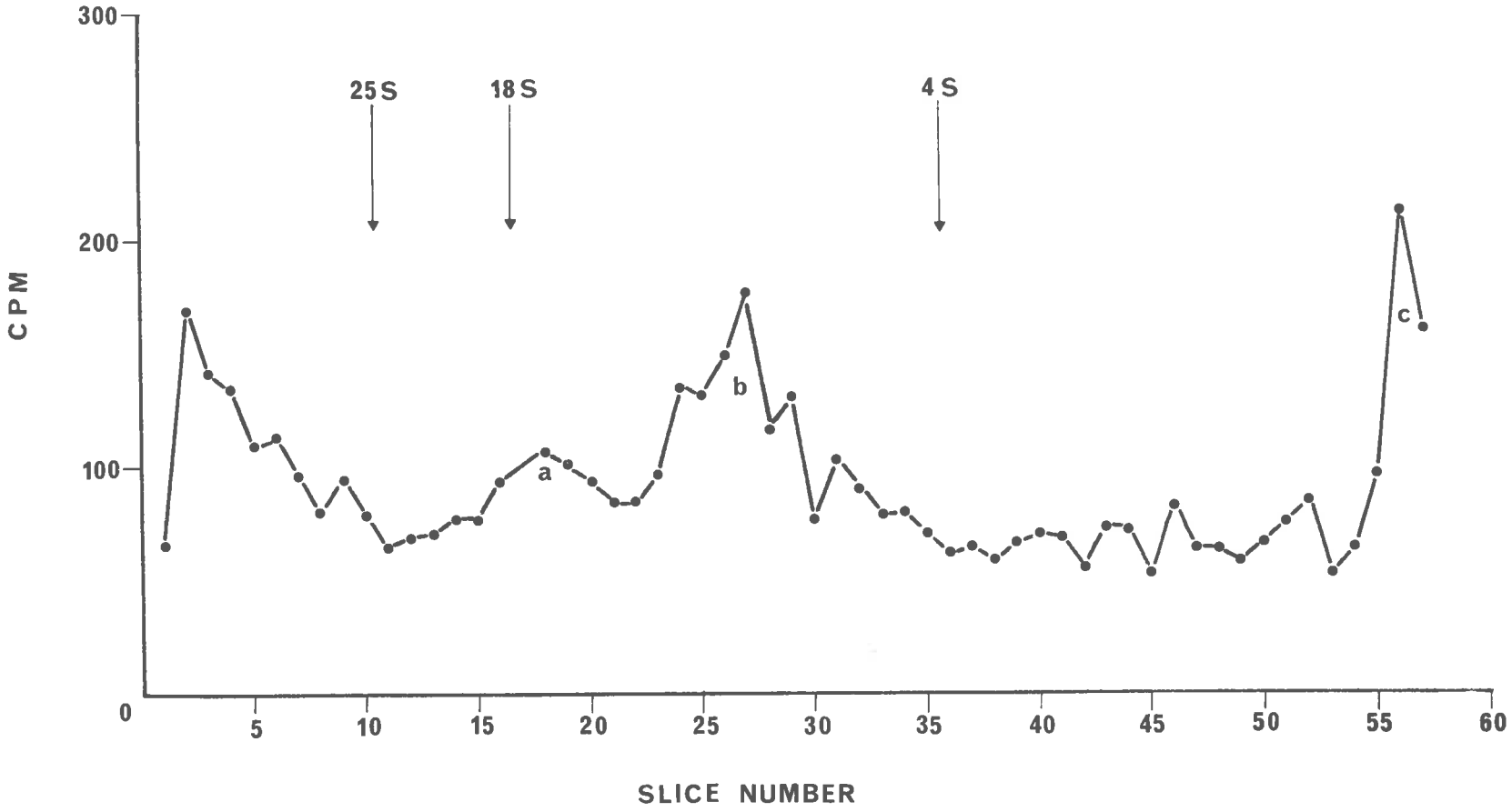
These observations indicate that the ss-RNA transcribed from FDV-RNA was of low molecular weight and heterogeneous and was not released from subviral particles following synthesis, but was separated from the ds-RNA of FDV on deproteinization.

CONCLUSIONS

Studies reported in this chapter demonstrate that:

1. RNA-dependent RNA polymerase activity was detected in concentrated extracts of gall tissue from FDV-infected sugarcane leaves but not in similar extracts from healthy leaf tissue.
2. The polymerase activity was correlated with FDV antigen indicating that the enzyme is virion associated.
3. Some polymerase activity was detected in preparations of FDV subviral particles.
4. Optimal polymerase activity occurred at about $35^{\circ}C$, at pH between 8.5 and 9.0, and in the presence of 8 mM $MgCl_2$ and 200 mM NH_4Cl. This enzyme activity was independent of an ATP regenerating system, actinomycin D and DNase.
5. The polymerase product was ss-RNA which was apparently transcribed from FDV ds-RNA.

Fig. 30. Analysis of in vitro reaction product on polyacrylamide gel electrophoresis. The extract was prepared by Method 1, suspended in TAM buffer and incubated at 30°C for 20 min in the standard polymerase assay medium as described in Materials and Methods section. The incubated mixture was deproteinised by phenol-SDS procedure and polysaccharides in these preparations were removed as described in Chapter II. Electrophoresis in an 8-cm 2.4% polyacrylamide gel was performed at 6°C and 4 mA/gel for $1\frac{2}{3}$ h. The gel was sliced and dissolved for counting as described in Materials and Methods section. Arrows indicate the position to which marker leaf RNAs migrated in a sister gel.


300
200
100
0
CPM
25S
18S
4S
a
b
c
5
10
15
20
25
30
35
40
45
50
55
60
SLICE NUMBER

CHAPTER VII

GENERAL DISCUSSION

1. Purification of FDV subviral particles

In my initial attempts to purify FDV, I was faced with some problems. The most serious was the lack of a satisfactory virus assay method. The usual technique used for the assay of viruses which are transmitted by insect vectors, but not by mechanical inoculation, is to inject the insects with extracts to be assayed and then to test their infectivity on plants. There are difficulties in assaying FDV using the vector *P. saccharicida*. Sinclair and Osborn (1970) reported that common methods of anaesthetizing insects such as low temperature CO_2 and nitrogen caused a high mortality of *P. saccharicida*. Furthermore, the vectors injected with extracts or fractions to be assayed had a high mortality (Francki and Grivell, personal communication). Francki and Jackson (1972) were able to detect ds-RNA immunochemically in FDV-infected sugarcane leaves but not in healthy tissues leading to the conclusion that FDV was a ds-RNA virus. In this study, the amount of viral ds-RNA was titrated by immunodiffusion against antiserum to poly [I] : poly [C] and the assumption was then made that the amount of ds-RNA in tissues or tissue fractions was proportional to the amount of FDV. In addition to being a rapid and relatively simple technique it had the advantage that FDV losses during purification could be estimated in insoluble as well as soluble fractions.

Another problem was the observation that FDV particles are apparently confined to cells of the small leaf galls (Table 2) putting serious limits on the amounts of tissue rich in FDV which could easily be obtained for experimental use. Furthermore, it was not practicable to raise adequate quantities of infected material in the glasshouse and I had to reply to some extent on naturally infected field plants.

The particles purified by the method described in Chapter III show a typical nucleoprotein spectrum (Fig. 7) and were 55-60 nm in diameter when stained in PTA. It has been reported that whole MRDV virions were destroyed by PTA negative stain (Lesemann, 1972; Milne *et al.*, 1973). When the particles purified from FDV-induced galls were stained in UAc, they were about 50 nm in diameter. I have not determined if these particles are infectious. In further studies infectivity of these particles must be tested.

Particles observed in crude leaf-dip preparations from FDV-induced galls (Teakle and Steindl, 1969; Hutchinson and Francki, 1973) and those observed in thin sections of infected cells (Teakle and Steindl, 1969; Francki and Grivell, 1972) measured about 70 nm in diameter. Thus it would appear that preparations of purified particles from FDV-infected tissue are somewhat degraded. These particles are similar to the subviral particles purified from MRDV-infected plants (Wetter *et al.*, 1969; Lesemann, 1972; Milne *et al.*, 1973), RBSDV-

infected plants (Kitagawa and Shikata, 1969) and reovirus cores (Shatkin, 1969). Some reports on the infectivity of subviral particles have been published. Nonoyama *et al.* (1970) reported that particles from reoviruses digested by chymotrypsin showed a low level of infectivity. Kitagawa and Shikata (1969) reported that RBSDV subviral particles, about 60 nm in diameter, were infectious. However, Milne *et al.* (1973) reported that MRDV subviral particles did not display infectivity.

I have not examined at which stage of the purification procedure virus particles lose their outer shell. However, Shikata (1973) reported that freshly prepared leaf extracts in phosphate buffer or distilled water contain RBSDV particles about 60 nm in diameter without obvious outer shells, similar to those seen in purified preparations.

Some virus capsids have been stabilized by the addition of protein or formaldehyde. Crawford (1960) reported that Rous sarcoma virus was stabilized by 0.02% bovine plasma albumin, and Cramer (1959) reported the use of 0.2% defatted and decomplemented horse serum albumin. Bovine serum albumin has been used for stabilization of BTV (Verwoerd, 1969). Francki and Habili (1972) reported that cucumber mosaic virus (Q strain) could be stabilized by 0.02% formaldehyde. Threadlike particles associated with the citrus tristeza disease were purified by density-gradient centrifugation in caesium chloride after fixation with formaldehyde (Bar-Joseph *et al.*, 1972). However, the

fixation of unpurified FDV with these reagents was unsuccessful. Boccardo also failed in purifying intact MPDV particles after fixation with formaldehyde (personal communication). In future studies, the purification of intact FDV particles would be very desirable.

The success in purification of FDV subviral particles was due to the addition of EDTA to the suspension buffer and the use of the nonionic detergent, Nonidet P40, to solubilize the contaminating host membranes. The use of EDTA apparently minimized the aggregation of FDV antigen (Table 8). In contrast to EDTA, the addition of Mg^{2+} to the suspension buffer resulted in the sedimentation of FDV antigen into the pellet during low-speed centrifugation (Table 8). Mg^{2+} lowers the net negative surface charge and may increase the rate of aggregation.

Anionic and cationic detergents usually cause denaturation and precipitation of proteins (Morton, 1955). However, nonionic detergents do not possess strong polar groups and therefore have no strong electrostatic interaction with proteins. Nozu and Yamaura (1971) reported that successful use of a nonionic detergent such as Triton X-100 in the extraction of TMV from *Zinnia* and spinach. Van Oosten (1972) reported the effectiveness of a nonionic detergent in the purification of plum pox (sharka) virus. Since these reports, nonionic detergents are frequently used in the purification of plant viruses.

2. Serology of FDV subviral particles

The results shown in Table 11 and Fig. 8 illustrate that FDV subviral particles are immunogenic and can be used for the preparation of FDV-specific antisera. The results shown in Table 11 illustrate the usefulness of mice for the preparation of antisera to a nucleoprotein available in very small amounts. However, the problem of collecting large amounts of serum in mice remained. Munoz (1957) and Sommerville (1967) observed that mice injected intraperitoneally with antigens mixed with Freund's incomplete adjuvant developed large amounts of ascitic fluid containing specific antibody in high concentrations. Gambel and Kinsley (1963) applied this technique to produce antiserum to animal viruses. In my experiments mice injected with preparations of FDV subviral particles and adjuvant did not develop significant amounts of ascitic fluid. However, a large volume of fluid could be induced by injecting the mice with Ascites tumour cells. Although the concentration of antibody in ascitic fluid was lower than that in blood serum (Table 11), very much larger volumes were obtained.

