PHYSICO-CHEMICAL AND SUBSTRUCTURAL STUDIES ON *NUDAURELIA CAPENSIS* β VIRUS.

Thesis

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J.K. STRUTHERS

Department of Botany and Microbiology

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CONTENTS

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CHAPTER I

INTRODUCTION.

The pine emperor moth, Nudaurelia cytherea capensis Stoll is an insect which, during the larval stage, causes extensive defoliation of the pine tree, Pinus radiata in the Cape province. These insects are susceptible to a virus disease, which on occasions causes large scale mortality. Five nonoccluded viruses have been shown to infect the pine emperor moth, and of these, one found in the greatest concentration, Nudaurelia capensis β virus (NβV) has been characterised to the greatest extent. This virus has been shown to contain RNA, to be isometric with a diameter of 36 nm, and to have a molecular weight of 16 million. The virus occurs in all stages of the insect's development, and by fluorescent antibody staining has been shown to develop in the cytoplasm of the host's cells.

There have in recent years been a number of reports describing nonoccluded RNA viruses which appear to be similar to NβV. These are the viruses isolated from the moths Gonometa podocarpi and Antheraea eucalypti, and the one from the citrus red mite, Panonychus citri. These viruses have not been as extensively characterised as NβV, so the extent of the similarity between them and NβV is not known. However it would appear as if their discovery collectively heralds the emergence of a distinct new grouping within the nonoccluded RNA viruses of insects.

This work reports the isolation and further characterisation of N.capensis β virus, its protein and nucleic acid.

CHAPTER II

REVIEW OF LITERATURE.

A. THE NONOCCLUDED RNA CONTAINING ARTHROPOD VIRUSES.

The nonoccluded viruses of the arthropods fall into two major groups, depending on their type of nucleic acid. The most extensively studied group consists of those viruses containing DNA, the majority of members comprising the Iridescant virus group. The viruses containing RNA make up a relatively small group to date and they have not been extensively studied, most of the members having been discovered after 1965. This review is confined to these RNA containing nonoccluded viruses of which Nudaurelia capensis β virus (NβV) is a member.

Sacbrood is a disease of the honey-bee, Apis mellifera, being the first insect disease shown to be of viral origin. Steinhaus (1949) demonstrated the presence of spherical particles 60 nm in diameter in diseased larvae, but Brcak et al (1963) claimed these particles to be paraspherical with a diameter of 28-30 nm. Bailey et al (1964) found that the infective agent of the disease had an S_{20W} value of 160S. Brcak and Kratek (1965), using the electron micrographic rotation technique of Markham et al (1963) proposed that this virus had 5 : 3 : 2 symmetry and consisted of 42 capsomeres. Lee and Furgala (1965) isolated the nucleic acid from this virus using phenol reagent. Their isolation technique was questionable, as the published ultraviolet absorption spectrum had a peak at 270 nm and a 260/280 ratio of 1.4. This nucleic acid preparation did however give a positive orcinol reaction and a negative diphenylamine reaction and was thus identified as RNA. Bailey et al (1964) have shown that virus development takes place in the cytoplasm of the host's cells.

The first virus disease discovered in the Orthoptera, was that of the cricket Gryllus bimaculatus. Diseased insects showed symptoms of

paralysis and trembling of the legs and virus particles 30 nm in diameter, which upon injection into healthy insects caused the disease to develop, were isolated from diseased insects. Reinganum et al (1970) reported the isolation of a virus from two species of field crickets, Teleogryllus oceanus and T.commodus, the disease having similar characteristics to the one above. Early to mid instar nymphs were most susceptible to this disease, while the adults showed some degree of resistance, as healthy adults were found to contain low levels of the virus. This virus had a diameter of 27 nm and contained RNA. Virus development was shown to occur in the cytoplasm of infected cells.

Although belonging to the Arachnida, the citrus red mite, Panonychus citri is infected by a virus which is very similar to the others discussed here. Smith et al (1959) showed that the disease was transmissible and that diseased mites contained virus particles 35 nm in diameter. Estes and Faust (1965) purified the virus by differential ultracentrifugation and isolated its nucleic acid by the perchloric acid method. This nucleic acid was identified as being RNA, giving a positive orcinol reaction. They calculated the nucleic acid content of the virus to be 10.6%. Reed and Hall (1972) have however shown that diseased mites contain rod shaped viruses (194 x 58 nm enclosed in an envelope 266 x 111 nm) which develop in the nucleus of the cells. They claim that the spherical particles mentioned above occur in both healthy and unhealthy mites. There is thus a controversy concerning the causal agent of this disease in P.citri, as both Smith et al (1959) and Estes and Faust (1965) produced evidence for a spherical virus-like particle containing RNA being able to produce the same disease.

The first RNA containing virus of the Lepidoptera was discovered by Grace and Mercer (1965). A large number of larvae of the emperor gum moth, Antheraea eucalypte Scott (Saturniidae), reared in their laboratory became infected at all instars and died. Unhealthy larvae usually dropped from the leaves on which they fed and lay in a moribund

state, or hung by the last pair of prolegs from the branches. Apparently healthy larvae became sick and died within 12 hours. On examination of the infected insects they found no evidence of inclusion bodies characteristic of the polyhedroses or granuloses. When the larvae were homogenised and subjected to differential ultracentrifugation followed by density gradient ultracentrifugation, a discrete opalescent band was obtained, which when examined under the electron microscope revealed many spherical particles 50 nm in diameter containing a central core 30 nm in diameter. Sections of diseased cells of the midgut showed numerous virus particles in the nucleus.

Further work in the same laboratory was done by Brzostowski and Grace, (1970),who showed that infected larvae of A.eucalypti contained two sizes of particles, one 32 nm in diameter, the other 14 nm in diameter. Presumably, although they make no mention of this, their estimation of particle size in the previous paper was incorrect. They purified the two agents together, and using phenol reagent isolated the nucleic acid which was identified as RNA. These RNA preparations, when examined under the electron microscope, were shown to be free of undegraded virus, and when aliquots were injected into healthy larvae, all developed the disease and died within eleven days. Sections of these diseased larvae examined under the electron microscope revealed the presence of both sizes of particles in large concentrations. Brzostowski and Grace (1970) also claim that the RNA is infectious when administered orally. No relationship between the two particles found in diseased A.eucalypti larvae has been reported, and it is not known whether only one or both types are infective.

The occurrence of an intestinal disease of the wild silkworm, Hyalophora cercropia, reared in the laboratory was first reported by Riddiford (1967). Peters and Staal (1968) investigated a similar disease from their laboratory populations of this insect. They found that low temperatures, such as 25°C invariably caused the disease with 100% mortality, and even at 32°C, the most "healthy" temperature, the insects were

fastidious. Wilting leaves, shortage of food and wet food induced the disease, whereas overcrowding was not important. The various symptoms of the disease included sluggish behaviour, diarrhoea and abnormal morphological development. Larvae were homogenised with chloroform and subjected to isopyenic ultracentrifugation. This resolved a number of nucleoprotein zones, the main one containing many isometric particles with a mean diameter of 39 nm from apex to apex, and a sedimentation coefficient of 209S. A smaller component with a sedimentation coefficient of 20S and a diameter of 11 nm was also present in purified extracts of diseased larvae. As this small component had an absorption spectrum typical of a protein, Peters and Staal (1968) assume the larger particle to be the infective agent. This small component may be the equivalent of the small particle found in diseased A.eucalypti larvae (Brzostowski and Grace, 1970). Peters and Staal (1968) did not identify the nucleic acid, but the similarity of this virus to the others discussed in this section warrants its inclusion.

Harrap et al (1966) reported a virus disease of the moth Gonometa podocarpi. They obtained larvae of this insect which had died during heavy infestations of Pinus radiata plantations in Uganda. They found no evidence of rickettsiae, bacteria or polyhedra in the dead larvae. However, following homogenisation of the larvae and differential ultra-centrifugation, the "high speed" pellet contained many isometric viruses with a mean diameter of 38 nm from apex to apex. The nucleic acid gave a positive orcinol reaction identifying it as RNA. They estimated that 75% or more of the population studied was infected.

The work on this virus was extended by a recent report by Longworth et al (1973). They showed by electron microscope studies that virus development occurred in the cytoplasm, the nucleus containing no detectable virus products. The groups of virus particles in the cytoplasm were commonly bounded by a membrane. The size of the virus particles was recalculated to be 32 nm from apex to apex. RNA was extracted from

purified preparations of the virus, and the sensitivity of this RNA to ribonuclease, the melting profile and reactivity with formaldehyde indicated that this RNA of G.podocarpi virus was single stranded. The RNA was extracted using either the method of Kirby (1965) or Nair and Lonberg-Holm (1971), but was never of a uniform high molecular weight, and was also not infective when tested on five European lasiocampids or on several other Lepidoptera larvae. It was thus likely that the RNA had become degraded.

In sedimentation studies the bouyant density of the virus in caesium chloride was determined to be 1.35 g/cm^3 at pH 7.6, and there was no change in this bouyant density after dialysis against buffers over the pH range 5.0 to 7.6. The sedimentation coefficient (S_{20W}) of the purified virus was calculated to be 180S, with no dependance on concentration in the range 0.4 to 1.2 mg/ml.

The characteristics of the virus proteins are given in Table 1. Molecular weights were determined by electrophoresis in SOS-containing polyacrylamide gels. The cryptogram ascribed to this virus was thus: R/1; */37; S/S; I/0. Longworth et al (1973) propose that this virus has many properties in common with the enterovirus group of the picornaviruses.

They also isolated a virus from the sympatric lasiocampid, Pachymetana sp. and using G.podocarpi virus antiserum, this virus showed a reaction of identity with G.podocarpi virus. The polyacrylamide gel electrophoretic patterns of their proteins were also identical.

A disease of the larvae of the pine tree emperor moth Nudaurelia cytherea capensis Stoll, was first reported by Tooke and Hubbard (1941). They described diseased larvae as becoming flaccid and dark in colour. The disease on occasions reached epidemic proportions, causing as much as 90% mortality. They called the disease polyhedral wilt.

Hendry et al (1968) found however that a nonoccluded virus was present in diseased larvae. The virus was purified by trituration of larvae in the presence of organic solvents, followed by differential ultracentrifugation

TABLE 1.

Characteristics of the proteins of G.podocarpi virus (Longworth et al, 1973).

Polypeptide	Mol. Wt.	Molar proportions
VP_0	47,500	-
VP_1	36,500	1,23
VP_2	32,000	1,10
VP_3	29,000	0,83
VP_4	12,000	0,40

and density gradient zone electrophoresis. Ultraviolet spectroscopy of this purified virus yielded a spectrum typical of a nucleoprotein, containing about 12% nucleic acid (Warburg and Christian, 1946). The 280/260 ratio was 0.7. Hendry (1969) determined a number of biophysical properties of this virus. A sedimentation coefficient of 220.2S was calculated at infinite dilution, and using the model H electrophoretic apparatus, the virus was shown to migrate as a single species with a mobility of -11.8×10^{-5} cm^2/volt/sec at pH 7.2. The virus was reported to have a diameter of 36 nm.

Using density gradient zone electrophoresis, Tripconey (1970) determined an RØ value (van Regenmortel, 1967) of 1.01 for Nudaurelia virus. She calculated a sedimentation coefficient of 210S at infinite dilution and a particle density of 1.298 g/ml by centrifugation in a preformed density gradient. From electron microscope studies, Tripconey (1970) claimed that the virus was made up of 162 capsomeres, being a regular icosahedron with 5 subunits along each edge of the triangular faces. She also showed that the virus occurred in all stages of the insect's development, as well as adapting it to the larval forms of the silkworm Bombyx mori, the tiger moth Dionycopis amasis and the spookworm, Metahepialus xenoctenis. The virus was also cultured in the ovarian cells of B.mori and N.cytherea and using fluorescent antibody techniques she showed that virus development occurred in the cytoplasm of infected cells.