Ds-RNA alone can elicit antibodies in animals (Steinberg *et al.*, 1969). However, this response can be greatly enhanced by the addition of adjuvant and still further by coupling the ds-RNA with protein (Van Boxel *et al.*, 1972). Thus it would seem possible that when an animal is immunized with a ds-RNA virus such as FDV, which is partly

degraded after injection to expose some of its ds-RNA, then antibodies will be produced to both the viral proteins and ds-RNA. Antibodies to both FDV-protein and FDV-RNA were detected in anti-FDV sera (Fig. 8 and Table 11). It may well be that the ds-RNA acts as an adjuvant for the production of antibodies to viral protein and that the viral protein may similarly enhance the production of antibodies to ds-RNA. Antibodies to ds-RNA were also detected in antisera to RDV and MRDV (Figs. 9 and 10). Milne and Lovisolo (1974) and Luisoni *et al.* (1975) have confirmed the presence of antibodies to ds-RNA in anti-MRDV serum by immune electron microscopy, gel diffusion and slide precipitin tests. The presence of antibodies to ds-RNA in sera immunized with ds-RNA viruses may be widespread or even universal.

Two contradictory reports on the serological relationship of WTV and reovirus have been published. Streissle and Maramorosch (1963) reported preliminary evidence for the presence of "a common antigen" as shown by complement fixation tests. Gomatos and Tamm (1963) and Gamez *et al.* (1967) found no cross reactivity between the two viruses. These contradictory reports may be due to the former workers having used antisera containing antibodies to ds-RNA.

As the antisera to FDV, RDV and MRDV contained antibodies to ds-RNA it is not surprising that they produced precipitin bands when tested against FDV nucleic acid preparations, but positive reactions

with sap from FDV-infected leaves were unexpected. However, the results can be reconciled if FDV-infected leaf cells contain significant amounts of free ds-RNA or if FDV particles in crude extracts are degraded sufficiently to expose the ds-RNA. The results shown in Tables 12 and 13 and Figs. 12 and 13 show some antigenic specificity among the ds-RNAs studied. The determinants of specificity among nucleic acids are little understood (Stollar, 1973). It has been considered that antigenic specificity of ds-RNAs may be determined by several factors.

1. The purine or pyrimidine bases may determine specificity. Garro *et al.* (1971) reported that anti-cytosine antibodies did not react with denatured T2 phage DNA which is deficient in cytosine whereas anti-thymine antibodies did. The complement fixation reaction of an anti-cytidine antibody and denatured DNA could be inhibited to the extent of 66% by 0.05 μmoles of cytidine, whereas a hundred-fold higher concentration of guanylic acid was required for a similar inhibition. Even these high amounts of thymidine, uracil or adenosine did not cause significant inhibition (Erlanger and Beiser, 1964). Reactions of anti-N^2-methylguanosine antibodies were inhibited by 10-50 μmoles of this base but were unaffected by hundred-fold higher concentrations of 1-methylgaunosine or 7-methylguanosine or the unmodified base (Levine *et al.*, 1971).

2. The sugar-phosphate backbone along one chain of polynucleotide may determine specificity. The poly GLU, LYS-[G]MP5' (the product of GMP-5' photo-oxidation in the presence of a random copolymer composed of glutamic acid and lysine residures) immune system was equally and effectively inhibited by all of ribonucleoside-5'-phosphates (GMP5', AMP5', UMP5' and CMP5') (Van Vunakis *et al.*, 1968). However, the deoxynucleoside-5'-phosphates reacted to a lesser extent than the ribonucleotides (Van Vunakis *et al.*, 1968). Guanosine was not effective but ribosome-5'-phosphate did have an effect (Van Vunakis *et al.*, 1968).

3. The macromolecular structural character, e.g. single-stranded polynucleotide, double, or triple-stranded helics may also determine the specificity. Immunization with the triple-stranded complex poly [A] - 2 poly [U]- methylated bovine serum albumin gave antibodies which reacted specifically with the homologous antigen poly [A] - 2 poly [U]. No reaction was noted with the constituent homopolymers poly [A] or poly [U]. In addition, anti-poly [A] - 2 poly [U] antibodies did not react with any of the double-stranded complexes (Stollar and Stollar, 1970). From these observations, Lacour *et al.* (1968) concluded that the specificity of these antisera is determined by the macromolecular conformation of the antigen which stimulated the immunogenic process. Some sera against poly [G] - poly [C] showed specificity for a form of secondary helical structure which is present in the r-RNA of chicken

cells, bacteriophage Qβ RNA and reovirus RNA (Nahon-Merlin *et al.*, 1971; Michelson *et al.*, 1971). The data reported in Tables 12 and 13 suggest that the antigenicity of ds-polyribonucleotides may be dependent mainly on conformational determinants since serum from an animal immunized with the synthetic ds-RNA poly [I] : poly [C] contains antibodies reactive with viral ds-RNAs which lack I residues but contain A, U and G which are absent from the synthetic antigen. Although destruction of antigenicity by heat denaturation (Table 12) may also show that the conformation of ds-RNA determine the specificity, I can't exclude the possibility that heat-denatured ds-RNA was degraded by RNase in the gel before a reaction.

3. The RNA of FDV subviral particles

All data presented in Chapter V confirm that FDV contains ds-RNA. Van Griensven *et al.* (1973) reported that there was a linear relationship between the density and the G-C content of ds-RNA similar to that between the density and G-C content of DNA. In Fig. 31A, data from the literature on the buoyant densities of ds-RNAs are plotted against their G-C content. The buoyant density of FDV ds-RNA corresponds to a G-C content of about 0.45. The correlations between Tm of ds-RNA and its G-C content have been well studied (Van Griensven *et al.*, 1973). In Fig. 31B, data obtained from the literature on the Tm's of ds-RNAs are plotted against their G-C contents. Although it is difficult to determine the G-C content of FDV-RNA from Fig. 31B

because of the discrepancy between the values reported by the various workers, FDV-RNA appears to have a G-C content between 0.39 and 0.45. I have not determined the base ratio of FDV-RNA chemically because of the difficulty of obtaining sufficient nucleic acid.

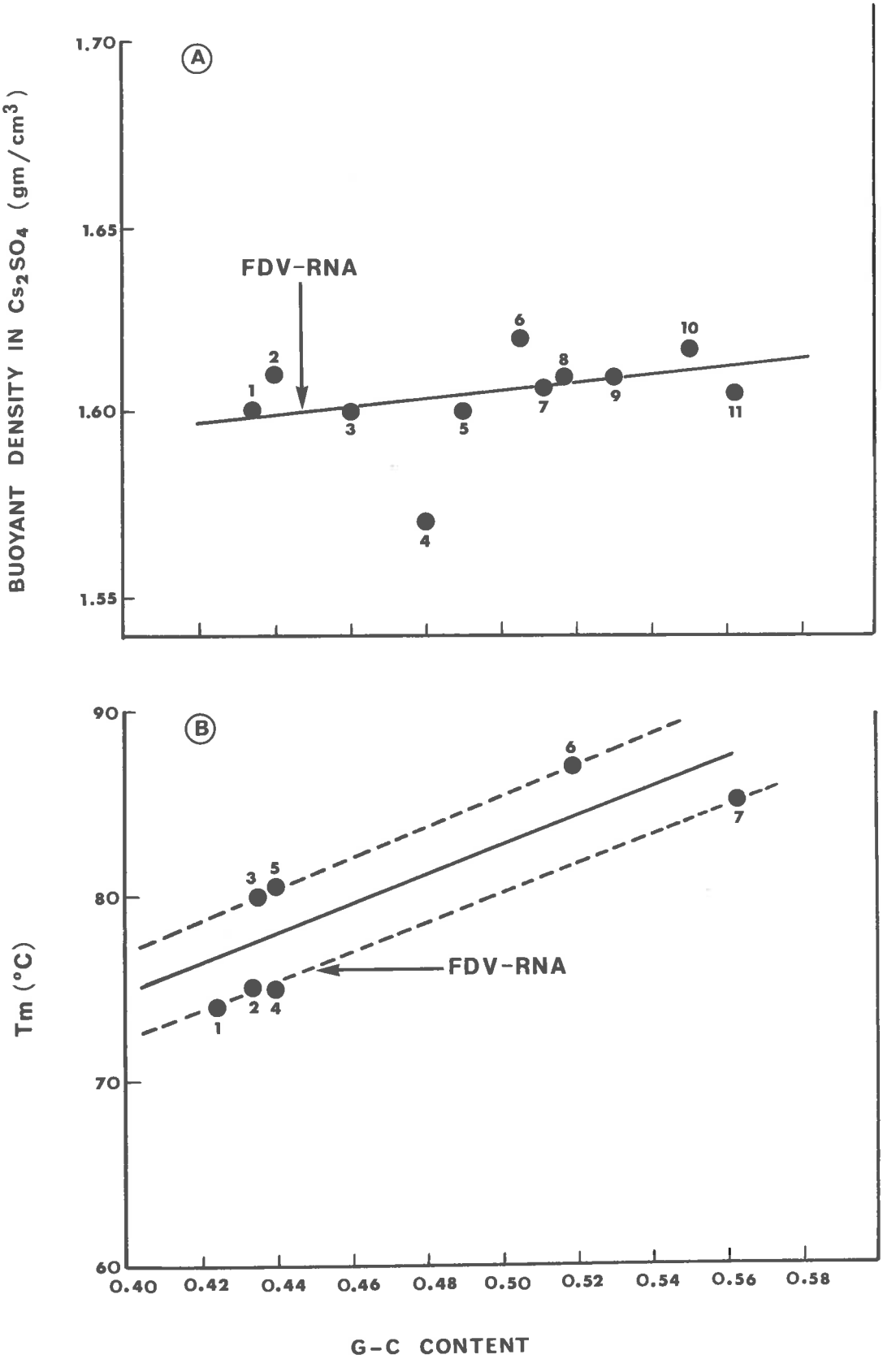
Various workers have been able to establish, using ultracentrifugation, electron microscopy and gel electrophoresis, that the ds-RNA of viruses is not homogeneous in size (Kleinschmidt *et al.*, 1964; Bellamy *et al.*, 1967; Miura *et al.*, 1968; Fujii-Kawata *et al.*, 1970; Verwoerd *et al.*, 1970; Lewandowski and Millward, 1971; Reddy and Black, 1973b; Reddy *et al.*, 1975). Data presented in Figs. 19 and 20 and Table 14 indicate that FDV contains nine segments of ds-RNA. The presence of numerous segments seems to be a common feature of ds-RNA from viruses. The electrophoretic profile was reproducible whether the RNA was examined immediately after preparation or after storage for about a month in 70% ethanol. This reproducibility and the discrete size distribution of RNA segments suggest that the pieces of RNA genome are not products arising from the random scission of viral RNA. The existence of ten unique molecules in the reovirus virion itself has been clearly demonstrated. Banerjee and Shatkin (1971) examined the 5' termini of the ten fragments and discovered that they were all ppGpPyp, suggesting that each had been synthesized as a separate entity. Millward and Graham (1970) reported that all ten pieces of RNA were uniformly labelled when the 3' terminal nucleoside residues of reovirion RNA were labelled *in situ* by reduction with

	G-C content	ρ^{20} (gm/cm^3)	Reference
1. TMV ds-RNA	0.434[a]	1.601[b]	a) Reddi (1964) b) Burdon *et al.* (1964)
2. Reovirus ds-RNA	0.44[a]	1.61[b]	a) Bellamy *et al.* (1967) b) Shatkin (1965)
3. Poliovirus ds-RNA	0.46	1.60	Baltimore *et al.* (1964)
4. Encephalomyocarditis ds-RNA	0.48	1.57	Montagnier & Sanders (1963)
5. Poliovirus ds-RNA	0.49	1.60	Bishop and Koch (1967)
6. Semliki forest ds-RNA	0.505	1.62	Martin & Sounabend (1967)
7. Phage R17 ds-RNA	0.512	1.607	Lozeron & Szybalski (1966)
8. Phage MS2 ds-RNA	0.517	1.609	Billeter *et al.* (1966)
9. Phage fr ds-RNA	0.53	1.609	Kaerner and Hoffman-Berling (1964)
10. Turnip yellow mosaic virus ds-RNA	0.55	1.617	Bockstahler (1967)
11. ϕ6 bacteriophage ds-RNA	0.562	1.605	Semancik *et al.* (1973)

Fig. 31A. The buoyant density (ρ^{20}) of various ds-RNAs in Cs_2SO_4 versus its G-C content.

	Tm (°C)	G-C content	Reference
1. BTV ds-RNA	74	42.4	Verwoerd *et al.* (1970)
2. TMV ds-RNA	75	43.4	Burdon *et al.* (1964)
3. CPV ds-RNA	80	43.5	Miura *et al.* (1968)
4. Reovirus ds-RNA	75	44	Bellamy *et al.* (1967)
5. RDV ds-RNA	80.5	44	Miura *et al.* (1966)
6. MS2 ds-RNA	87[a]	51.9[b]	a) Weissman & Borst (1963) b) Burdon *et al.* (1964)
7. ϕ6 bacteriophage ds-RNA	85	56.2	Semancik *et al.* (1973)

Fig. 31B. The melting temperature (Tm) of ds-RNAs plotted versus their G-C content. The Tm's were determined in 0.01 x SSC by measuring the hyperchromicity at 260 nm or their RNase resistance.


A
BUOYANT DENSITY IN Cs_2SO_4 (gm/cm^3)
1.70
1.65
1.60
1.55
FDV-RNA
1
2
3
4
5
6
7
8
9
10
11
B
Tm (°C)
90
80
70
60
FDV-RNA
0.40
0.42
0.44
0.46
0.48
0.50
0.52
0.54
0.56
0.58
G-C CONTENT

^{3}H borohydride following periodate oxidation.

Recently Reddy *et al.* (1975) reported that FDV-RNA consists of ten segments and not nine as reported from our laboratory (Ikegami and Francki, 1975). Table 23 shows the molecular ratio of each RNA segment of FDV, MRDV and reovirus which was calculated from data reported by Reddy *et al.* (1975). The total mean molecular ratio of segments 2-4 of FDV-RNA is 2.29 which is in approximate agreement with the total molecular ratio of class 2-3 as detected in this work. The molecular ratio of classes 2-4 of MRDV-RNA which are considered to account for 3 segments is 2.63 (Table 23). That of class 2-3 of reovirus is 2.13 which separated into 2 bands (Table 23). Furthermore, Reddy *et al.* (1975) reported that although MRDV-RNA separated into 11 bands, the area under the last peak comprising two segments (see Fig. 2 in Reddy *et al.*, 1975) indicates one molecular weight equivalent, and the two segments are thought to be two forms of component 10. Thus it is difficult at present to conclude if material electrophoresing as class 2-3 Fig. 20 accounts for 2 or 3 segments.

An RNA-dependent RNA polymerase was detected in extracts of FDV-induced galls and appears to be a transcriptase which transcribes an ss-RNA from FDV ds-RNA. Furthermore, the correlation of transcriptase activity with FDV antigen and detection of the polymerase in purified FDV subviral particles suggests that the enzyme is an

TABLE 23. Molecular ratios of FDV, MRDV and reovirus-RNA segments calculated from data published by Reddy *et al.* (1975).

Band No.[a]	FDV-RNA				MRDV-RNA				Reovirus-RNA		
	1[b]	2[c]	3[d]	Mean	1[b]	2[c]	3[d]	Mean	1[b]	2[c]	Mean
1	0.94	1.06	0.80	0.93	0.75	1.14	0.97	0.95	1.09	1.07	1.08
2									2.07	2.13	2.10
3	2.16	2.60	2.12	2.29	2.63	2.90	2.35	2.63			
4									0.96	0.86	0.91
5	0.83	0.94	0.88	0.88	0.75	0.86	0.84	0.82	0.96	0.86	0.91
6	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7	0.89	0.77	1.17	0.94	0.88	0.95	1.03	0.95	0.78	0.93	0.86
8	0.89	1.11	1.24	1.08	0.92	1.10	1.15	1.06	0.76	0.93	0.85
9	2.11	2.16	2.21	2.16	0.92	0.95	1.00	0.96	0.91	1.07	0.99
10					1.21	1.00	1) 0.62 2) 0.53	1.12	0.95	1.13	1.04

a) Separated RNA bands in a gel are numbered from the largest molecular weight band to the smallest one as in Figs. 3-5 reported by Reddy *et al.* (1975). Molecular ratio is calculated using bands 6.

b) Calculated from data in Fig. 3 of Reddy *et al.* (1975).

c) Calculated from data in Fig. 4 of Reddy *et al.* (1975).

d) Calculated from data in Fig. 5 of Reddy *et al.* (1975).

integral part of subviral particles as found in other ds-RNA viruses such as reovirus, CPV, WTV, BTV and RDV (Borsa and Graham, 1968; Shatkin and Sipe, 1968; Lewandowski *et al.* 1969; Black and Knight, 1970; Verwoerd and Huismans, 1972; Martin and Zweerink, 1972; Kodama and Suzuki, 1973).

The FDV transcriptase is most active at 35°C, pH 8.5 - 9.0, and 8 mM $MgCl_2$. Although the characteristics of FDV transcriptase are similar to those reported from the transcriptases of the other ds-RNA viruses (Table 24), the most striking difference between the enzymes of reovirus and FDV are the optimum temperatures for their activity (Table 24). The FDV transcriptase was also stimulated by NH_4^+, about 200 mM being optimum. Relatively little attention has been paid to the effect of monovalent cations on transcriptases of ds-RNA viruses. However, Levin *et al.* (1970) reported that both K^+ and NH_4^+ are able to stimulate the polymerase of reovirus. Van Etten *et al.* (1973) have also shown that the RNA polymerase of the ds-RNA bacteriophage $\phi 6$ can be stimulated by the additon of NH_4^+, the optimum concentration being 75-100 mM.

Although the FDV polymerase appears to transcribe FDV-RNA with a high degree of base sequence fidelity (Table 22), it appears neither to release the transcripts from subviral particles (Fig. 28), nor to transcribe FDV-RNA segments completely (Fig. 30). If the FDV-RNA segments had been transcribed completely, like those of

TABLE 24. Optimal conditions of transcriptase activity associated with virus particles belonging to Reoviridae

Virus	pH	Temperature (°C)	$MgCl_2$ (mM)	NH_4Cl (mM)
Reovirus	7.8-8.8[a)]	47-52[b)]	8-10[c)]	500[c)]
BTV[d)]	8.1-8.5	28	8	-
CPV[e)]	8.0	27	4-8	-
RDV[f)]	8.5-9.0	35	-	-
WTV[g)]	8.5	28-30	-	-
FDV[h)]	8.5-9.0	35	8	200

a) Skehel and Joklik (1969)
b) Kapuler (1970)
c) Levin *et al.* (1970)
d) Verwoerd and Huismans (1972)
e) Lewandowski *et al.* (1969)
f) Kodama and Suzuki (1973)
g) Black and Knight (1970)
h) Present work.

reovirus and CPV (Skehel and Joklick, 1969; Shimotohno and Miura, 1973), ss-RNAs of molecular weights between 0.54 to 1.3 x 10^6 daltons should have been detected. The apparently incomplete *in vitro* transcription of FDV-RNA may well be due to the short *in vitro* life of the enzyme.

4. Taxonomic position of FDV

The International Committee on Taxonomy of Viruses approved that a family be known as Reoviridae to include all viruses which (a) possess genomes consisting of several segments of ds-RNA with molecular weights ranging from 0.3 x 10^6 to 3 x 10^6, all segments being encapsidated within a single virus particle; and (b) possess a quasi-spherical capsid 60-80 nm in diameter which exhibits icosahedral symmetry (Fenner *et al.*, 1974). On the basis of shape, morphological characteristics of particles, properties of RNA and the presence of RNA-dependent RNA polymerase associated with the particles FDV is similar to the other members and should be included in the family.

Serological studies are widely used to assist in virus classification. However, the demonstration that antisera to ds-RNA viruses can contain antibodies to ds-polyribonucleotides indicates that extreme caution must be exercised when tracing relationships among such viruses. Two obvious precautions that can be taken are either to use antisera absorbed with RNA from the virus used for immunization,

or to carry out serological tests using viral protein preparations devoid of all RNA. Luisoni *et al.* (1975) reported serological relationship between MRDV and RBSDV after taking such precautions.

FDV is similar to MRDV and to a lesser extent to WTV and RDV (lovisolo, 1971a; Hutchinson and Francki, 1973; Table 25). Lesemann (1972), Milne *et al.* (1973) and Milne and Lovisolo (1974) reported that MRDV particles have two protein shells and that on the surface there are 12 symmetrically placed "projections"; these "projections" are apparently absent on WTV and RDV particles (Bils and Hall, 1962; Fukushi *et al.*, 1962; Toyoda *et al.*, 1965; Streissle and Granados, 1968; Kimura and Shikata, 1968). Although the morphological characteristics of FDV particles have not been examined in detail, intact FDV particles appear to consist of two protein shells with some "projections" (Dr. Hatta, personal communication) and the subviral particles appear to each have 12 "projections" on their surfaces (Figs. 4 and 5). The ds-RNA segmentation of FDV and MRDV appears to be different from that reported for WTV and RDV (Table 25). FDV and MRDV are transmitted by planthoppers (Delphacidae), whereas WTV and RDV are transmitted by leafhoppers (Cicadellidae) (Black, 1970; Lovisolo, 1971a,b; Hutchinson and Francki, 1973; Iida *et al.*, 1972). FDV and MRDV both cause the development of neoplastic tissue in graminaceous hosts and both produce similar cytopathological structures in infected plant cells. In contrast, RDV is uniformly