Polson et al (1970), using the method of particle counting on electron micrographs (Williams et al, 1951), obtained a value of 16.3×10^6 dattons for the particle weight of Nudaurelia virus. This figure was supported by values obtained using a variety of hydrodynamic parameters. Combining in the Svedberg equation the sedimentation coefficient of 210S (Tripconey, 1970) and the diffusion coefficient of 1.08×10^{-7} cm^2/sec, obtained by the thin layer technique of Polson and Parkyn (1969), they calculated the particle weight to be 15.9×10^6.

Juckes (1970) re-examined isolates from infected pupae of N.cytherea and showed the presence of 5 different nonoccluded viruses which he

TABLE 2.

Properties of the viruses isolated from N.cytherea capensis (Juckes, 1970).

Particle	Diameter	RØ[a]	S_{20W}	Nucleic acid
α	60	0.50	-	-
β	38	0.93	212	1 RNA [b]
γ	31	0.16	160	-
δ	21	0.33	-	-
ε	38	0.67	212	-

a = van Regenmortel (1967)

b = Identified by staining procedures.

named α, β, γ, δ and ε (Table 2). The virus previously described was the β virus and was the most predominate member of this group in isolates.

Struthers (unpublished results) investigated certain chemical aspects of NβV. Using the degradative methods described by Knight (1963), attempts were made to produce soluble native protein. The virus could however not be degraded by the cold and warm salt, the 67% acetic acid and mild alkaline methods, or modifications of these. Methods such as the hot salt procedure produced denatured protein. The virus showed a high degree of acid stability, as in pH 5.0 buffers it only failed to react visibly in immunodiffusion tests with virus antiserum after it had been heated to 90°C. The virus could be degraded by heating to 100°C in the presence of 1% SOS or to 50°C with 8 M urea, both in the presence of mercaptoethanol. The protein dissolved in urea was examined by the "high speed" sedimentation equilibrium technique of Yphantis (1964). The molecular weight values ranged from 58 to 81 x 10^3 over 5 determinations. All the plots of ln C *versus* r^2 showed upward concavity, indicating solute heterogeneity. The best plot as judged by the least deviation from linearity, yielded a result of 69,000.

Struthers (unpublished results), showed that the nucleic acid isolated by the phenol method described by Knight (1963) gave a positive reaction with orcinol reagent, identifying it as RNA. The isolated RNA had a 260/280 ratio of 2.0 to 2.1, but the yields were low, being in the order of 20-30% of the total theoretical yield, assuming the virus to contain 12% RNA (Hendry, 1969).

Juckes *et al* (1973) have studied the serological relationship between some of the viruses discussed above. *Nudaurelia* β virus and *Antheraea* virus showed a reaction of identity. *Nudaurelia* ε virus and the viruses of *G.podocarpi* and *P.citri* showed no serological relationship with each other or the two above (*Nudaurelia* β and *Antheraea* viruses).

Other viruses which fall into this group are bee acute paralysis virus, which contains single stranded RNA and has a diameter of 28 nm

TABLE 3.

Properties of the nonoccluded insect RNA virus described.

Virus of.	RNA %	RNA Strand	Diameter (nm)	Local.in Cell	Host	Reference
Antheraea +	-	-	32	N*	Lepidoptera	a
Bee acute	-	1	28	C	Hymenoptera	b
Gonometa	37	1	32	C	Lepidoptera	c
NβV +	12	1	38	C	Lepidoptera	d,e,f
Red Mite	10.6	-	35	C	Arachnida	g
Sacbrood	-	-	28	C	Hymenoptera	h
Teleogryllus	-	-	27	C	Orthoptera	i
Transparency	-	-	60-70	C	Coleoptera	j

\+ = Serological relationship (Juckes et al, 1973).

* = Juckes, (personal communication) does not agree.

C = Virus development occurs in cytoplasm.

N = Virus development occurs in nucleus.

a = Grace and Mercer (1965)

b = Vago (1968)

c = Longworth et al (1973)

d = Hendry (1969)

e = Tripconey (1970)

f = Juckes (1970)

g = Estes and Faust (1965)

h = Lee and Turgala (1965)

i = Reinganum et al (1970)

j = Krieg and Huger (1960)

(Vago, 1968). Krieg and Huger (1960), have shown that the disease transparency of scarabeidae is caused by an RNA-containing virus 60 - 70 nm in diameter.

All the viruses, with the exception of transparency, are in the size range 28-39 nm diameter, contain RNA and all, with the possible exception of Antheraea virus, develop in the cytoplasm, (see Table 3). The work on these viruses to date is of a very preliminary nature and more detailed studies are required before comparisons can be made. Antheraea virus and Nudaurelia β virus appear to be very similar as they show a strong reaction of identity in serological tests.

B. THE DETERMINATION OF PROTEIN MOLECULAR WEIGHTS BY POLYACRYLAMIDE GEL ELECTROPHORESIS.

Electrophoresis in polyacrylamide gels (PAG) in the presence of the anionic detergent sodium dodecyl sulphate (SDS) has proven to be a useful tool for the separation and identification of polypeptide chains. Maizel (1966) used SDS to dissociate adenovirus type 2 followed by electrophoresis in SDS-PAG and showed the capsid consisted of 10 different proteins. Shapiro et al (1966) used SDS-PAGE to identify and quantify heavy and light chains of γ globulins during polyribosome synthesis. Shapiro et al (1967) showed that SDS-PAGE could be used for the estimation of the molecular weights of proteins. They used as markers eleven well characterised proteins such as ribonuclease A, lysosyme and pepsin. These proteins were denatured and reduced at pH 7.1 in 1% SDS and 1% 2- mercaptoethanol, (2-Me) and after dialysis into 0.1% SDS and 0.1% 2-Me, were electrophoresed in 5% PAG containing 0.1% SDS. When the relative migration of the proteins was plotted against the log of the molecular weight, a straight line could be fitted to the points over the molecular weight range 15 to 170 x 10^3. When their results were plotted rectilinearly an almost linear relationship between rates of migration and molecular weight over the range 15 to 90 x 10^3 was obtained. Over the entire range of molecular weights studied however, the true function was hyperbolic. Thus even though the proteins used had isoelectric points ranging from 4 to 11, all points approximated the fitted curve, suggesting that SDS minimized the native charge differences, and all the proteins migrated as a function of their molecular weight, all being similarly charged anions as a result of complex formation with SDS. This method is very useful in that it results in the quantitative solubilisations of many relatively insoluble proteins, by the extensive disruption of hydrogen, hydrophobic and disulphide linkages by the SDS and 2-Me. In the case of ovalbumin and bovine serum albumin, Shapiro et al (1967) showed that during electrophoresis a second slow moving component was present. Carboxymethylation of reduced samples resulted in the disappearance of the

slow component in each case, suggesting that aggregation into dimers by disulphide bonding occurred during electrophoresis. The migration of these dimers fitted the standard curve.

The reliability of this technique was tested to a greater extent by Weber and Osborn (1969). Utilizing 37 well characterised proteins ranging from 11 to 70 x 10^3 in molecular weight, they showed that when the migration of all 37 was plotted against the log of the molecular weight, the points fell very close to a straight line. They pointed out that while the accuracy of SDS-PAEE is not comparable with well defined physico-chemical methods, an accuracy of at least 10% can be obtained in the determination of the molecular weight of an unknown protein.

The major advantage of this method is the speed and ease of determinations, and the resolving power. Sensitivity can be increased by the use of radioactive proteins, enabling the estimation of molecular weights of minute amounts of material.

A second method devised to determine the molecular weights of proteins by PAGE was that of Hedrick and Smith (1968). Their investigation arose from the discovery that phosphorylase a, with a molecular weight of 360 x 10^3 had a greater mobility than phosphorylase b with a molecular weight of 180 x 10^3. This was unexpected as the larger protein should have been retarded by the "sieving action" of the gel to a relatively greater extent than the smaller protein. Hence phosphorylase a should have moved slower than phosphorylase b, especially as the two forms of this protein did not seem to differ sufficiently in net charge to explain this phenomenon. Hedrick and Smith (1968) thus investigated the effect of charge on the migration of proteins in PAG. Utilising a large number of well characterised proteins, they obtained for each a ratio of migration (R_m), this being the distance the protein migrated over the distance a bromophenol blue marker migrated at a particular gel concentration. R_m value were obtained for a range of gel concentrations (from 3% to 15%) for each protein. A plot of log R_m of one protein against gel concentration

yielded a straight line with a negative slope. They showed that proteins having the same charge, but different molecular weights, e.g. the monomer, dimer and trimer of bovine serum albumin, resulted in three non-parallel lines in plots of log R_m versus gel concentration. These lines all intersected at a common point at about 2% gel concentration. Proteins having the same molecular size but different charges, e.g. the lactate dehydrogenase isoenzymes yielded parallel lines in such plots. When two proteins differed in both size and charge, the results were somewhat different. Here they used two proteins, ferritin having a molecular weight of 450×10^3 and being highly charged and ovotransferrin of molecular weight 87×10^3 but with a small charge. At a gel concentration of less than 5.7%, ferritin had a greater R_m value than ovotransferrin. At 5.7% gel concentration the R_m values were identical but at gel concentrations greater than 5.7% the R_m value of ferritin was less than that of ovotransferrin. Thus although these two proteins differed dramatically in both size and charge, only above 5.7% does the gel retard ferritin due to its size. From plots of log R_m versus gel concentration, Hedrick and Smith (1968) calculated the slopes of the lines for proteins over a molecular weight range of 50 to 500×10^3, and found an excellent correlation between these slopes and the molecular weights, the deviation being $\pm$ 4%. The advantage of this technique over that of Shapiro *et al* (1967) is that the proteins are electrophoresed in their native form and thus a protein's position in a gel can be established using a specific biological activity. The molecular weight of a protein which in its natural form consists of two or more identical or different polypeptide chains can also be established. The technique of Shapiro *et al* (1967) destroys not only secondary and tertiary structure but also any quaternary structure.

The technique of Shapiro *et al* (1967) has been in most common use in recent years (Koenig *et al*, 1970; Lesnaw and Reichman, 1970; Michelin-Lausarot *et al*, 1970), but has in some cases lead to discrepant

results. The reported molecular weight of AMV protein determined using this technique has varied by as much as 23% (Hull, 1971; Kruseman et al, 1971). Using the same technique, Hull (1971) obtained a value of 30,900 for the molecular weight of CMV protein whereas Hill and Shepherd (1972a) reported a value of 24,200. Tung and Knight (1972) working on the protein of two strains of Cucumber virus 3 (Japan and Berck) reported that both strains had a protein molecular weight of 17×10^3 based on amino acid analysis, but using SDS-PAGE, the one strain gave a value of 16×10^3 and the other a value of $14{,}2 \times 10^3$, an error of some 13%. They concluded that electrophoretic migration of proteins of similar size in SDS-PAG was a closely related function of their molecular weight only when they had the same charge-mass ratio.

In an earlier paper Tung and Knight (1971) demonstrated the significance of charge effects in SDS-PAGE. They obtained a straight line of mobility versus log molecular weight using five proteins. Using this standard line they determined the molecular weights of various other proteins before and after treatment with maleic anhydride. Maleic anhydride reacts with unionised amino groups to yield malyl derivatives which are stable at neutral pH. They showed the molecular weights of the malyl proteins, estimated from electrophoretic runs with known unmalyated markers, were in general higher than the values for the comparable unmalyated or demalyated proteins, and in general the added weights of the malyl groups attached were not great enough to account for the apparent higher molecular weight obtained. Pepsin ($Mw = 35 \times 10^3$) after malylation had an apparent molecular weight of 42×10^3 based on SDS-PAG experiments, while the expected molecular weight should only have been 35.8×10^3 due to the attachment of 7-8 malyl groups. Since demalylated proteins had molecular weights similar to unmalylated proteins, the observed changes in electrophoretic mobility can be attributed to the introduction of the malyl groups. Tung and Knight thus showed that migration in SDS-PAG was not only dependent on chain length (i.e. molecular size) but that some

other factor was important and had to be considered with this technique. They stated that it was therefore advisable to check the value of a protein's molecular weight determined by SDS-PAGE with some other well known physico-chemical method e.g. amino acid analyses.