TABLE 25. Comparison of RNA genomes of plant viruses with ds-RNA

Band No.	RDV-RNA[a]	RDV-RNA[b]	WTV-RNA[c]	WTV-RNA[b]	MRDV-RNA[d]	MRDV-RNA[e]	FDV-RNA[e]	FDV-RNA[f]
1	2.80	3.10	2.55	2.90	2.58	2.85	2.93	2.60
2	2.35	2.50	2.20	2.48	2.33	2.52	2.53	2.23
3	2.05	2.25	1.98	2.25	2.24	2.50	2.50	2.17
4	1.75	1.90	1.66	1.80	2.06	2.48	2.48	1.90
5	1.72	1.80	1.66	1.80	1.73	2.25	2.25	1.68
6	1.03	1.15	1.02	1.20	1.42	1.82	1.90	1.36
7	1.03	1.13	1.02	1.15	1.21	1.50	1.50	1.17
8	0.77	0.86	0.83	0.90	1.19	1.32	1.32	1.08
9	0.71	0.78	0.58	0.63	1.11	1.24	1.22	1.08
10	0.71	0.75	0.58	0.61		1.13	1.22	
11	0.44	0.52	0.58	0.60				
12	0.44	0.52	0.58	0.35				
13			0.58					
14			0.34					
15			0.34					
Total molecular weight of genome ($\times 10^6$)	15.8	17.3	16.5	16.7	15.9	19.6	19.9	15.3

a) Fujii-Kawata *et al.* (1970)
b) Reddy *et al.* (1974)
c) Wood and Streissle (1970)
d) Redolfi and Boccardo (1974)
e) Reddy *et al.* (1975)
f) Present work.

distributed throughout the leaf tissue, causing disintegration of chloroplasts and thereby inducing chlorotic symptoms (Iida *et al.*, 1972). Nonetheless, FDV and MRDV appear to be distinct viruses. There appears to be no serological relationship between the proteins of these viruses (Fig. 8) and Tm's of their RNAs in 0.01 x SSC appear to differ (Redolfi and Pennazio, 1972), indicating a significant difference in their G-C ratios. Although FDV and MRDV infect maize, repeated efforts to infect sugarcane with MRDV have failed (Harpaz, 1972). At present the exact or relative taxonomic significance of these differences is uncertain.

It is interesting to note that the transcriptases of Reoviridae are clearly distinguished from the transcriptases of viruses belonging to other families in the Mg^{2+} concentration required for optimal activity. For example, virus-like particles with ds-RNA isolated from *Penicillium* sp. require only 5 mM Mg^{2+} for maximum activity (Alaoui *et al.*, 1974; Chater and Morgan, 1974), Rhabdoviruses about 4-6 mM Mg^{2+} (Baltimore *et al.*, 1970; Francki and Randles, 1972) and Myxoviruses are dependent on Mn^{2+} and not Mg^{2+} (Penhoet *et al.*, 1971; Chow and Simpson, 1971).

The FDV transcriptase differs from those of other Reoviridae in that it maintained activity for only 15-20 min at 30°C (Fig. 22) whereas enzymes of other Reoviridae have been observed to incorporate labelled nucleoside triphosphates over periods of hours. With reovirus, such incorporation continued at a constant rate for at least

10 h (Levin *et al.*, 1970). Unlike reovirus, but like CPV (Lewandowski *et al.*, 1969), WTV (Black and Knight, 1970) and RDV (Kodama and Suzuki, 1973), FDV does not require chymotrypsin or heat treatment to activate its polymerase. These treatments appear to remove the outer layer of the virions to expose enzymatically active cores or subviral particles (Shatkin and Sipe, 1968; Banerjee and Shatkin, 1970; Shatkin and Lafiandra, 1972). The formation of reovirus cores appears to be a reversible reaction and the enzyme activity is masked in the reassembled virions (Astell *et al.*, 1972). FDV requires no activation, probably because the outer layer of its particles is easily lost during extraction from plant tissues; I could not prevent this loss during purification (Chapter III). All these observations point to the conclusion that FDV is very much less stable than most of the other Reoviridae studied.

There seems no doubt that FDV and MRDV are sufficiently similar to be assigned to the same group. RBSDV also resembles MRDV and FDV regarding transmission by insects and cytopathological structures in infected cells. These viruses are transmitted by planthoppers (Delphacidae) MRDV and RBSDV having the same vector, *Laodelphax striatellus* Fallen. Milne and Lovisolo (1974) and Luisoni *et al.* (1975) reported serological relationship between RBSDV and MRDV. Thus, RBSDV may be classified into the same group as FDV and MRDV.

MWEV resembles FDV and MRDV in the symptoms it induces on maize and the segmentation of its ds-RNA (Grylls, 1975; Mr. Grylls, personal communication). However, these viruses appear to be distinct. There appear to be no serological relationships between FDV and MWEV (Fig. 11).

Kitajima and Costa (1970) have reported particles 60-70 nm in diameter with cores of about 40 nm in leaf tissue of the Pangola grass, *Digitaria decumens* Stent infected with Pangola stunt virus and tissues of viruliferous planthoppers. Milne *et al.* (1974) reported that *Arrhenatherum* blue dwarf disease appear to be caused by a reo-like virus, similar to MRDV. Milne *et al.* (1975) also reported that cereal tillering disease virus and oat sterile dwarf virus are similar to MRDV but not identical. Inclusion of these lesser known viruses in the Reoviridae may well be justified by further work.

APPENDIX

PAPERS PUBLISHED

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Presence of Antibodies to Double-Stranded RNA in Sera of Rabbits Immunized with Rice Dwarf and Maize Rough Dwarf Viruses

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Positive serological reactions were observed in immunodiffusion tests between Fiji disease antigen and antisera to rice dwarf and maize rough dwarf viruses. However, the reactions were shown to result not from the precipitation of viral proteins but of double-stranded RNA.

Rice dwarf virus (RDV) and clover wound tumour virus (WTV) have been provisionally classified with the Reoviruses (*1*). These viruses have polyhedral particles 75–80 nm in diameter which contain double-stranded RNA (dsRNA). Maize rough dwarf virus (MRDV) (*2*), rice black-streaked dwarf virus (*3*), pangola stunt virus (*4*), and Fiji disease virus (FDV) (*5*) may also belong to the same group. While investigating the possibility of serological relationships between FDV, RDV, and MRDV, we observed that in immunodiffusion tests antisera to RDV and MRDV produced precipitin bands when tested against plant extracts containing FDV. However, experiments which we report in this communication demonstrate that the serological reactions were not due to relationships among the viral proteins, but resulted from reactions of FDV RNA with antibodies to dsRNA. It is well known that antibodies to dsRNA can be specific to dsRNAs but not to dsDNA or single-stranded polynucleotides (*6–9*).

Antisera to RDV and MRDV were kindly supplied by Drs. I. Kimura and E. Luisoni, respectively, and that to polyinosinic:polycytidylic acid [poly(I):poly(C)] was the same as previously used (*9*). Antisera to tobacco mosaic virus and potato virus X were from our own laboratory stocks. Normal sera were obtained from rabbits prior to any experimental immunization. The following preparations were used as antigens:

(a) *FDV-infected sap.* Excised galls from FDV-infected sugar cane leaves (var. NCO-310) were cut into small pieces and ground in a pestle and mortar in 2.5 times their weight of STE buffer (0.1 *M* NaCl, 0.05 *M* Tris–HCl, 0.005 *M* EDTA, pH 7.5). The brei was centrifuged at 1,500*g* for 5 min, and the supernatant recovered.

(b) *FDV nucleic acid.* FDV nucleic acid was prepared from excised galls as described by Francki and Jackson (9) and suspended in STE buffer.

(c) *Poly(I):poly(C).* Poly(I):poly(C) was purchased from Nutritional Biochemicals Corp. (Cleveland, OH) was dissolved in STE buffer at a concentration of 100 μg/ml.

(d) *Healthy sap.* The healthy sap was prepared as in (a) but from healthy sugar cane leaf tissue.

(e) *Host nucleic acid.* The host nucleic acid was prepared as in (b) but from healthy sugar cane leaf tissue. All serological tests were carried out by two-dimensional immunodiffusion tests as described by Francki and Jackson (9). Photographs were taken 7 days after the tests were set up.

When tested by immunodiffusion against antisera to RDV and MRDV, nucleic acid preparations and sap from FDV-diseased tissue as well as poly(I):poly(C) produced precipitin bands all of which were confluent

(Fig. 1). However, even after two weeks of incubation, no reactions were observed between the two antisera and similar preparations from healthy leaf tissue (Fig. 1). Failure by Francki and Jackson (*9*) to detect any serological reaction between FDV-infected sap and antiserum to poly(I):poly(C) was probably due to the fact that FDV was not as concentrated in their preparations as in those used for the present studies. The titers of antisera to RDV, MRDV, and poly(I):poly(C) were similar when tested against FDV-infected sap, FDV nucleic acid, and poly(I):poly(C) (Table 1). However, no reactions were observed between any of the antigens when tested against two normal sera and antisera to the single-stranded RNA viruses, tobacco mosaic virus and potato virus X.

The above observations indicated that the antisera to RDV and MRDV contained antibodies to dsRNA. This was confirmed by intragel cross-absorption tests (*10*) using poly(I):poly(C) to absorb antibodies to dsRNA. From the results summarised in Fig. 2 it can be seen that all the antibodies in antisera to RDV and MRDV which reacted with FDV-infected sap could be absorbed

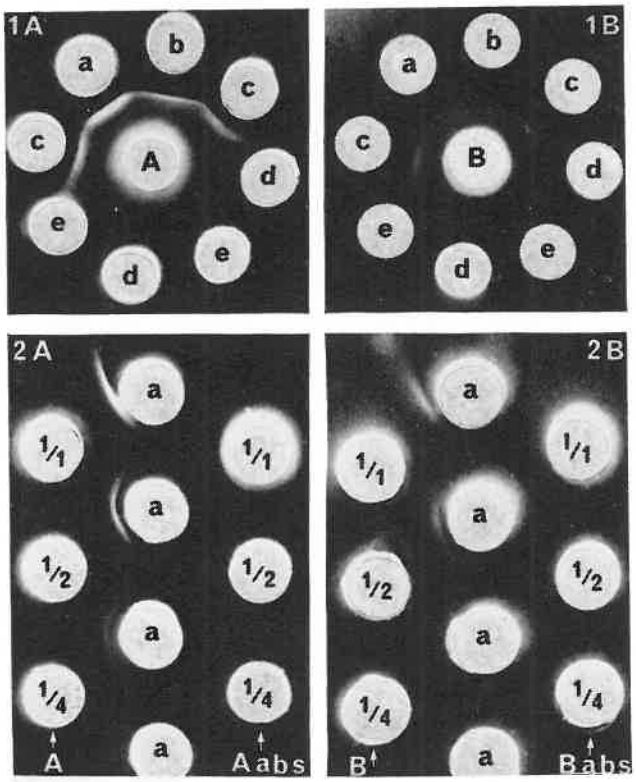

FIG. 1. Two-dimensional immunodiffusion of antisera to RDV and MRDV with various antigen preparations. (A) Antiserum to RDV was placed in the center well and antigen preparations in the outer wells. Well a, FDV-infected sap; Well b, FDV nucleic acid; Well c, poly(I):poly(C); Well d, healthy sap; Well e, host nucleic acid. All the antigens were prepared as described in text. (B) Antiserum to MRDV (B) was placed in the center well and antigen preparations in outer wells as in (A).

FIG. 2. The effect of cross-absorbing antisera to RDV and MRDV with poly(I):poly(C) on the serological reactions with FDV-infected sap. (A) Antiserum to RDV in a twofold dilution series was placed in wells marked A and in wells marked Aabs. To the wells marked Aabs, poly(I):poly(C) (at a concentration of 100 μg/ml) had been added 24 hr previously. FDV-infected sap was added to wells marked a. (B) Antiserum to MRDV (B) was used but otherwise the arrangement of reactants was similar to that in (A).

TABLE 1

REACTIONS BETWEEN FDV AND Poly(I):poly(C) ANTIGENS WITH ANTISERA TO RDV, MRDV, AND Poly(I):poly(C)

Antigen preparation	Titer of antiserum to:[a]		
	RDV	MRDV	Poly(I):poly(C)
FDV-infected sap	4–8	2	4
FDV nucleic acid	4	2	4
Poly(I):poly(C)	4	2	4

[a] Titers are expressed as reciprocals of highest dilution producing a visible precipitin line in gel diffusion tests.

with poly(I):poly(C). Thus it is concluded that no serological relationships can be inferred from the above experiments between the viral proteins of FDV and RDV or FDV and MRDV.

As the antisera to RDV and MRDV contained antibodies to dsRNA, it is not surprising that they produced precipitin bands when tested against FDV nucleic acid preparations, but positive reactions with FDV-infected sap were unexpected. However, the results can be reconciled if FDV-infected leaf cells contain significant amounts of free dsRNA or if FDV particles in crude extracts are degraded sufficiently to expose the viral dsRNA.

Although dsRNAs alone can elicit antibodies in animals (*11*), the response can be greatly enhanced by the addition of adjuvant and still further by coupling the dsRNA with protein (*12*). It is also known that double-stranded polyribonucleotides can act as adjuvants (*13*, *14*). Thus it would seem likely that, when an animal is immunized with a dsRNA virus such as RDV or MRDV which is partly degraded after injection to expose some of its dsRNA, then antibodies will be produced to both the viral protein and dsRNA. It may well be that the dsRNA acts as an adjuvant for production of antibodies to viral protein and that the viral protein may similarly enhance the production of antibodies to dsRNA.

The demonstration that antisera to ds-RNA viruses can contain antibodies to double-stranded polyribonucleotides indicates that extreme caution must be exercised when investigating the serological relationships of these viruses. Two obvious precautions that can be taken in such studies are, either to use antisera absorbed with RNA from the virus used for immunization, or to carry out all serological tests using viral protein preparations devoid of all RNA.

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We thank Drs. I. Kimura and E. Luisoni for generous gifts of antisera, Dr. P. B. Hutchinson for supplies of FDV-infected sugar cane material, Mrs. L. Wichman for photography, and Mr. K. Jones for maintaining plants in the glasshouse. One of us (M.I.) is supported by an Adelaide University Research Grant Scholarship, and the project has been supported in part by a generous grant from the Colonial Sugar Refining Co.

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Purification and Serology of Viruslike Particles from Fiji Disease Virus-Infected Sugar Cane

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Roughly spherical particles 55–60 nm in diameter have been purified from leaf galls of sugar cane infected with Fiji disease virus (FDV) by differential centrifugation, treatment with the nonionic detergent Nonidet P40 and sucrose density-gradient centrifugation; they are probably subviral particles. Immunization of mice with preparations of these particles resulted in the production of antibodies to both protein and RNA of FDV. These antibodies were present in both the blood serum and ascitic fluid produced in response to injection with Krebs 2 Ascites tumor cells. However, both the antisera and ascitic fluids were free of antibodies to host plant antigens.

INTRODUCTION

Polyhedral particles, about 70 nm in diameter, have been observed in leaf galls of sugarcane infected with Fiji disease virus (FDV) (Giannotti *et al.*, 1968; Teakle and Steindl, 1969; Francki and Grivell, 1972). It seems probable that these particles are the causal agent of Fiji disease. Francki and Jackson (1972) were able to detect double-stranded (ds) RNA in gall tissue from FDV-infected plants but not in tissues of healthy sugarcane leaves by using an antiserum to polyinosinic: polycytidylic acid (poly(I):poly(C)) with specificity for ds-polyribonucleotides. This, together with the observation that FDV particles are similar to those of reovirus and other viruses with ds-RNA genomes, led to the provisional conclusion that FDV is a ds-RNA virus (Hutchinson and Francki, 1973).

In attempts to purify FDV we have been faced with two problems: (1) the lack of a satisfactory virus assay method; and (2) the observation that FDV particles are apparently confined to cells of the small leaf galls (Francki, unpublished results) putting serious limits on the amounts of tissue rich in FDV which can easily be obtained for experimental use. As we have been primarily interested in preparing an FDV-specific antiserum we have at present concerned ourselves with the purification of particles from FDV-infected galls to use as an antigen. In this paper we describe a method of preparing FDV-specific antigen from small amounts of gall tissue which we have used to elicit antibody production in mice. We also describe the specificity of antibodies to this antigen obtained from both the blood serum and ascitic fluid of immunized mice.

MATERIALS AND METHODS

Virus-Infected Plant Material

Infected sugarcane plants (var. NCO-310) grown in the field or in a glasshouse were used. Galls were excised from leaves of infected plants with razor blades taking care to include as little surrounding normal tissue as possible. The excised galls were used as starting material from which FDV particles were purified.

Assay of FDV

The amount of FDV in tissue extracts was assumed to be proportional to the amount of ds-RNA present. The ds-RNA was assayed by immunodiffusion against an antiserum to poly(I):poly(C) previously

used by Francki and Jackson (1972). The tissue fraction or extract to be assayed was extracted with phenol, precipitated with ethanol and resuspended in a small volume of ST buffer (0.1 *M* NaCl, 0.05 *M* Tris–HCl, 1 *mM* EDTA, pH 6.7) as described by Francki and Jackson (1972). Twofold dilutions of this preparation were used for immunodiffusion tests and the reciprocal of the dilution end-point was taken as the relative concentration of ds-RNA and hence FDV.

Electron Microscopy

Purified preparations of FDV were mounted on copper grids, stained with 2% phosphotungstic acid (PTA) adjusted to pH 6.8 with KOH and examined in a Siemens Elmiskop I electron microscope.

Preparation of Anti-FDV Serum

At each immunization, an FDV preparation purified from 10 g of leaf galls and emulsified with 2 vol of Freund's complete adjuvant was injected intraperitoneally into two Swiss white mice of an outbred strain. The mice were bled through the retro-orbital plexus and at the conclusion of the experiment by heart puncture under anaesthesia. The serum was processed as described by Francki and Habili (1972). Both the injection and bleeding schedule are later described in Table 1.

When it was required to induce antibody production in ascitic fluid, the mice were given a booster injection of antigen as described above followed immediately by an intraperitoneal injection of about 1 ml of Krebs 2 Ascites Tumor cells freshly obtained from a tumerogenic mouse. A similar technique has been used for preparation of antisera to animal viruses (Gamble and Kingsley, 1963). After about 6 days the mice developed markedly distended abdomens. Ascitic fluid and tumor cells were tapped by puncturing the abdomen with an 18 gauge hypodermic needle allowing the fluid to drain into a beaker by applying gentle pressure to the animal's abdomen. As with blood, the harvested fluid was centrifuged at 2,500 *g* for 20 min to remove cells and the clear fluid was stored at −15°.

Preparation of Crude Antigens for Immunodiffusion Tests

The following antigens were prepared as described by Ikegami and Francki (1973). "FDV-infected sap" was prepared by grinding excised galls in 2.5 times their weight of STE buffer (0.1 *M* NaCl, 0.05 *M* Tris–HCl, 5 m*M* EDTA, pH 7.5) and the brei was centrifuged at 1,500 *g* for 5 min. The supernatant fluid was recovered and used without further treatment. "FDV nucleic acid" was prepared from excised galls by phenol extraction (Francki and Jackson, 1972), ethanol precipitation and resuspension of STE buffer. "Poly(I):poly(C)" was purchased from Nutritional Biochemicals Corp. (Cleveland, OH) and dissolved in STE buffer at a concentration of 100 μg/ml. "Healthy sap" and "host nucleic acid" were prepared as for "FDV-infected sap" and "FDV nucleic acid" respectively, but from healthy sugarcane leaf tissue.

Serological techniques. All serological tests were carried out by two-dimensional immunodiffusion tests at 25° as described by Francki and Habili (1972). The tests were done in 10 cm diameter petri dishes containing 12 ml of 0.75% agar in 0.14 *M* NaCl, 0.02 *M* phosphate buffer, pH 7.2, containing 0.02% sodium azide. Holes 3 mm in diameter and 3.5 mm apart were cut and 10 μl of serum or antigen were placed in each. The plates were observed and photographed after 7-day incubation.

RESULTS

Purification and Properties of FDV Particles

In all our experiments FDV particles were purified from gall tissue excised from leaves of FDV-infected sugarcane since serological tests demonstrated that only this tissue contained detectable amounts of ds-RNA.

Preliminary experiments were carried out to determine if FDV could be purified by schedules recommended for other plant viruses containing ds-RNA, such as wound tumor virus (Black and Knight, 1970; Reddy and Lesnaw, 1971), rice dwarf virus (Suzuki, 1969) and maize rough dwarf virus (Wetter *et al.*, 1969). However, none

of these methods proved satisfactory. Removal of host materials from gall extracts during the early stages of FDV purification proved difficult. The use of organic solvents as clarifying agents, such as chloroform (Suzuki, 1969; Wetter *et al.*, 1969), carbon tetrachloride (Kitagawa and Shikata, 1969; Black and Knight, 1970; Milne *et al.*, 1973), ether and Freon 113 (Streissle and Granados, 1968; Milne *et al.*, 1973) or absorbants such as charcoal and celite (Wetter *et al.*, 1969) resulted in serious losses of FDV and did not remove host materials efficiently. Electron microscopic examination of crude FDV preparations disclosed that most of the contaminating material consisted of membrane fragments. Therefore, we used a nonionic detergent to solubilize membranous material so that it would not sediment with FDV particles when ultracentrifuged.

It was concluded from preliminary experiments that a pH between 7.0 and 7.5 was required to prevent FDV losses during low-speed centrifugation. It was found that FDV sedimented during low-speed centrifugation at pH above 7 when Mg^{2+} was added to the suspending buffer as recommended for the purification of wound tumor virus (Black and Knight, 1970). Significant losses of FDV into the low-speed pellet were also observed when SSC buffer (recommended by Smith *et al.*, 1969, for the purification of reovirus) was used, but addition of EDTA to resuspending buffers minimized these losses.