The accuracy of the technique of Shapiro et al (1967) appears to be enhanced when it is carried out embracing the method of Hedrick and Smith (1968). The first example of this has been done by van Regenmortel et al (1972). Using SDS-PAGE to determine the molecular weight of CMV protein, R_m values were calculated for the virus and standard proteins at a number of gel concentrations. The slopes obtained were plotted against molecular weight, yielding a straight line from which a value of 25,200 was determined for the CMV protein. This value agrees well with the value determined by amino acid analysis of 24,000 with 215 amino acid residues from the data of van Regenmortel (1967b). This confirms the value of 24,200 from SDS-PAGE determined by Hill and Shepherd (1972a).

C. THE DETERMINATION OF RNA MOLECULAR WEIGHTS BY POLYACRYLAMIDE GEL ELECTROPHORESIS.

The constant charge: mass ratio of ribonucleic acids severely limits the possibilities of resolving species of different molecular weights by conventional electrophoretic methods (Olivera et al, 1964). Polyacrylamide gels resolve however not only according to electrophoretic mobility but also according to molecular size and shape (Richards et al, 1965). Thus as the charge: mass ratio of all RNA species is the same, polyacrylamide gel electrophoresis would be expected to resolve RNA on the basis of molecular weight alone. Initial work using PAGE to separate RNA was done by Richards and Gratzer (1964), who used the gels to purify yeast soluble RNA. Richards et al (1965) showed that the electrophoretic mobilities of soluble RNA from various sources was directly related to their molecular weights and sedimentation coefficients.

Peacock and Dingman (1967) used 3.5% PAG to separate high molecular weight cytoplasmic RNA from the liver, kidneys and brain of rats, and showed how superior PAGE was in its resolving power compared to sucrose density gradient centrifugation. The low concentration gels used however were extremely difficult to handle, and to obviate this difficulty Dingman and Peacock (1968) made the gels 0.5% with respect to agarose, which made them more firm and had no effect on the resolving power of the PAG. The most extensive use of PAGE involving RNA has been its application for determining molecular weights. Bishop et al (1967) studied ten IRNA species from plant and bacterial viruses, as well as Escherichia coli ribosomal RNA. The molecular weight range covered was from 2.3×10^6 (TYMV-RNA) to 3.4×10^5 (BMV-RNA, component 3). They showed that the distance of migration of these RNA types was directly proportional to the log of their molecular weights. This relationship only existed over a gel concentration of 2.6 to 3.6%, as higher gel concentrations prevented migration of the larger molecular weight species.

This technique has been used extensively in recent years for molecular weight determinations of IRNA (Koenig, 1971; Gonsalves and Shepherd, 1972). The high resolving power of this method was demonstrated by the work of Lane and Kaesberg (1971). They studied the RNA of BMV, which has three sedimenting species of 25, 20 and 15S. Analysed by PAGE it was found that the 25S component could be resolved into 2 components of molecular weights 1.01 and 0.99×10^6 respectively. This method has also been used to purify RNA. Gonsalves and Shepherd (1972) working with pea enation virus showed that its RNA consisted of 3 species of molecular weight 1.74, 1.44 and 0.28×10^6. To test which of these was infective, the RNA was separated by PAGE, each band cut out, the RNA eluted from the gel and tested for infectivity on the host plant. They showed that the band of molecular weight 1.44×10^6 was the only one to produce lesions and thus was infective.

However this technique has been shown to give erroneous results in

molecular weight determinations. The mobility of RNA depends not on its molecular weight but on its molecular volume, and RNA species with the same molecular weight have been shown to have different molecular volumes (Gesteland and Boedtker, 1964). The work of Doty et al (1959) and Cox (1966) has shown that IRNA consists of about 60% double stranded hydrogen bonded regions due to back foldings in the chain and the base pairing possibilities which then exist. The size of these double stranded regions will depend on the base sequence of the RNA. Thus even though two different types of RNA have the same molecular weight, their effective volumes may be vastly different depending on their base sequences. This fact has not been considered until recently in the PAGE of RNA. The helical structure of IRNA can be destroyed by heating, thereby converting it to a random coil, and thus changing its molecular volume. Groot et al (1970) reported that the molecular weight of rat mitochondrial RNA, determined by PAGE, varied with temperature. Thus it became evident to Boedtker (1971) that a conformational independant method for molecular weight determination of RNA was necessary. Formaldehyde at a concentration of 1M or above converts IRNA to the random coil form (Boedtker, 1968). Boedtker (1971) thus incorporated formaldehyde in all stages of her PAG system. As standards she used the well characterised RNA species from TMV (2.0×10^6) and E.coli ribosomal RNA (1.1 and 0.56×10^6). She then determined the molecular weights of the RNA from the bacteriophages R_{17} and Q β using this system and found that the molecular weight obtained was considerably larger than the value of 1.0×10^6 commonly assumed to be correct for these two RNA species. She found in this system that the molecular weights for R_{17} and Q β RNA were 1.3 and 1.5×10^6 respectively. These values do depend on the accuracy of the molecular weights of the standards used, but the results show the importance of molecular volume in the determination of molecular weights, and that the formaldehyde method should be used if accurate determinations are required.

PAGE has been used to separate the 2RNA of reovirus and bluetongue

virus. Shatkin (1969) has shown that reovirus contains 10 2RNA species, and that their migration rate is directly proportional to the log of their molecular weight. Verwoerd (1972) and Huismans (1973) have shown the same with bluetongue virus.

PAGE of RNA has been used extensively in the study of RNA species produced in the infective cycles of viruses, as well as many other studies in higher organisms. Like its use for protein study, PAGE of RNA is today an invaluable technique.

CHAPTER III

VIRUS ISOLATION AND CHARACTERISATION.

A. MATERIALS.

1. Nudaurelia capensis β virus.

Source.

NβV was obtained from the diseased larvae of the pine emperor moth, N.cytherea capensis (Lepidoptera : Saturniidae), collected from Pinus radiata plantations in the George - Knysna area. The larvae were collected in the period September - October, when the majority of larvae were in or nearing their final instar. The infected larvae were stored frozen until used for virus purification.

Isolation.

One hundred grams of frozen larvae were used in an isolation procedure. The larvae were thawed overnight at room temperature, and triturated with 500 ml of 0.1M-phosphate, pH 7.2, in an MSE Ato Mix blender. The homogenate was expressed through cheese cloth, and the insect material retained by the cloth was subjected to a further cycle of homogenisation with 500 ml of the above buffer, and re-expressed through cheese cloth. The combined filtrates were centrifuged at 10,000 rev/min for 10 minutes. The supernatant was retained and adjusted to pH 5.0 with 10% acetic acid in order to precipitate more host material, which was removed by centrifugation at 10,000 rev/min for 10 minutes. The virus was concentrated from the supernatant by the addition of polyethylene glycol (PEG) (Juckes, 1970). Struthers (unpublished results) showed that 15% PEG and an NaCl concentration of 1M was necessary for the complete precipitation of NβV. The supernatant treated in this fashion was incubated at $4^{o}C$ overnight. The dense precipitate of virus and host material was collected by centrifugation at 10,000 rev/min for 10 minutes. The pellets were resuspended in 100 ml 0.1M-phosphate, pH 7.2, and in order to ensure complete solution of the

virus, this material was placed on a magnetic stirrer for several hours. Insoluble material was removed by centrifugation at 10,000 rev/min for 10 minutes. The virus was concentrated by centrifugation at 45,000 rev/min for 1 hour. Following several cycles of differential ultracentrifugation, the PEG step was repeated. The virus obtained at this stage was regarded as being reasonably pure (see Chapter III B).

For experiments where purity was critical (dry weight determination, molecular weight determination, serology), the virus was further purified by zone electrophoresis on a sucrose density gradient (Polson and Russel, 1967). Approximately 10 mg of virus in a loading volume of 2 ml were electrophoresed at any one time. Electrophoresis was at 160 volts (20 mA) for 24-36 hours at room temperature. After this period, the rapidly migrating opalescent zone of NβV was collected by drainage through the bottom capillary.

2. Brome mosaic virus.

Source.

Brome mosaic virus (BMV) was obtained from Dr M.B. Von Wechmar, Department of Microbiology and Virology, University of Stellenbosch.

Propogation and isolation.

BMV was propogated in barley plants, Hardeum vulgare, maintained insect free in sterilised soil in a constant environment room at 23°C. Batches of 200 plants were inoculated with the virus at a concentration of 0.1 mg/ml in 0.03M-acetate, pH 6.0, while in the one leaf stage. These leaves were 5-10 cm high. The plants were harvested 10-12 days later and stored frozen.

Virus isolation was performed according to the method of Von Wechmar (1967). The frozen leaves were allowed to thaw at room temperature, and were then triturated in 0.03M-acetate, pH 4.0, in an MSE Ato Mix blender. The homogenate was expressed through cheese cloth, and then adjusted to pH 4.0 with 10% acetic acid. After clarification at 10,000 rev/min

for 10 minutes, the virus was concentrated by centrifugation at 45,000 rev/min for 1 hour, and further purified by a cycle of differential ultracentrifugation.

An alternative procedure developed involved bringing the supernatant, after the initial low speed spin, to 10% with respect to PEG. The precipitate was then collected after 2 hours, resuspended and clarified by centrifugation at 10,000 rev/min for 10 minutes. This procedure circumvented the need for a number of high speed spins to concentrate the virus from the large volume of clarified homogenate.

3. Tobacco mosaic virus.

Source.

Tobacco mosaic virus (TMV) was obtained from Dr M.B. Von Wechmar, Department of Microbiology and Virology, University of Stellenbosch.

Propogation and isolation.

TMV was propogated in young tobacco plants, Nicotiana tobacum cv White Burley. The virus was inoculated at a concentration of 0.1 mg/ml in 0.05M-phosphate, pH 7.2. Leaves showing severe systemic symptoms were harvested 4-8 weeks after inoculation and were stored frozen.

The virus was isolated according to the method of Von Wechmar and van Regenmortel (1970). The leaves were allowed to thaw in 0.05M-EDTA, pH 9.5, and ground in a food grinder. After extracting the juice through cheese cloth, the pulp was homogenised in an MSE Ato Mix blender in the presence of EDTA buffer (100 ml buffer/100 grams of tissue). Juice was again extracted through cheese cloth, and added to the first batch of juice. The pH of the juice was then adjusted to 7.5 with N NaOH. The activated charcoal step described by Von Wechmar and van Regenmortel (1970) was omitted, and following clarification by centrifugation at 10,000 rev/min for 10 minutes, the virus was precipitated by the addition of PEG and NaCl (5% and 4% respectively). The precipitate was collected by low speed centrifugation and resuspended in 0.01M-EDTA,

pH 7.5. After clarification, the PEG step was repeated and the virus finally resuspended in 0.05M-phosphate, pH 7.2.

4. Turnip yellow mosaic virus.

Source.

Purified turnip yellow mosaic virus (TYMV) was a gift from Dr J.J. Joubert, Department of Microbiology and Virology, University of Stellenbosch.

B. ESTIMATION OF PURITY AND CONFIRMATION OF IDENTITY OF NUDAURELIA CAPENSIS β VIRUS - METHODS.

1. Ultraviolet spectroscopy.

Ultraviolet spectra of diluted virus samples were recorded using a UNICAM SP 800 spectrophotometer. From the spectra obtained, the approximate nucleic acid contents and virus concentrations were determined using the method of Warburg and Christian (1946). The method employs the ratio of the absorbancies at 260 and 280 nm. No correction for light scattering was made.

2. Analytical ultracentrifugation.

(a) Sucrose density gradient centrifugation. Linear sucrose gradients (5-40% sucrose concentration) were prepared by layering successively heavier sucrose solutions into the tubes for the Spinco 25.1 rotor. This was done by means of a Pasteur pipette, topped by a funnel, extending to the bottom of the tube (3.5 ml of each sucrose concentration, increasing by 5%). The gradients were allowed to diffuse overnight at $4^{\circ}C$. Quantities (0.1 ml) of virus in 0.05M-phosphate, pH 7.2, at a concentration of 1 mg/ml, were layered on the surface of the gradients. Centrifugation was at 20,000 rev/min for 2 hours, after which the rotor was stopped without the brake. Tubes were removed, punctured in the bottom, and ultraviolet absorbing zones were detected

using an LKB Uvicord and recorded by means of an Hitachi recorder. A constant flow rate was maintained by an LKB Re Cy Chrom peristaltic pump attached to the effluent side of the Uvicord.

(b) The Beckman L2-65B preparative ultracentrifuge equipped with the schlieren optical accessory was used to examine virus preparations. Virus at concentrations of 5-8 mg/ml were examined, using a double sector analytical cell equipped with quartz windows, in an AN-D rotor at 10-15 C. Speeds between 12,000 and 15,000 rev/min were utilized. In order to calculate the sedimentation coefficient, photographs were taken at regular time intervals using a Polaroid camera equipped with Polaroid projection film type 46-L. For each photograph the log. of the distance in cm from the boundary to the axis of rotation was measured using a travelling microscope. The maximum ordinate of the schlieren peak was used as the boundary position in these measurements. The log. of the distances were plotted against the time interval, and the slope calculated. The sedimentation coefficient was calculated from this slope by equation (i):

$$s = \frac{\text{slope} \quad \frac{2.303}{60}}{2 \left(\frac{\text{rpm}}{60}\right)^2} \text{ seconds}$$

$$= \frac{\text{slope} \quad \times 3.499 \text{ seconds}}{(\text{rpm})^2} \quad \text{----- (i)}$$

The sedimentation coefficient obtained using this equation was converted to standard conditions at 20°C using equation (ii):

$$S_{20w} = s \left(\eta\right) \left(\eta_o\right) \quad \text{----- (ii)}$$

where η = the viscosity of the solvent relative to that of water

η_o = the viscosity of distilled water at the temperature at which the experiment was performed.

(c) Isopynic density gradient centrifugation. This was performed using the Spinco SW 50.1 rotor. A 0.1 ml volume of virus at a concentration of 0.5 mg/ml was layered on top of a 0-40% CsCl density gradient (density range 1.00 to 1.42 gm/ml). Centrifugation was at 45,000 rev/min for 2 hours, after which the rotor was allowed to coast to a stop. The tubes were punctured and ultraviolet absorbing zones were detected as discussed in Chapter III, section B 2(a).

3. Electrophoretic mobility (Rø value).

The electrophoretic mobility of the purified virus on a sucrose density gradient relative to a phenol red marker was determined by zone electrophoresis (van Regenmortel, 1967). Two ml of virus at a concentration of 2 mg/ml and containing 0.05% phenol red were applied to the column and electrophoresis was performed at 160 volts (20 mA) for 24 hours at room temperature. After this period, the column was drained through the capillary and ultraviolet absorbing zones detected as in Chapter III, B 2(a). Rø values were taken as being the distance from the origin of the position of the 50% concentration leading edge of the virus zone, divided by the distance of migration of phenol red.

4. Serology.

Antiserum prepared against his isolates of NßV was kindly donated by I.R.M. Juckes. Using the Ouchterlony technique preparations of NßV prepared in this laboratory were tested at dilutions down to 1/32 against dilutions down to 1/512 of Juckes' antiserum. Well patterns were cut in agar, the central well filled with a dilution of Juckes' antiserum, and the 6 outer wells with 6 dilutions of the virus. The dishes were stored at room temperature for 24 hours in a humid environment and were then examined for precipitin lines.

C. DETERMINATION OF N.CAPENSIS β VIRUS MOLECULAR WEIGHT BY SEDIMENTATION EQUILIBRIUM.

Equilibrium ultracentrifugation was performed according to the "high speed" method of Yphantis (1964) using a model E analytical ultracentrifuge equipped with interference optics. The standard 12 mm double sector centrepiece or the Yphantis 6 channel centrepiece were used. The cell was equipped with sapphire windows. Virus for these determinations was subjected to two cycles of density gradient zone electrophoresis and then dialysed exhaustively against 0.05M-phosphate, pH 7.0. The assembled cell was filled according to the method described by Van Holde (1967). The left hand reference channel (filling holes up, cell top towards filter) was filled with 0.01 ml FC43 flourocarbon oil and 0.12 ml of solvent. The right hand channel was filled with 0.02 ml FC43 and 0.10 ml of virus solution at a concentration of 0.5 to 2.0 mg/ml. The FC43 functioned to shift the liquid columns away from the outside edge of the cell more into the centre of the field of vision. Owing to the low rotational speed employed to obtain equilibrium with the virus, solvent ion sedimentation was not encountered, so meniscus matching of the two sectors was not critical.

To obtain maximum stability at the low rotational speed employed, the AN-J rotor was utilised. The rotor was oversped at 5,000 rev/min for 45 minutes in order to shorten the time to attain equilibrium. The speed required to obtain equilibrium of the virus with a molecular weight of 16.3×10^6 (Polson et al, 1970) was calculated from equation (iii) (Yphantis, 1964).

$$5 = \frac{\omega^2 M (1 - \bar{v} \rho)}{RT} \qquad \text{----- (iii)}$$

where M = molecular weight
$\bar{v}$ = partial specific volume of the virus
ρ = density of the solute
$\omega = \frac{\pi \, rpm}{30}$

This gave a value of 1,533 rev/min. Speeds between 1,400 and 1,600 rev/min were used in the experiments. The time required to attain equilibrium was calculated roughly from equation (iv) (Van Holde and Baldwin, 1958).

$$t = \frac{0.65\ (b - a)^2}{D} \qquad \text{-----} \quad \text{(iv)}$$

where $(b - a)$ = the column height
D = diffusion coefficient of the solute.

For N βV this gave a value of 52 hours. Photographs were taken 60 hours after the start of the run using type II-G Kodak plates. The photograph having the optimum exposure time as judged by the facility of fringe discernment was used for measuring. Plates were read, according to the method described by Hendry (1969) using a Nikon 6C shadowgraph.

The plot of ln. of vertical fringe displacement vs. the square of the distance from the axis of rotation resulted in a straight line. The slope was calculated and substituted into equation (v).

$$M = \frac{\text{slope} \quad 2RT}{\omega^2\ (1 - \bar{v}\rho)} \qquad \text{-----} \quad \text{(v)}$$

where M = molecular weight
$\bar{v}$ = partial specific volume of the virus
ρ = density of the solute.

The partial specific volume ($\bar{v}$) was calculated from equation (vi).

$$\bar{v} = \frac{1}{\rho_o} \quad 1\frac{\rho_s - \rho_o}{c} \qquad \text{-----} \quad \text{(vi)}$$

where ρ_o = solvent density
ρ_s = solution density
c = solute concentration (g/ml)

Virus was exhaustively dialysed against 0.05M-phosphate, pH 7.0, and densities of outer dialysates and virus suspensions were determined

at 20°C by three separate determinations in 4.5 ml Springel type pyonometers. Values of $\bar{v}$ were not corrected tc the temperature of the equilibrium experiments. Virus concentration was determined by dry weight (see Chapter V). The value for $\bar{v}$ was calculated to be 0.7022 ± 2.3%, agreeing with the value of 0.703 calculated by Polson et al (1970).

D. RESULTS AND DISCUSSION.

1. Purity of virus preparations.

The ultraviolet absorption spectrum of NβV is shown in Fig. 1. The 280/260 ratio obtained was 0.68, agreeing with the value of 0.70 obtained by Hendry (1969). The ultraviolet absorption spectra of BMV and TMV are shown in Fig. 2. The 280/260 of BMV was 0.62 agreeing with that obtained by Von Wechmar (1967) and for TMV it was 0.85. Using the method of Warburg and Christian (1946) the nucleic acid contents of NβV, BMV and TMV were 12.0%, 17.5% and 5.3% respectively agreeing with previously reported values (Hendry, 1969; Von Wechmar, 1967; Knight and Woody, 1958). The BMV and TMV preparations were regarded as being reasonably pure on the basis of their ultraviolet spectra.

The sedimentation velocity pattern of NβV using schlieren optics is shown in Fig. 3. A single sedimenting peak was obtained. This finding was supported by the sedimentation profile on a 0-30% sucrose gradient (Fig. 4a), and by isopycnic centrifugation on a 0-40% CsCl gradient (Fig. 4b), both showing the presence of a single component. These results indicated that the preparations of NβV were sufficiently pure, being free from host material, and more important from the other viruses known to infect diseased larvae of N.capensis (Juckes, 1970). Juckes (1970) reported that these viruses α, γ, δ and ε are always present in very low concentrations compared to NβV.

2. Identification of NβV.

The virus isolated from diseased N.capensis larvae was identified

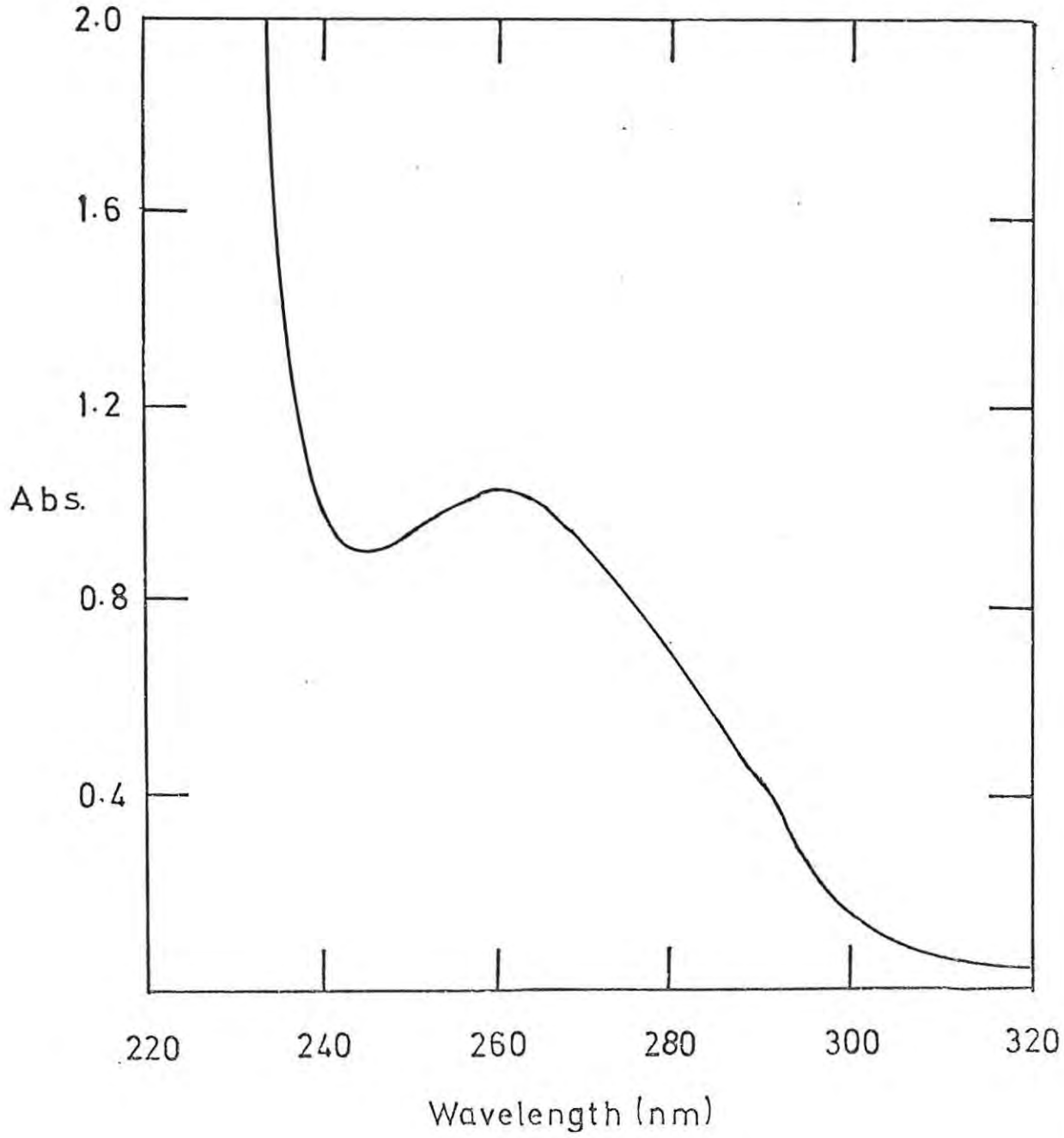


Figure 1. Ultraviolet absorption spectrum of NβV.