The procedure finally adopted for the purification of FDV was as follows. Gall tissue was pulverized at 4° with a pestle and mortar in 0.1 *M* glycine, 5 m*M* EDTA, pH 8.5 (1 ml/g of tissue), and a little acid washed sand. The brei was squeezed through two layers of cheese cloth and the fibre was reextracted twice more with the same extracting medium. The extracts were pooled, centrifuged at 1,500 *g* for 5 min, the pH of the supernatant fluid was adjusted to 7.5 with 0.1 *N* NaOH and centrifuged at 5,000 *g* for 10 min; the supernatant fluid was then centrifuged at 165,000 *g* for 30 min to sediment FDV. The pellet was suspended in STE buffer, dispersed in a ground-glass homogenizer and left for 30–60 min at 4° before 20% Nonidet-P40 in STE buffer was added to a final concentration of 1% and the mixture was stirred for 5 min at 4°. The preparation was centrifuged at 5,000 *g* for 10 min and the supernatant fluid was layered over 2 ml cushions of 10% sucrose in STE buffer in 12 ml plastic tubes and centrifuged at 165,000 *g* for 40 min. The pellets were allowed to resuspend in STE buffer for at least 60 min at 4° before clarification at 5,000 *g* for 10 min. Samples of the pale green supernatant (0.5 ml) were layered on 30–60% (w/v) linear sucrose density-gradients (prepared in STE buffer) in 5 ml tubes and centrifuged at 40,000 rpm for 60 min in a Spinco SW 50.1 rotor. The tube contents were scanned and fractionated with an ISCO model D density-gradient fractionator and flow densitometer assembly. Typical density-gradient scans of preparations from gall tissue and healthy sugarcane leaves are presented in Fig. 1. A peak about

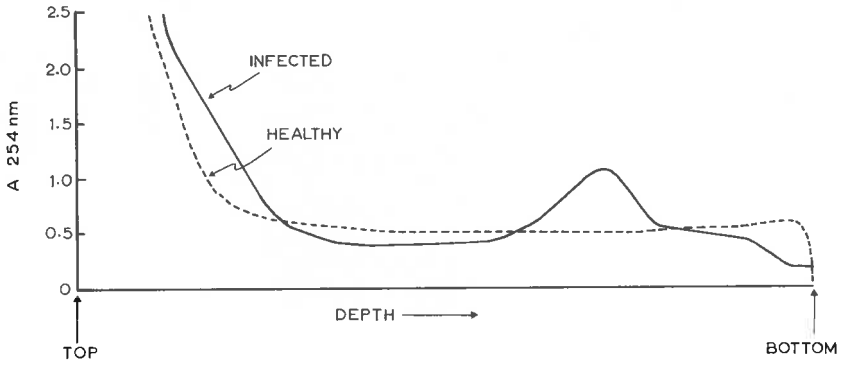

FIG. 1. Sucrose density-gradient profiles of material purified by differential centrifugation from leaf galls of FDV-infected sugarcane leaves (——), and healthy leaves (-----).

one-quarter of the way up from the bottom can be observed in the tube containing a preparation of FDV but not in the tube containing material from healthy leaves. Material producing this FDV-specific peak was recovered, dialysed against STE buffer overnight and concentrated by centrifugation at 165,000 *g* for 30 min. The pellet (final FDV preparation) was resuspended in either distilled water or buffer depending on what the preparation was to be used for.

FDV preparations purified by the above procedure contained ds-RNA as determined serologically and had ultraviolet absorption spectra characteristic of nucleoprotein (Fig. 2) with a maximum at about 260 nm, a minimum at 245 nm and 260:280 nm and 260:245 nm ratios of 1.38 and 1.11 respectively. Preparations from about 10 g of isolated galls finally suspended in 1 ml of distilled water had an optical density at 260 nm between 0.15 and 0.25. Electron microscopic examination of these preparations revealed the presence of numerous roughly spherical particles with a mean diameter of 55–60 nm without significant amounts of contaminating materials (Fig. 3).

Properties of Antiserum to FDV

Two precipitin lines were observed when sera of mice immunized with purified FDV preparations were tested against FDV-infected sap; a heavy line was always observed near the antigen well and a fainter one nearer the antiserum well (Fig. 4). The line nearer the antiserum well was confluent with the single line produced between the antiserum and phenol-extracted FDV antigen (FDV nucleic acid)

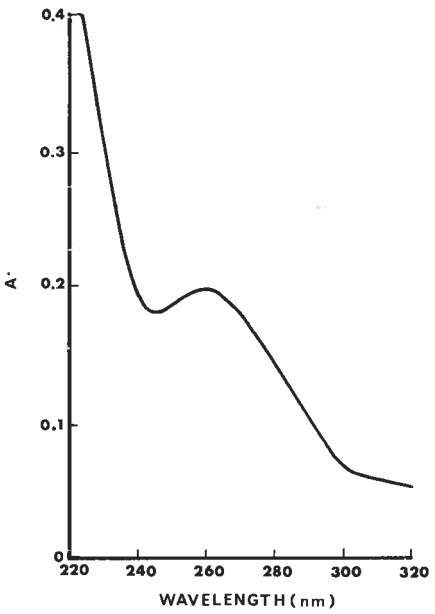

FIG. 2. Ultraviolet-absorption spectrum of a typical preparation of nucleoprotein from excised galls of FDV-infected sugarcane leaves.

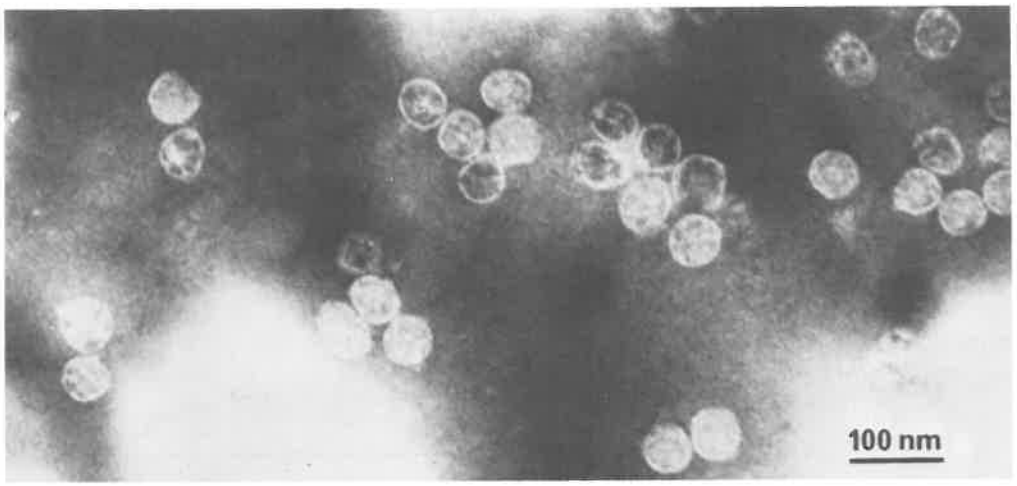

FIG. 3. Electron micrograph of virus-like particles purified from excised galls of FDV-infected sugarcane leaves.

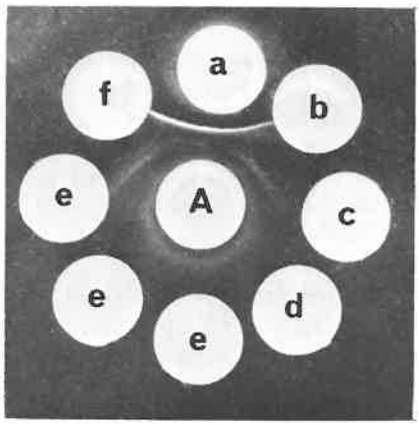

FIG. 4. Two-dimensional immunodiffusion of antiserum to preparations of FDV particles (A) and the following antigens: a, FDV-infected sap; b, FDV nucleic acid; c, host nucleic acid; d, healthy sap; f, poly(I):poly(C). (Wells marked e remained empty.)

(Fig. 4). In most but not all of our tests, there was also a weak precipitin line between the anti-FDV serum and poly(I):poly(C) (Fig. 4). Antigens prepared from healthy sugarcane leaves (both sap and nucleic acid) invariably failed to produce any reactions when tested with the antisera (Fig. 4). From these experiments we conclude that antiserum produced in response to injection of our FDV preparations was free of host plant antigens, but contained antibodies to both the protein of FDV particles (producing a precipitin line nearer the antigen well) and to FDV-RNA (producing a precipitin line nearer the antiserum well).

Details of the immunization schedule and titres of sera from both mice immunized with preparations of FDV are summarized in Table 1. The results show that

TABLE 1

IMMUNIZATION SCHEDULE AND ANTIBODY PRODUCTION IN MICE IMMUNIZED WITH PREPARATION OF FDV PARTICLES

Days after initial immunization	Treatment of mice[a]	Antibody titre[b]							
		Mouse 1				Mouse 2			
		Blood serum against:		Ascitic fluid against:		Blood serum against:		Ascitic fluid against:	
		Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA
0, 6 and 13	1st–3rd injection								
19	1st bleeding	4–8	2			32	n.d.[c]		
32	2nd bleeding	n.d.	n.d.			64	32		
39	3rd bleeding	4–8	2–4			128–256	64		
57	4th injection								
67	5th injection, 4th bleeding and injection with Ascites Tumor	2–4	2						
73	1st tapping			2	1			32	16
75	2nd tapping			n.d.	n.d.			32	16
77	5th bleeding and 3rd tapping	4	2	2	1			64–128	32
80	6th bleeding and 4th tapping	n.d.	n.d.	n.d.	n.d.	256	64	128	32

[a] Bleeding refers to collection of blood for assay of blood serum antibodies and tapping refers to collection of ascitic fluid for antibody assay.

[b] Titres are expressed as reciprocals of highest dilutions producing a visible precipitin line in gel-diffusion tests.

[c] Not determined.

mouse 2 was very much more immunoresponsive to FDV than mouse 1 and that antisera from both mice invariably contained antibodies to both FDV protein and FDV-RNA. It is also evident that antibody titres to both FDV protein and RNA were higher in the blood serum than in the ascitic fluid. However, only 50–150 μl of blood serum was obtained from each bleeding whereas yields of as much as 5 ml of ascitic fluid could be obtained by tapping a mouse with Ascites tumor. In the case of mouse 2 a total of 10.5 ml of ascitic fluid was obtained from the four tappings taken (Table 1). The data summarized in Table 1 also show that both the blood serum and ascitic fluid always had higher antibody titres against FDV protein than against FDV-RNA.

Some of the FDV-specific antiserum samples produced faint precipitin bands against poly(I):poly(C) (Fig. 4), the titres were always very much lower than those produced against FDV-RNA. However, an antiserum to poly(I):poly(C) had the same titre to both homologous antigen and FDV-RNA. Intragel cross-absorption tests demonstrated that whereas all the homologous antibodies in anti poly(I):poly(C) serum could be absorbed with FDV-RNA (Fig. 5A), antibodies to FDV-RNA in the anti FDV serum were not absorbed to any great extent with poly(I):poly(C) (Fig. 5B).