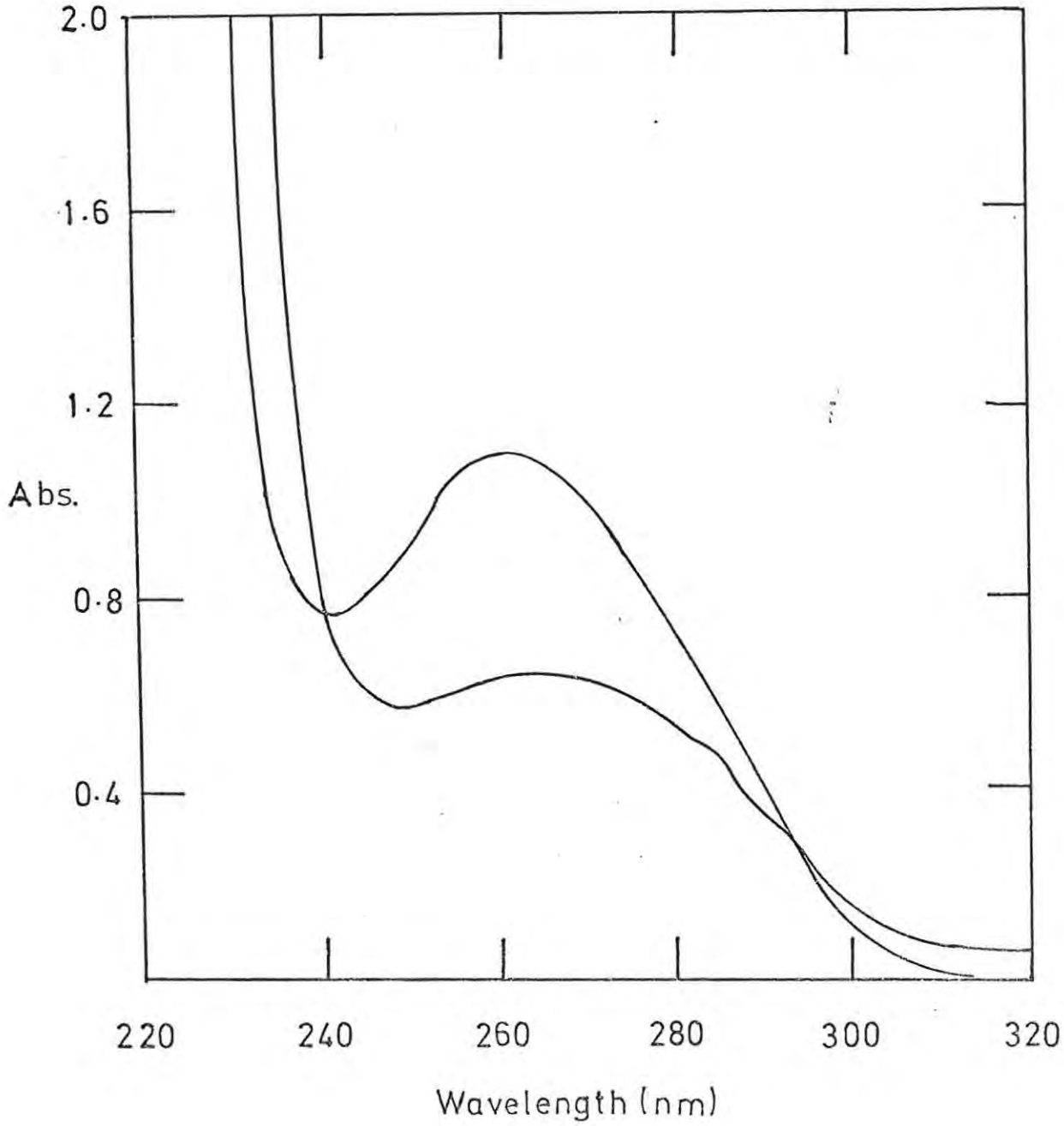


Figure 2. Ultraviolet absorption spectra of BMV (upper curve) and TMV (lower curve).

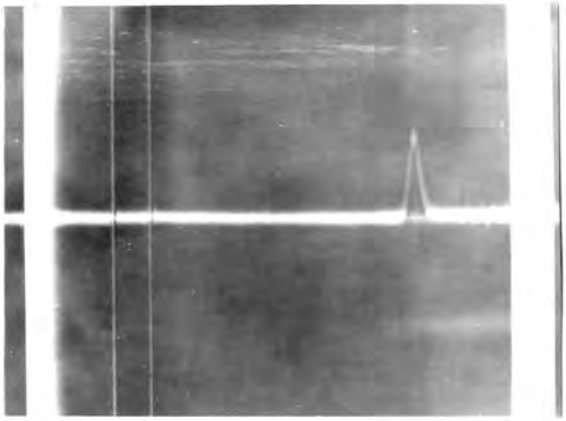


Figure 3. Sedimentation velocity pattern of NβV at 12°C in 0.05M- phosphate, pH 7.2. Centrifugation was at 12,090 rev/min. Sedimentation from left to right.

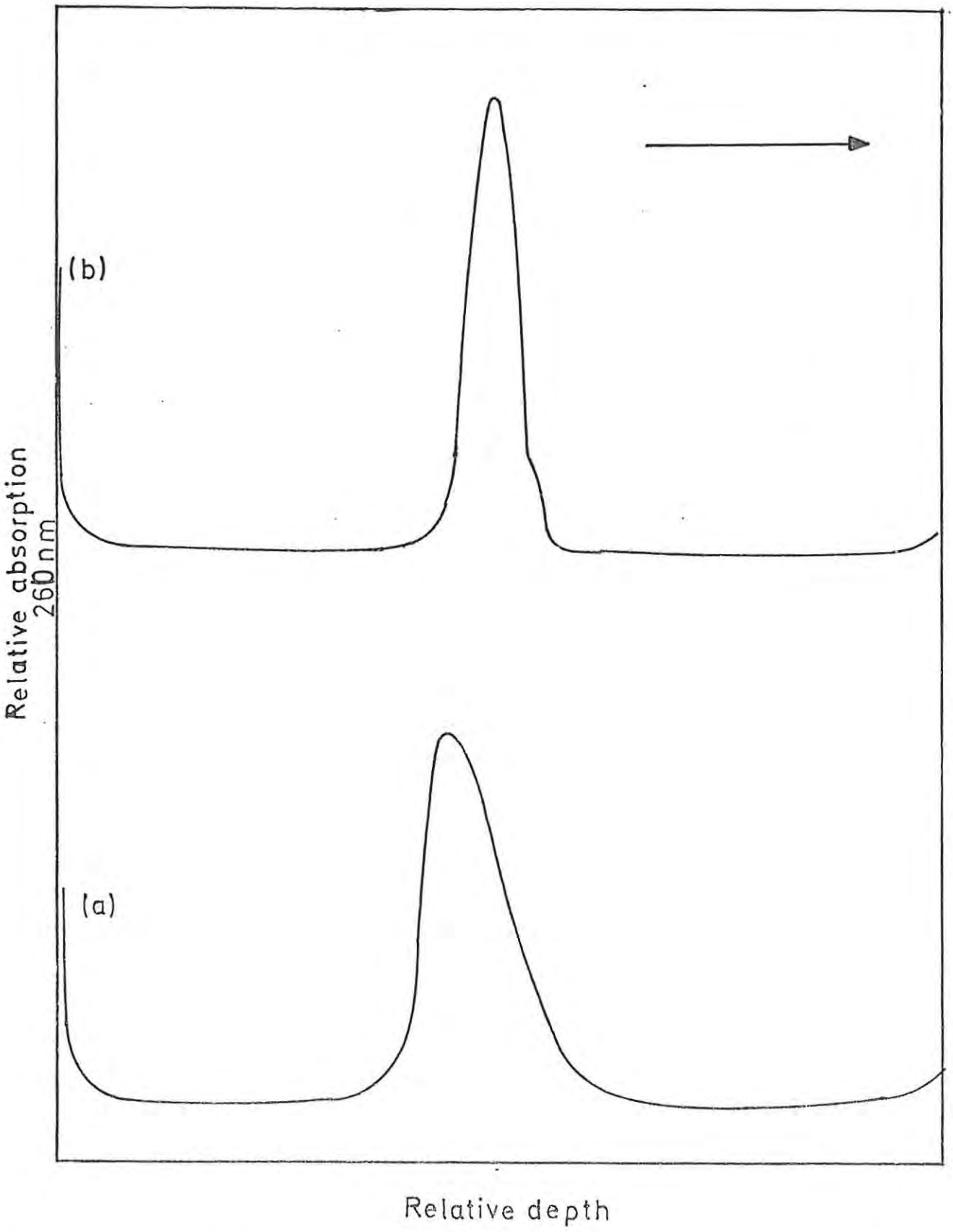


Figure 4. Optical density profiles of: (a) NβV after centrifugation for 2 hours at 20,000 rev/min. in a 5 - 40% sucrose density gradient. (b) NβV after centrifugation for 2 hours at 45,000 rev/min. in a 0 - 40% CaCl density gradient. The arrow indicates the direction of centrifugation.

as NβV by the following criteria: The sedimentation coefficient was in the range 220-225 S, agreeing with the value of 220.2 S obtained by Hendry (1969). The ultraviolet absorption spectra were always identical and the virus was calculated to contain 12%. The Rø value was calculated to be 0.94 ± 0.04, agreeing with the reported value of 0.93 obtained by Juckes (1970). The virus prepared in this laboratory showed a strong reaction of identity with the antiserum obtained from Juckes, which he prepared against his isolates of NβV, reacting down to 1/128 (Fig. 5).

3. Purification procedure.

In the isolation procedure it was found unwise to use large quantities of infected larvae in a single purification procedure. One kilogram of larvae after trituration resulted in 2-3 litres of homogenate, and the final return was 0.5 to 1.0 gram of virus (0.05 - 0.1% wt. virus/wt. larvae). When 100 grams of larvae were used, from an initial homogenate volume of 1.2 litres, 300 - 500 mg of virus were obtained (0.3 - 0.5% wt. virus/wt. larvae). This indicates that a high weight larvae/volume of homogenate ratio resulted in a large amount of virus being lost, most likely in the first low speed spin, due to coprecipitation with the large quantities of suspended host materials. This virus loss could largely be obviated by starting with less infected material and by re-extracting virus from the insect material.

4. The molecular weight of the virus, determined by sedimentation equilibrium.

The complete results of the sedimentation equilibrium experiments are given in Table 4. A plot of ln c versus r^2 is shown in Fig. 6. The shape of this plot is typical of what was obtained with all the experiments. There was no deviation from linearity between values of ln c 2.0 to 5.0, indicating the relative absence of any concentration effect or heterogeneity in the system. The absence of concentration dependance is supported by the data in Fig. 7. Here it is obvious

Figure 5. Reaction of identity between Juckes's NβV antiserum and NβV used in this work. A dilution of antiserum (central well) is surrounded by 6 dilutions of virus (outside wells).