DISCUSSION

Results presented in this paper demonstrate that the particles purified from galls of infected sugarcane leaves by the method described were 55–60 nm in diameter. Particles observed in crude leaf-dip preparations from FDV-induced galls (Teakle and Steindl, 1969; Hutchinson and Francki, 1973) and those observed in thin sections of infected cells (Teakle and Steindl, 1969; Francki and Grivell, 1972) measured about 70 nm in diameter. Thus it would appear that our preparations of purified FDV particles were somewhat degraded. They are remarkably similar to the subviral particles purified from plants infected with maize rough dwarf virus (Lesemann, 1972; Milne *et al.*, 1973). We have not yet deter-

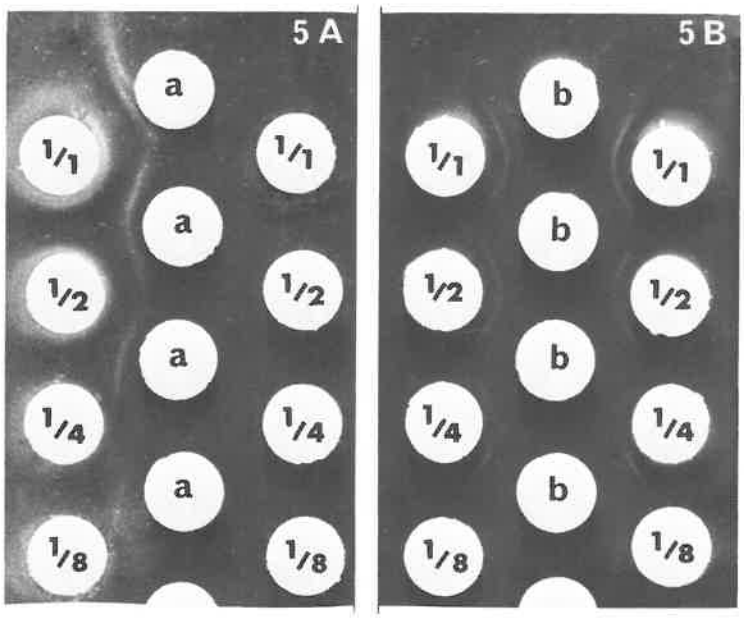

FIG. 5. The effect of cross-absorbing antisera to FDV particles and poly(I):poly(C). (A) Antiserum to poly(I):poly (C) in a twofold dilution series was loaded to wells marked A and in those marked AabsB. To the well marked AabsB, FDV nucleic acid was added 24 hr previously. Poly(I):poly(C) was added to wells marked a. (B) Antiserum to FDV particles in a twofold dilution was loaded in wells marked B and BabsA. To the wells marked BabsA, poly(I):poly(C) was added 24 hr previously. FDV nucleic acid was added to wells marked b.

mined if the 55–60 nm FDV particles are infectious, but our results demonstrate that they are immunogenic and can be used for the preparation of FDV-specific antisera.

Ikegami and Francki (1973) showed that antisera to both maize rough dwarf virus and rice dwarf virus contain antibodies to ds-polyribonucleotides which react with RNA from extracts of FDV-induced galls. It was pointed out that caution must be exercised when investigating serological relationships among these viruses. It has now been demonstrated that FDV-specific antisera contain antibodies to both ds-RNA and protein. The specificity of antibodies directed against FDV-RNA is interesting in that relatively few of the antibodies are able to react with poly(I):poly(C). However, antibodies directed against poly(I):-poly(C) in sera prepared against conjugates of poly(I):poly(C) and methylated bovine serum albumin (Francki and Jackson, 1972) all appear to be able to react with FDV-RNA as shown by the intragel cross-absorption tests (Fig. 5).

Our experiments illustrate the usefulness of mice for the preparation of antisera to a nucleoprotein available in very small amounts (Table 1). The blood serum of these animals can be used when only small amounts of reagent are required. However, when larger volumes are needed, antibodies of ascitic fluid produced in response to Ascites tumor infection can be used.

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Some Properties of RNA from Fiji Disease Subviral Particles

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Nucleic acid isolated from subviral particles of Fiji disease virus (FDV) was identified as double-stranded (ds)-RNA by the following properties: (1) Positive orcinol reaction; (2) resistance to ribonuclease (RNase) in 1 × SSC (sodium chloride–sodium citrate buffer) but not in 0.1 × SSC; (3) susceptibility to RNase in 1 × SSC after thermal denaturation; (4) sharp thermal denaturation curve with a melting temperature of 76° in 0.01 × SSC; (5) buoyant density of 1.60 g/cm^3 in Cs_2SO_4; and (6) no increase in ultraviolet absorption on treatment with formaldehyde at 37°. On electrophoresis in polyacrylamide gel, FDV-RNA separated into nine RNA segments with a total molecular weight of 15.3×10^6.

INTRODUCTION

Recently, Ikegami and Francki (1974) purified polyhedral particles from Fiji disease virus (FDV)-infected sugarcane; similar particles could not be detected in healthy plants. The particles measured about 55–60 nm in diameter and it was concluded that they were derived by degradation of intact virus particles, 70 nm in diameter (Giannotti *et al.*, 1968; Teakle and Steindl, 1969; Francki and Grivell, 1972). Immunochemical tests, using antiserum to polyinosinic:polycytidylic acid (poly(I)·poly(C)), indicated that FDV contains double stranded (ds)-RNA (Francki and Jackson, 1972; Ikegami and Francki, 1973). We now report experiments in which we have isolated and characterized the nucleic acid from preparations of subviral particles of FDV.

MATERIALS AND METHODS

Nucleic acid preparations. Nucleic acid from purified subviral particles associated with FDV (Ikegami and Francki, 1974) was isolated either by phenol–sodium dodecyl sulphate (SDS) extraction (Francki and Jackson, 1972) or by pronase–SDS digestion (Murant *et al.*, 1972). RNA from tobacco mosaic virus (TMV) preparations (Gooding and Hebert, 1967) was isolated by phenol–SDS extraction; that from bacteriophage ϕ6 was supplied by Dr. J. van Etten (Department of Plant Pathology, University of Nebraska) and that from reovirus type 3, Dearing strain, by Dr. A. R. Bellamy (Department of Cell Biology, University of Auckland).

Nucleic acid absorption spectra were examined in a Unicam SP1800 Spectrophotometer equipped with a SP876 Series 2 Temperature Programme Controller and a Philips X-Y recorder. RNA concentrations were determined at 260 nm using $E_{1\text{ cm}}^{0.1\%} = 16.7$ (Ito and Joklik, 1972) for ds-RNA and $E = 25$ for single stranded (ss)-RNA.

Thermal denaturation and formaldehyde treatment of RNA preparations was done as described by Miura *et al.* (1966; 1968).

Isopycnic density-gradient centrifugation. RNA preparations were centrifuged to equilibrium in Cs_2SO_4 (Shatkin, 1965). Centrifuge tubes were punctured at the bottom and the contents of each tube were collected dropwise into 24 fractions. The densities of the fractions were determined gravimetrically and absorbance at 260 nm was determined after dilution of each sample with 0.8 ml of distilled water.

Polyacrylamide-gel electrophoresis. Nucleic acid preparations were subjected to electrophoresis in 5% gels prepared from

recrystallized acrylamide and bis-acrylamide (Loening, 1967) in Plexiglas tubes 6 mm in diameter and 140 mm long (Reddy and Black, 1973). The electrophoresis buffer (0.04 *M* sodium phosphate, 0.015 *M* Tris and 0.002 *M* disodium EDTA, pH 7.6) in each reservoir (300 ml) was changed every 5–6 hr. After electrophoresis at 4 mA/gel at 6°, the gels were rinsed in 0.4 *M* acetate buffer, pH 4.7, for 15 min, stained in 0.1% toluidine blue O in the same buffer for 30 min and destained in distilled water. The stained gels were scanned in a Joyce-Loebl Chromoscan at 620 nm.

RESULTS

Evidence for FDV Having ds-RNA

Nucleic acid preparations isolated from FDV subviral particles had ultraviolet spectra with 260/230-nm and 260/280-nm ratios of about 2.2 and 1.8, respectively, and gave positive orcinol reactions (Volkin and Cohn, 1954) indicating the presence of ribose. Tests on the susceptibility of FDV nucleic acid to pancreatic ribonuclease (RNase) indicate that it is a ds-RNA (Fig. 1). When suspended in 0.1 × SSC (SSC buffer contained 0.15 *M* NaCl and 0.015 *M* sodium citrate, pH 7), the rate of digestion of native FDV-RNA was comparable to that of phage ϕ6-RNA which is known to be double-stranded (Semancik *et al.*, 1973). However, after heat denaturation, it was comparable to that of TMV-RNA, a typical ss-RNA (Fig. 1A). Furthermore, when suspended in 1 × SSC, both native FDV-RNA and ϕ6-RNA were highly resistant to RNase digestion, unlike that of thermally denatured FDV-RNA or TMV-RNA (Fig. 1B). The ds nature of FDV-RNA was confirmed by the following observations.

(1) The thermal denaturation kinetics in 0.01 × SSC was typical of a ds nucleic acid with a sharp melting temperature (T_m) of about 76° and a hyperchromic shift of about 44% (Fig. 2). Under similar conditions the T_m of reovirus RNA has been shown to be 75° (Bellamy *et al.*, 1967).

(2) The FDV-RNA banded at a buoyant density of 1.60 g/cm^3 when centrifuged to equilibrium in Cs_2SO_4 (Fig. 3). This was easily distinguished from TMV-RNA which under the same conditions banded at a density of 1.66 g/cm^3. The buoyant density of reovirus RNA is 1.61 g/cm^3 (Shatkin, 1965).

(3) Formaldehyde treatment had very little effect on native FDV-RNA, but after heat denaturation it produced an increase

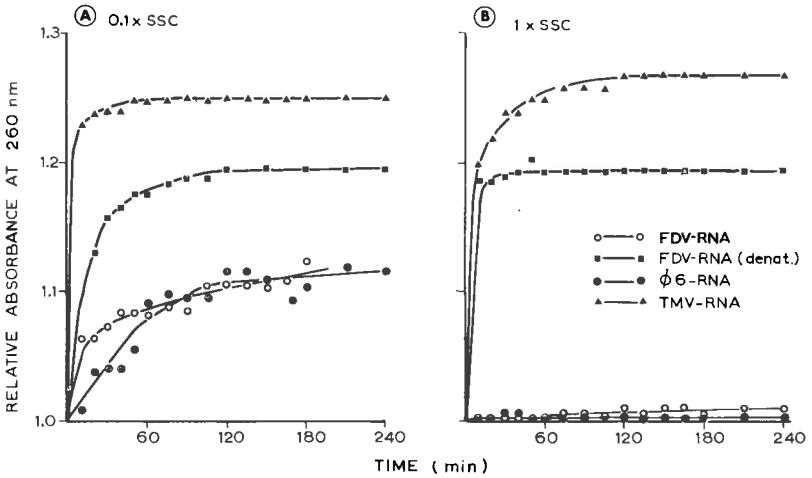

FIG. 1. Kinetics of digestion of FDV-RNA compared to that of ϕ6 phage ds-RNA and TMV ss-RNA in the presence of RNase as measured by the rate of increase in absorbance at 260 nm. The nucleic acids were isolated by phenol–SDS extraction and were suspended in either 0.1 × SSC (A) or 1 × SSC (B). FDV-RNA was denatured by heating in 0.1 × SSC for 10 min at 100° followed by rapid cooling in an ice–ethanol bath. About 15 μg/ml of each RNA preparation was incubated at 25° in the presence of 0.04 μg/ml (A) and 0.5 μg/ml (B) of RNase. The absorbance at zero time is that measured before the addition of the enzyme.