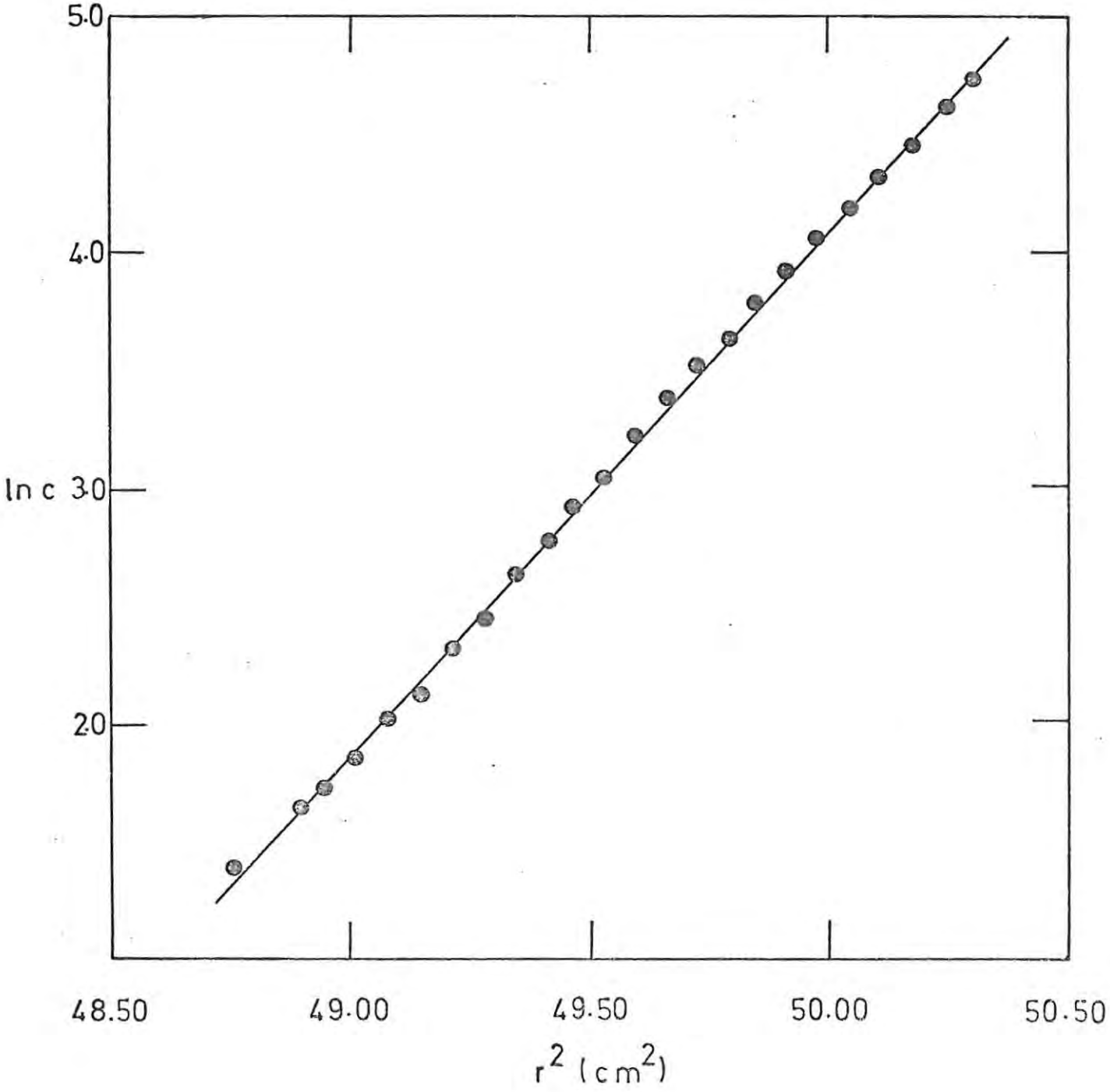


Figure 6. A plot of ln c vs. r^2 obtained in a sedimentation equilibrium experiment of NβV using the Yphantis "high speed" method.

TABLE 4.

Data obtained from sedimentation equilibrium experiments of N.capensis β virus. These results were obtained from three experiments using the Yphantis 6 channel centrepiece.

Run No.	Speed (rev/min)	Temp.	Conc. (mg/ml)	Slope (lnc vs. r^2)	M.W. ($\times 10^6$)
1 ▲	1509	16.9	0.6	2.64	17.9
1 ▲	"	"	1.2	2.55	17.3
1 ▲	"	"	1.8	2.57	17.4
2 ●	1411	16.6	0.6	2.23	17.3
2 ●	"	"	1.2	2.11	16.4
2 ●	"	"	1.8	2.32	18.0
3 □	1608	10.9	0.6	2.95	17.3
3 □	"	"	1.2	3.02	17.7
3 □	"	"	1.8	3.05	17.9

Average molecular weight = 17.4×10^6

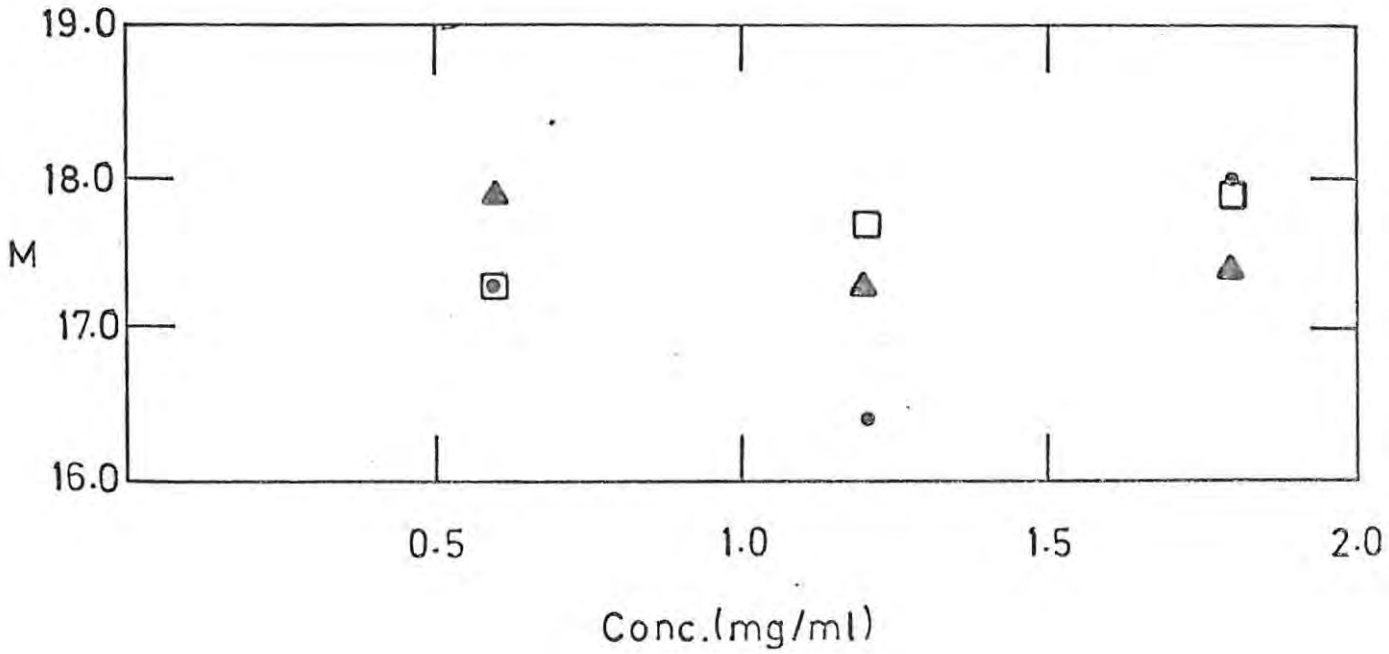


Figure 7. Values of the molecular weight of NβV (in millions) obtained in sedimentation equilibrium experiments as a function of concentration. The random distribution indicates the absence of concentration dependance.

that the distribution of apparent molecular weight against virus concentration is random.

The average molecular weight from 9 determinations was $17.4 \times 10^6 \pm 1.0 \times 10^6$, an error of 5.7%. This value is 6.7% higher than the value of 16.3×10^6 obtained by Polson et al (1970) by particle counting and 9.4% higher than the value these workers obtained by substituting into the Svedberg equation the S_{20W} value of 210S (Tripconey, 1970) and a D_{20W} value of 1.08×10^{-7} cm^2/sec. The difference between the value obtained in this work by the Yphantis "high speed" sedimentation equilibrium method and the values reported by Polson et al (1970) cannot be regarded as significant, if one bears in mind the discrepancies normally seen in the reported molecular weights of viruses. Juckes (personal communication) states that a repeat of the diffusion coefficient of N βV gave a value which substituted into the Svedberg equation with the other constants given by Polson et al (1970) gave a molecular weight for the virus in the region of 17×10^6. One can thus regard the molecular weight of N βV as being 16 - 17 million.

The "high speed" sedimentation equilibrium method is useful due to the fact that it can be used for molecular weight determinations of large viruses. Bancroft and Freifelder (1970) used this method to estimate the molecular weights of the T coliphages which have molecular weights in the region of 50×10^6. The low rotational speeds required for such determinations (800 rev/min) are constant due to electronic speed control devices in analytical ultracentrifuges.

CHAPTER IV

THE PROTEIN COMPONENT OF N.CAPENSIS β VIRUS.

A. MATERIALS AND METHODS

1. Production of soluble virus protein.

As discussed in Chapter II A, attempts to produce soluble virus protein by the methods described by Knight (1963), or modifications of them, were unsuccessful (Struthers, unpublished results). The formic acid method of Miki and Knight (1965) did however yield soluble protein. The procedure involved the addition of 1 volume of virus sample in distilled water at a concentration of 5-10 mg/ml to 2 volumes of 98% formic acid. The virus opalescence disappeared within 5 minutes and the solution was then incubated at 37°C for 24 hours. The solution was then dialysed for 24 hours at 4°C against 2 litres of distilled water, and the ultraviolet absorption spectra obtained at this stage were typical of a protein (see Results). Initially various buffers were tested to determine which would maintain the protein in a soluble form. The protein precipitated during dialysis against 0.1M-barbital, pH 2.6-6.5 and in neutral phosphate. 0.1M-acetate buffers of pH 3.6 to 4.5 did not cause precipitation, but acetate buffers of pH 5.0 and above did. Dialysis against repeated changes of distilled water also caused the protein to precipitate. It was thus concluded that the precipitation of the protein was due to its isoelectric point being reached and that this lay between pH 4.5 and 5.0.

A number of experiments were conducted to determine if the protein could withstand precipitation and resuspension. To one volume of protein in 0.1M-acetate, pH 4.0, was added 1 volume of saturated ammonium sulphate or 10% PEG. The protein was allowed to precipitate for 30 minutes at 4°C and was then collected by centrifugation at 10,000

rev/min for 10 minutes. The pellet was resuspended in 0.1M-acetate, pH 4.0, and dialysed for 24 hours against the same buffer. During this period the protein did not resolubilise.

Experiments were also conducted in an attempt to resolubilise the protein above its isoelectric point. To the protein in 0.1M-acetate, pH 4.0, 1M sodium acetate was added dropwise until a dense precipitate developed. This precipitate was collected by low speed centrifugation and could be redissolved in 8M urea or in a very high pH solution e.g. 0.2N NaOH. Upon dialysis into 0.05M- tris HCl, pH 7.0 to 9.0, only the protein solubilised in NaOH remained in solution, while that prepared using urea precipitated. The ultraviolet absorption spectrum of the NaOH solubilised protein in 0.05M- tris HCl, pH 7.0, was identical to that of the protein in 0.1M-acetate, pH 4.0.

The procedure described by Miki and Knight (1965) was used to show that formic acid treatment did not break peptide bonds. After formic acid treatment, 0.5 ml of the protein preparation was degassed for 2 hours at 37°C to remove the formic acid, and 20 µl aliquots were applied to 20 x 15 x 0.05 cm Silica gel G plates. The spots were allowed to dry and the plates were developed by ascending chromatography in n-butanol/glacial acetic acid/water; 8:2:2 (v/v/v) (Brenner et al, 1965). When the solvent front had moved about 15 cm, the plates were removed, dried, and sprayed with ninhydrin reagent (0.3 grams ninhydrin in 100 ml n-butanol plus 3 ml glacial acetic acid). The plates were incubated at 110°C for 10 minutes and examined for the presence of the dark blue ninhydrin positive spots.