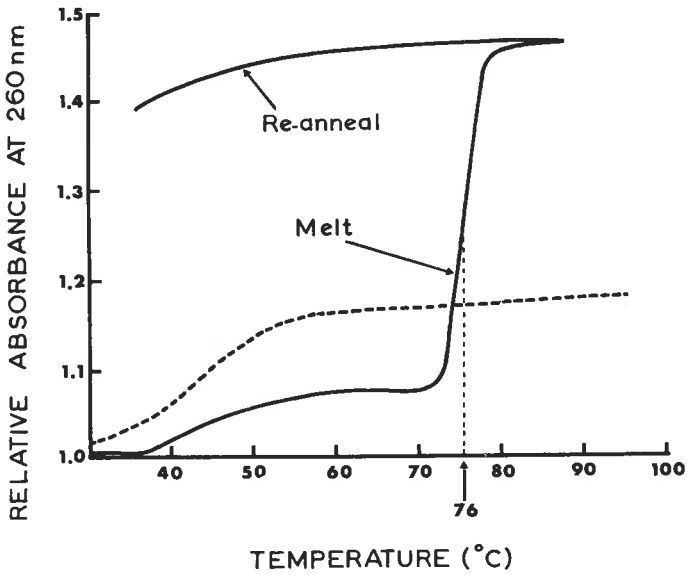

FIG. 2. Thermal denaturation kinetics of FDV-RNA (——) as compared to those of TMV ss-RNA (– – –). RNA's were isolated by phenol–SDS extraction and were suspended in 0.01 × SSC at a concentration of 12 μg/ml. Heating was at a rate of 0.50°/min and reannealing was allowed to take place at a room temperature of 25° after switching off the heater.

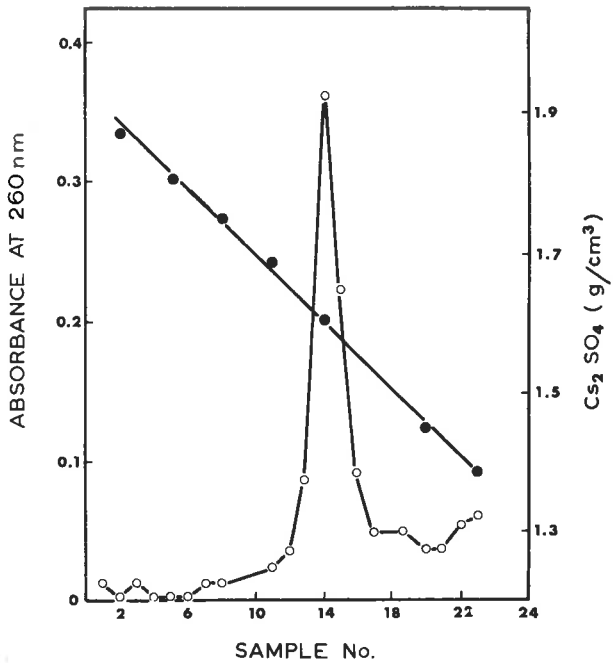

FIG. 3. Isopycnic density-gradient centrifugation of FDV-RNA in Cs_2SO_4. RNA (50 μg) prepared by pronase–SDS digestion in 1 ml of 1 × SSC was mixed with 4 ml of Cs_2SO_4 to produce a solution of density 1.6 g/cm³. The sample was centrifuged at 35,000 rpm for 88 hr in a Spinco SW 50.1 rotor at 5° (absorbancy, O——O; density ●——●).

of about 22% in maximum absorption and shifted both the absorption maximum and minimum to higher wavelengths by 4–6 nm (Fig. 4). Under similar conditions TMV-RNA behaved similarly to denatured FDV-RNA.

It is interesting to note that the relative absorbance of FDV-RNA increased to a greater extent on thermal denaturation (Fig. 2) than a digest of RNA denatured with RNase (Fig. 1), whereas the reverse was observed with TMV-RNA (Figs. 1 and 2). These data indicate that denatured FDV-RNA undergoes only partial RNase digestion, probably because of rapid partial renaturation.

Molecular Weight and Segmentation of FDV-RNA

Polyacrylamide-gel electrophoresis of FDV-RNA preparations for 30 hr resulted in the resolution of eight distinct bands (Fig. 5). The intensity of staining of the fastest-migrating band suggested that perhaps two RNA species of similar molecular

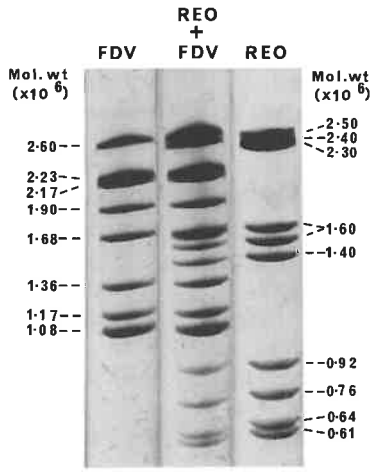

FIG. 5. Comparison of RNA molecules extracted from FDV and reovirus. FDV-RNA isolated by pronase-SDS extraction and reovirus RNA isolated by the phenol–SDS procedure (sample of about 15 μg in electrophoresis buffer) were separated by polyacrylamide-gel electrophoresis for 30 hr as described under Materials and Methods. Migration is from top to bottom. The molecular weights of the FDV-RNA segments were calculated using reovirus RNA and ϕ6-RNA segments as markers (see Fig. 6).

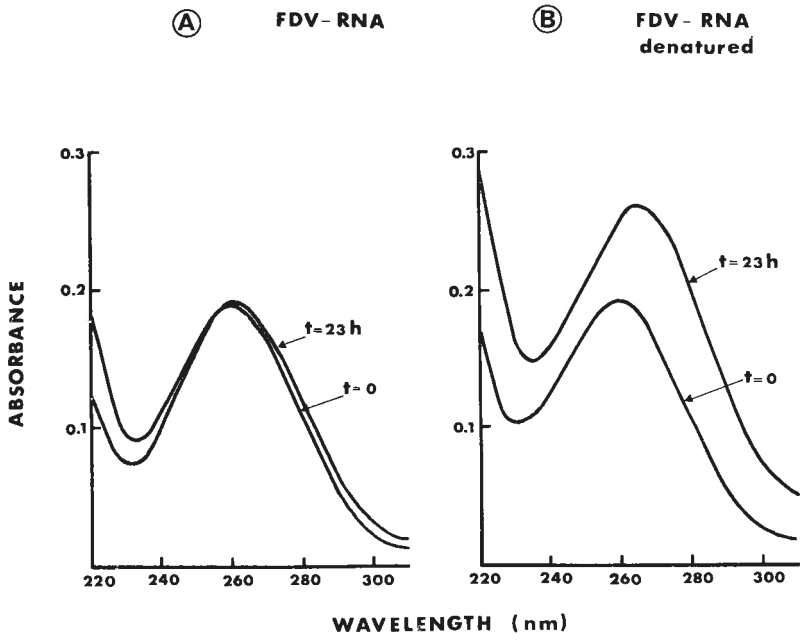

FIG. 4. Reaction of native (A) and heat denatured (B) FDV-RNA with formaldehyde. The ultraviolet spectra of phenol–SDS-prepared RNA in 0.1 *M* NaCl was examined after 23 hr incubation at 37° (t = 23) in the presence of 1.8% formaldehyde. The absorbance at zero time (t = 0) is that measured before the addition of formaldehyde.

weight may have been co-electrophoresing. Although when the time of electrophoresis was increased to 45 hr this band still appeared to be single and homogeneous, a densitometer trace of the gels revealed that the amount of material in the band was twice that expected if only one RNA species was present. Thus we conclude that the genome of FDV-RNA contains nine RNA segments, the two smallest being very similar in molecular size. When FDV-RNA was co-electrophoresed with reovirus RNA (Shatkin *et al.*, 1968) and ϕ6-RNA (Semancik *et al.*, 1973), it was demonstrated that the molecular weight of the FDV-RNA segments ranged from 2.60–1.08 $\times$ 10^6 and that the total FDV genome had a molecular weight of approximately 15.3 $\times$ 10^6 (Figs. 5 and 6 and Table 1).

DISCUSSION

All data presented here are consistent with the conclusion that FDV contains ds-RNA in nine segments whose total molecular weight is approximately 15.3 $\times$ 10^6. In this respect, as in several others already mentioned (Ikegami and Francki, 1973), FDV is similar to wound tumor virus (WTV), rice dwarf virus (RDV), and particularly maize rough dwarf virus (MRDV) (Table 1). WTV and RDV have been included as possible members of the reovirus group together with a number of viruses infecting animals (Wildy, 1971); it would appear that both FDV and MRDV warrant similar consideration.

Similarity between the RNA of FDV and MRDV is very striking (Table 1) although their T_m's in 0.01 $\times$ SSC appear to differ (Redolfi and Pennazio, 1972), indicating a significant difference in their G/C ratios. Although there appears to be no serological relationship between the viruses (Ikegami and Francki, 1973), their particles are very similar (Milne *et al.*, 1973; Ikegami and Francki, 1974), and they have many biological properties in common. Both viruses are transmitted by Delphacid planthoppers, both cause the development of neoplastic tissue in graminaceous hosts and both produce similar cytopathological structures in infected plant and insect cells (Lovisolo, 1971; Hutchinson and Francki, 1973). Although both viruses infect maize

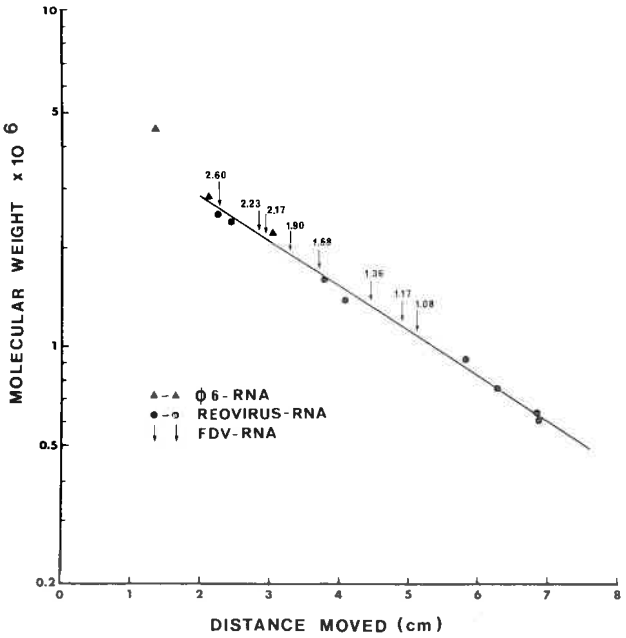

FIG. 6. Relationship between the electrophoretic mobilities of RNA's isolated from FDV, reovirus and ϕ6 phage.

TABLE 1

COMPARISON OF RNA GENOMES OF PLANT VIRUSES WITH DS-RNA

Band No.	Molecular weight ($\times 10^6$)			
	RDV-RNA[a]	WTV-RNA[b]	MRDV-RNA[c]	FDV-RNA[d]
1	2.80	2.65	2.58	2.60
2	2.35	2.20	2.33	2.23
3	2.05	2.00	2.24	2.17
4	1.75	1.68	2.06	1.90
5	1.72	1.65	1.73	1.68
6	1.03	1.05	1.42	1.36
7	1.03	0.95	1.21	1.17
8	0.77	0.88	1.19	1.08
9	0.71	0.57	1.11	1.08
10	0.71	0.56		
11	0.44	0.54		
12	0.44	0.33		
Total molecular weight of genome ($\times 10^6$)	15.8	15.1	15.9	15.3

[a] Fujii-Kawata *et al.* (1970).
[b] Reddy and Black (1973).
[c] Redolfi and Boccardo (1974).
[d] Present work.

(Lovisolo, 1971; Hutchinson *et al.*, 1972), repeated efforts to infect sugarcane with MRDV have failed (Harpaz, 1972).

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