2. Studies of the protein in 0.1M-acetate, pH 4.0.

(a) Polyacrylamide gel electrophoresis. 5-8% polyacrylamide gels were prepared by dissolving 1.25 to 2 grams of Cyanogum 41 in 0.1M-acetate, pH 4.0, and making the volume up to 25 ml. This solution was degassed under vacuum for 15 minutes, warmed to 25°C, and then 0.1 ml

DMAP and 0.25 ml 5% ammonium persulphate were added. This solution was well mixed and poured to a depth of 6 cm into 8.0 x 0.6 cm glass tubes, which had been sealed with parafilm. A flat starting surface was prepared by overlayering the solution with a few drops of 0.1M-acetate, pH 4.0, before polymerisation. Polymerisation occurred in 20-30 minutes at 37°C. The tray buffer was 0.02M-acetate, pH 4.0. Glycerol was added to the protein solution to a final concentration of 5% and 20-200 µg of protein were applied to a gel in a loading volume of 20-40 µl. Electrophoresis was at 10 mA/tube for 1-3 hours. After electrophoresis, gels were removed from the tubes by forcing water between the wall of the glass and the gel by means of a syringe. Staining was performed in 7% acetic acid containing 0.25% Amido Black 10B, for 6 hours. Excess stain was removed by soaking the gels in 7% acetic acid, or by destaining electrophoretically in 7% acetic acid in a destaining tank (Ward, 1970).

(b) Analytical ultracentrifugation. The sedimentation characteristics of the protein in 0.1M-acetate, pH 4.0, and in 0.05M-tris HCl, pH 7.0 were determined as discussed in Chapter III B2. The sedimentation coefficient of the protein in 0.1M-acetate, pH 4.0, was calculated by the same procedure. The sedimentation speed was 55,000 rev/min and the temperature was always kept below 5°C.

Due to the high degree of aggregation of the protein in 0.05M-tris HCl, pH 7.0 (see Results), and its inability to react with whole virus antiserum in gel precipitin tests, the protein was regarded as being denatured and was not studied further.

The apparent molecular weight of the formic acid-prepared protein was determined by the "high speed" sedimentation equilibrium technique of Yphantis (1964). This method employed the standard 12 mm double sector cell in the AN-D rotor. 0.1 ml of freshly prepared protein at a concentration of 1-2 mg/ml in 0.1M-acetate, pH 4.0 was placed in the

right hand sector of the cell (cell top towards filler), and 0.1 ml of 0.1M-acetate, pH 4.0 in the left hand sector. Care was taken to fill the sectors with identical volumes to ensure the menisci matched, to allow for redistribution during centrifugation of solvent molecules.

It was originally thought that this protein should have a molecular weight of 60,000 (see Results), and thus a speed of 20,000 rev/min was selected (Yphantis, 1964). However, at 20,000 rev/min the protein sedimented, as judged by the fringe pattern, and only at 10,000 rev/min did the pattern indicate equilibrium conditions. The rotor was centrifuged at this speed for 24 hours, and photographs were taken and read as described in Chapter III C.

3. Molecular weight determinations by SDS-PAGE.

SDS-PAGE was used to examine the homogeneity and size of the virus protein. The method of Shapiro et al (1967) was employed. 5% acrylamide gels were prepared by dissolving 1.25 grams of Cyanogum 41 in 0.1M-phosphate, pH 7.2 and then adjusting the volume to 25 ml. The solution was degassed for 30 minutes, poured into a beaker and to it were added 0.25 ml 10% SDS, 0.1 ml DMAP and 0.25 ml of 5% ammonium persulphate. The solution was well mixed and poured to a height of 8 cm into glass tubes with an internal diameter of 4 mm. In some experiments, 2-Me was incorporated into the gels at a concentration of 0.1% (Lesnaw and Reichman, 1970). Gels polymerised in 15-20 minutes. Viral protein was prepared by boiling the virus for 1-5 minutes in 0.01M-phosphate, pH 7.2, 1% SDS and 1% 2-Me. Glycerol was added to a final concentration of 20% (Michelin-Lausarot et al, 1970). In some cases dithiothreitol (DTT) was utilised as the reducing agent, at concentrations specified by Schafer et al, 1971). The marker proteins, ovalbumin dimer (Shapiro et al, 1967) ovalbumin monomer and cytochrome c were prepared in the same way.

A volume of 10-20 µl, containing 1 mg/ml of each protein, was

applied to a gel and electrophoresed at 10 mA/tube for 2-3 hours. The tray buffer was 0.01M-phosphate, pH 7.2, containing 0.1% SDS. After electrophoresis, the gels were removed from the tubes and stained for 12 hours in 0.25% Coomassie Brilliant Blue, in methanol-water-glacial acetic acid, 9:9:2. The gels were destained in 7% acetic acid, either electrophoretically or by soaking for 2-3 days. The position of the bands was then recorded using a Vitatron densitometer, and their distance of migration from the cathode end was measured off the densitometer traces. The distance of migration of the standard proteins were plotted against the log. of their molecular weights, yielding a straight line. From this calibration, the distance of migration of the virus protein enabled the calculation of its molecular weight.

The molecular weight of the virus protein was also calculated by modifying the method of Shapiro et al (1967) along the lines of the procedure develped by Hedrick and Smith (1968) (see van Regenmortel et al, 1972). In this experiment, β lactoglobin was also incorporated as a marker. This protein, as well as the virus protein and other three standards (see above), were electrophoresed together in SDS-containing gels of 5, 6½, 8 and 10% gel concentration. At each gel concentration the ratio of migration (R_m) of the proteins was calculated.

$$R_m = \frac{\text{distance of protein migration}}{\text{distance of cytochrome c migration.}}$$

Plots of log R_m versus gel concentration yielded a series of non parallel straight lines, which extrapolated to a common point at about 1% gel concentration. When the slopes of these lines were plotted against molecular weight, a calibration curve was obtained, enabling the estimation of the virus protein molecular weight.

B. RESULTS AND DISCUSSION.

The ultraviolet absorption spectrum of the protein prepared by the formic acid method is shown in Fig. 8. The spectrum is typical of a protein, with a 280/260 ratio of 1.4. This ratio gives a percent nucleic acid of 0.75, using the method of Warburg and Christian (1946). The 280/260 ratio of the protein in 0.05M- tris HCl, pH 7.0 was identical, indicating the absence of nucleic acid, as this protein had been concentrated by isoelectric precipitation, thus any RNA present would have been discarded with the supernatant when the precipitate was collected by centrifugation.

When the protein was analysed by thin layer chromatography, only one dark blue ninhydrin positive spot was present at the origin, indicating that the formic acid method had not degraded the protein subunits into smaller peptides. Miki and Knight (1965) found that formic acid did not split the peptide bonds of TMV protein.

The polyacrylamide gel electrophoretic pattern of the protein in 0.1M-acetate, pH 4.0 is shown in Fig. 9. When quantities of 200-50 µg of protein were applied to a gel, there was always one distinct band, but with the higher protein concentrations, there was evidence of trailing material, which had not however migrated as a discrete band. These results are similar to those obtained by Hill and Shepherd (1972b), who obtained a similar pattern with Turnip mosaic virus (TuMV), although these aggregates disappeared after carboxymethylation.

Electrophoresis of the protein in a 10% SDS-containing gel (Fig. 10), showed the presence of a single protein component. From these results it was concluded that the N βV capsid contains only one protein species.

The sedimentation profiles of the protein in 0.1M-acetate, pH 4.0, and in 0.05M- tris HCl, pH 7.0, are shown in Fig. 11 (a) and (b) respectively. Fig. 11 (b) shows the high degree of aggregation of the protein preparation in 0.05M- tris HCl, pH 7.0. The protein preparation in 0.1M-acetate,

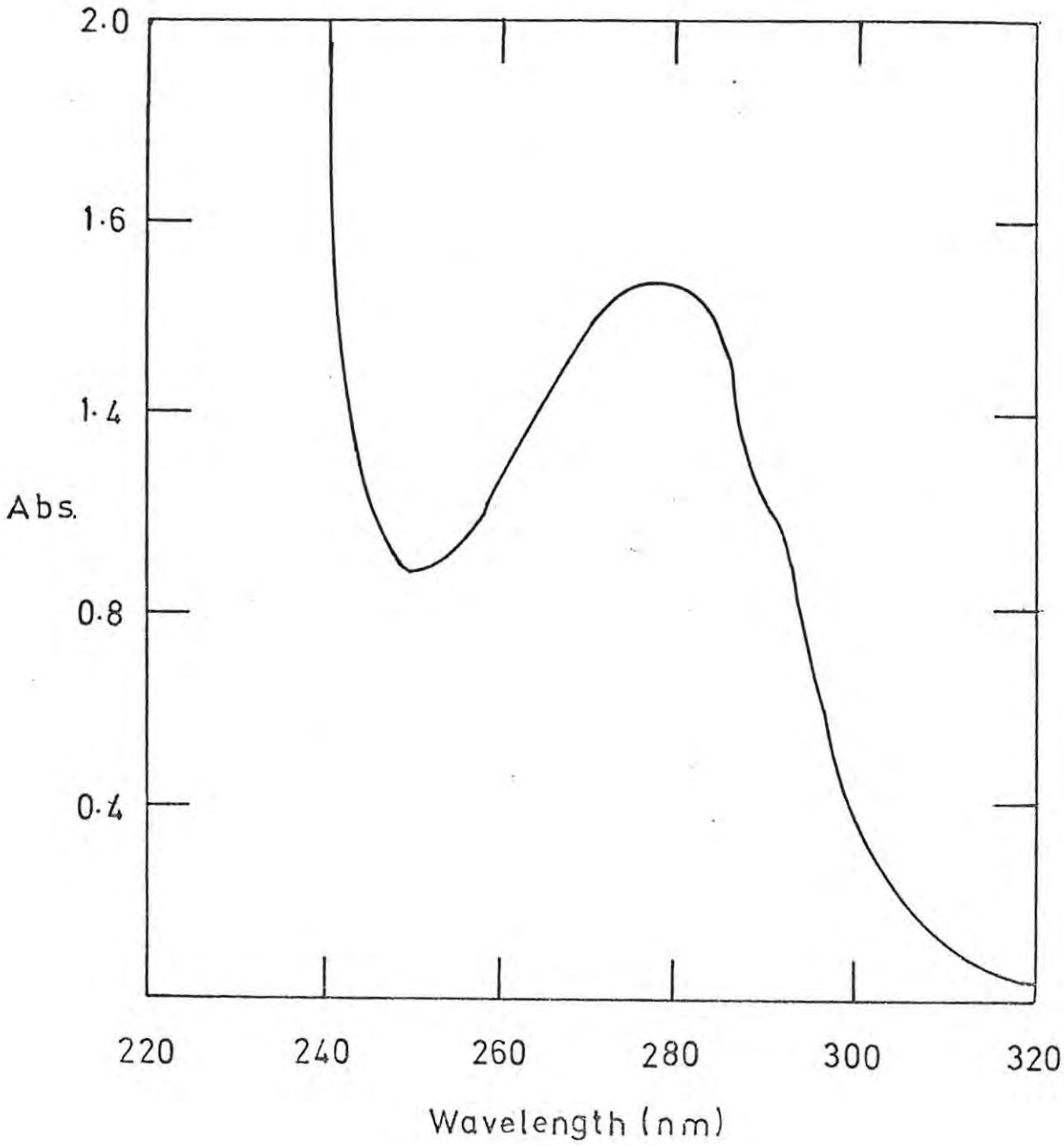


Figure 8. Ultraviolet absorption spectrum of NβV protein.

Figure 9. Electrophoretic pattern in a 5% polyacrylamide gel of NβV protein in 0.1M-acetate, pH 4.0, prepared by formic acid. Migration is from top to bottom, towards the cathode. 200 µg of protein were applied to this gel.

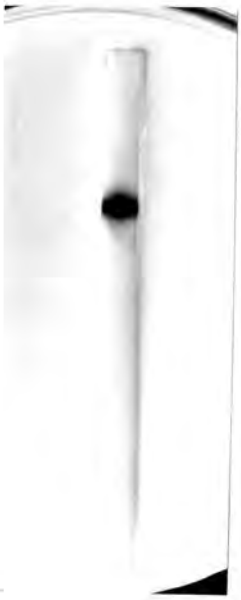
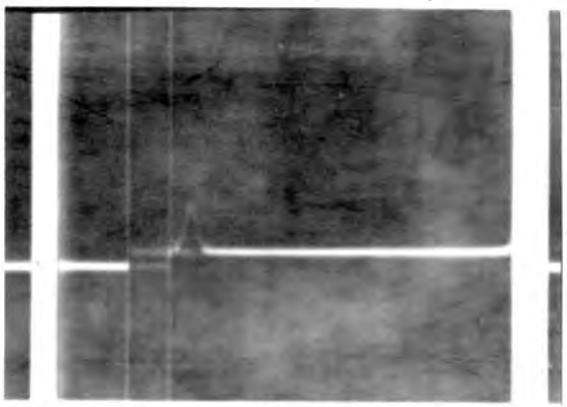


Figure 10. Electrophoretic pattern of NβV protein in a 10% SDS - containing polyacrylamide gel. Migration is from top to bottom.

(a)



(b)

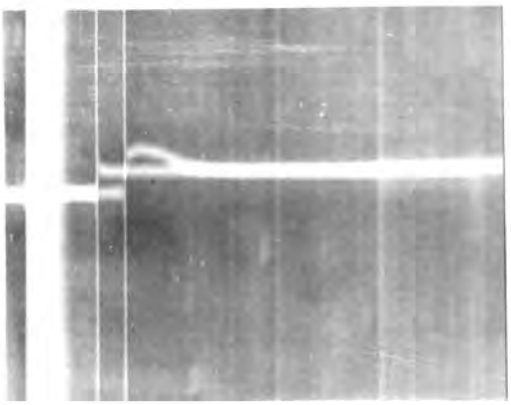


Figure 11. Sedimentation velocity patterns of NβV protein. Photographs were taken 4 minutes after a speed of 55,324 rev/min had been reached. Temperature was 4°C. (a) Protein in 0.1M - acetate, pH 4.0 and (b) protein in 0.05M - tris HCl, pH 7.0.

pH 4.0 (Fig. 11 (a)), however, showed a more symmetrical peak, which during sedimentation broadened rapidly and asymmetrically, again indicating solute heterogeneity. A plot of the log. of the distance of the peak from the axis of rotation versus time is shown in Fig. 12 (see Chapter III B2). This yielded a straight line, the slope of which when substituted into equations (i) and (ii) yielded an S_{20W} value of 14S (at 5 mg/ml). The results of a typical sedimentation equilibrium experiment are shown in Fig. 13. The plot of ln c versus r^2 showed upward concavity, which indicates solute heterogeneity (Yphantis, 1964). The arbitrarily chosen lines A-A' and B-B', when substituted into equation gave values of 228 and 510 x 10^3 respectively. This aggregation accounts for the high S_{20W} value obtained. In another experiment, the maximum weight obtained was 700 x 10^3. There is thus a high degree of aggregation exhibited by the protein in 0.1M-acetate, pH 4.0. This aggregation is difficult to reconcile with the polyacrylamide gel results of the same protein, which gave a single major band. No estimation of the molecular weight of this protein using PAGE was attempted, so it is not known whether this band represents a monomer or a multimer of the protein. It may be that the aggregates were dissociating under the conditions of electrophoresis.

The position of the protein relative to the markers using SDS-PAGE is shown in Fig. 14. Here it can be seen that the virus protein lies between the ovalbumin monomer and dimer. A densitometer scan of such a gel is shown in Fig. 15. The plot of log molecular weight of the standards against the distance of migration gave a calibration curve (Fig. 16), from which the molecular weight of the virus protein was calculated. The average of 12 determinations using both DTT and 2-Me as reducing agents was 62,000 $\pm$ 1000. This molecular weight was not altered with increased time of boiling of the virus during protein solubilisation.

The details of the Hedrick and Smith (1968) adaption of the

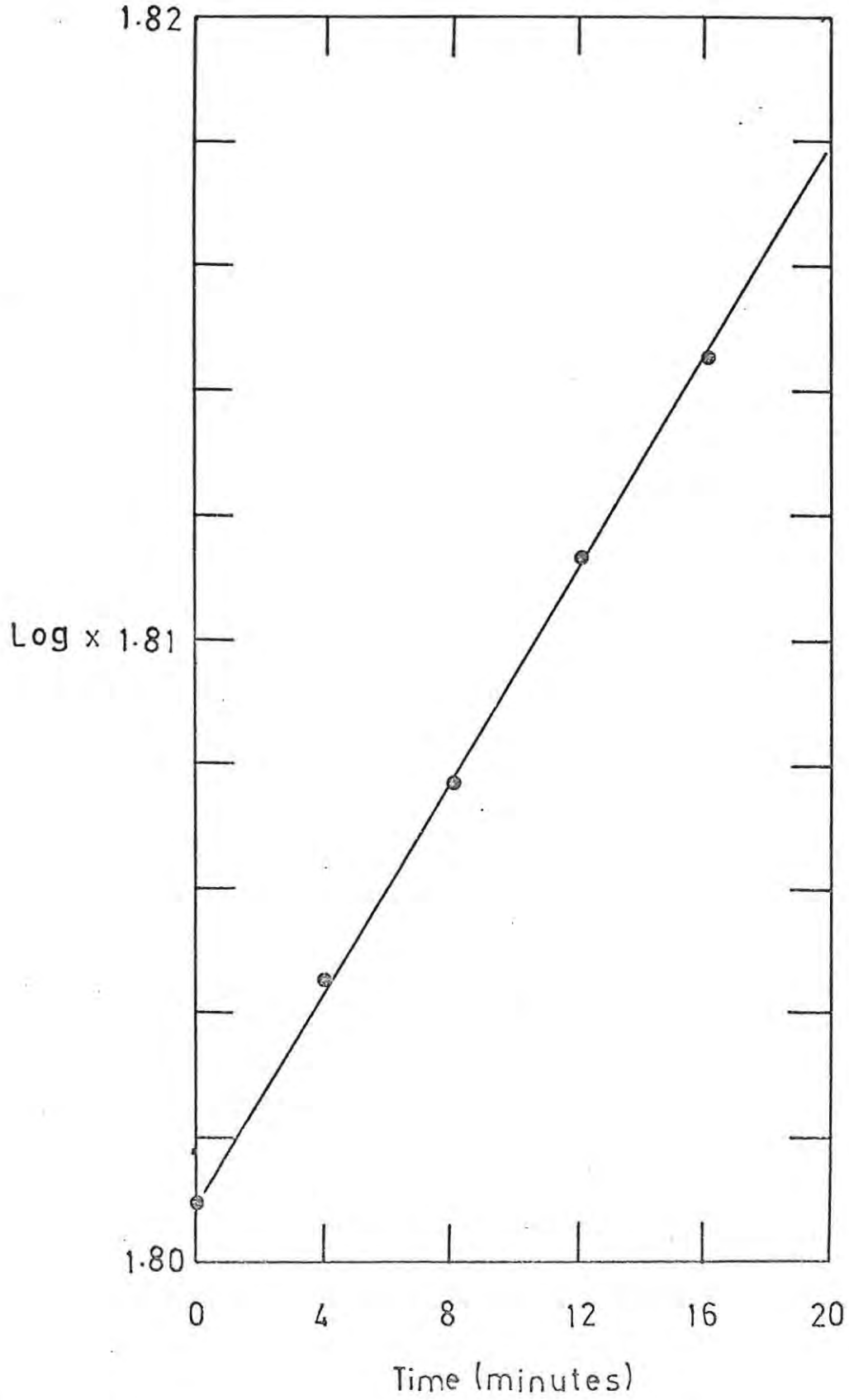


Figure 12. Sedimentation velocity experiment of NβV protein in 0.1M - acetate, pH 4.0. Pictures were taken at 4 minute intervals. Centrifugation was at 55,324 rev/min at 4°C.

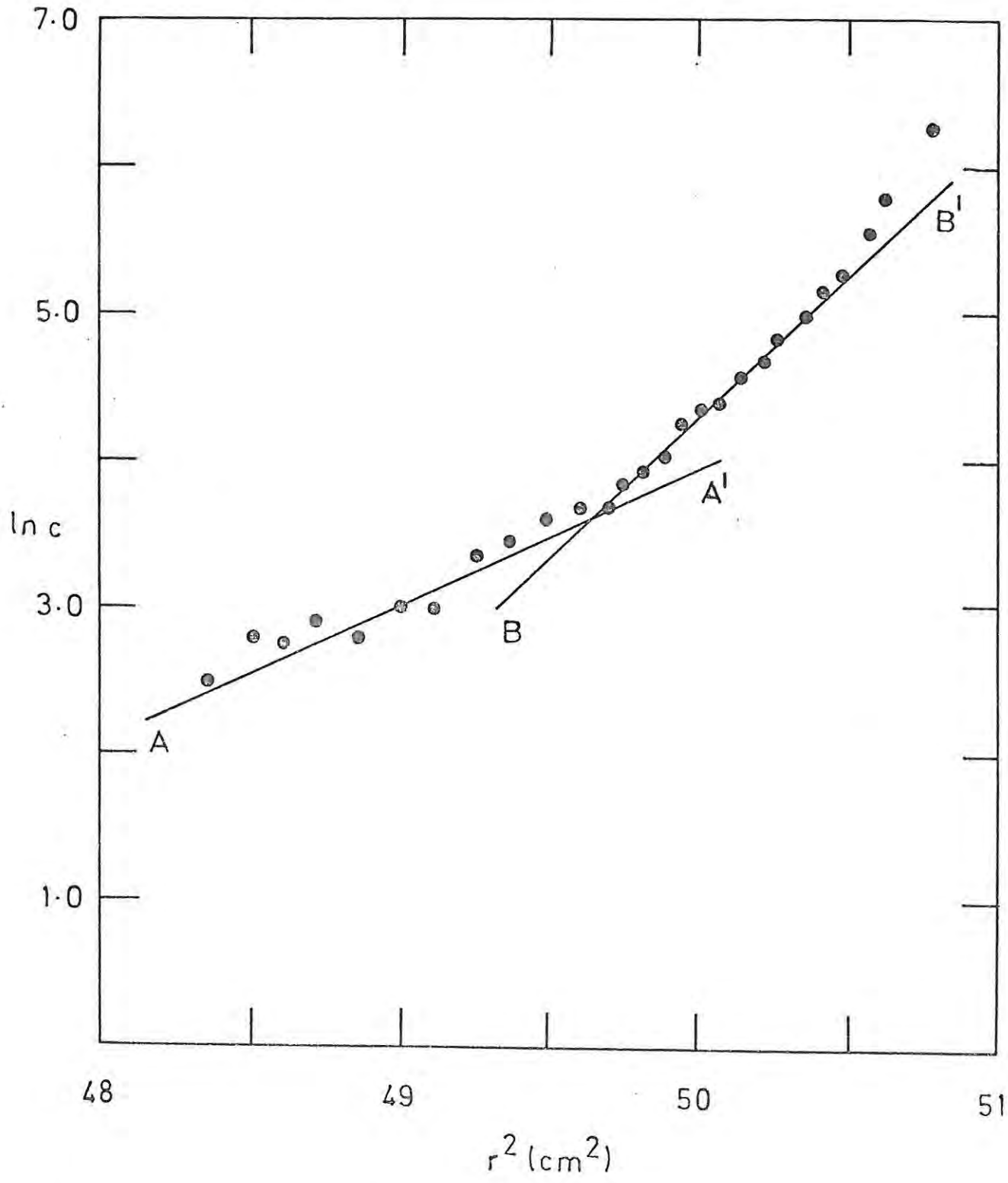


Figure 13. A plot of ln c versus r^2 in a sedimentation equilibrium experiment using the Yphantis "high speed" method with NβV protein in 0.1M - acetate, pH. 4.0. Lines A-A' and B-B' were arbitrarily chosen.

Figure 14. Electrophoretic pattern of NβV protein in SDS-PAG, in relation to the standards. Proteins are from top to bottom (left gel) NβV protein and cytochrome c and (right gel) ovalbumin dimer, NβV protein, ovalbumin monomer and cytochrome c. Migration is from top to bottom.

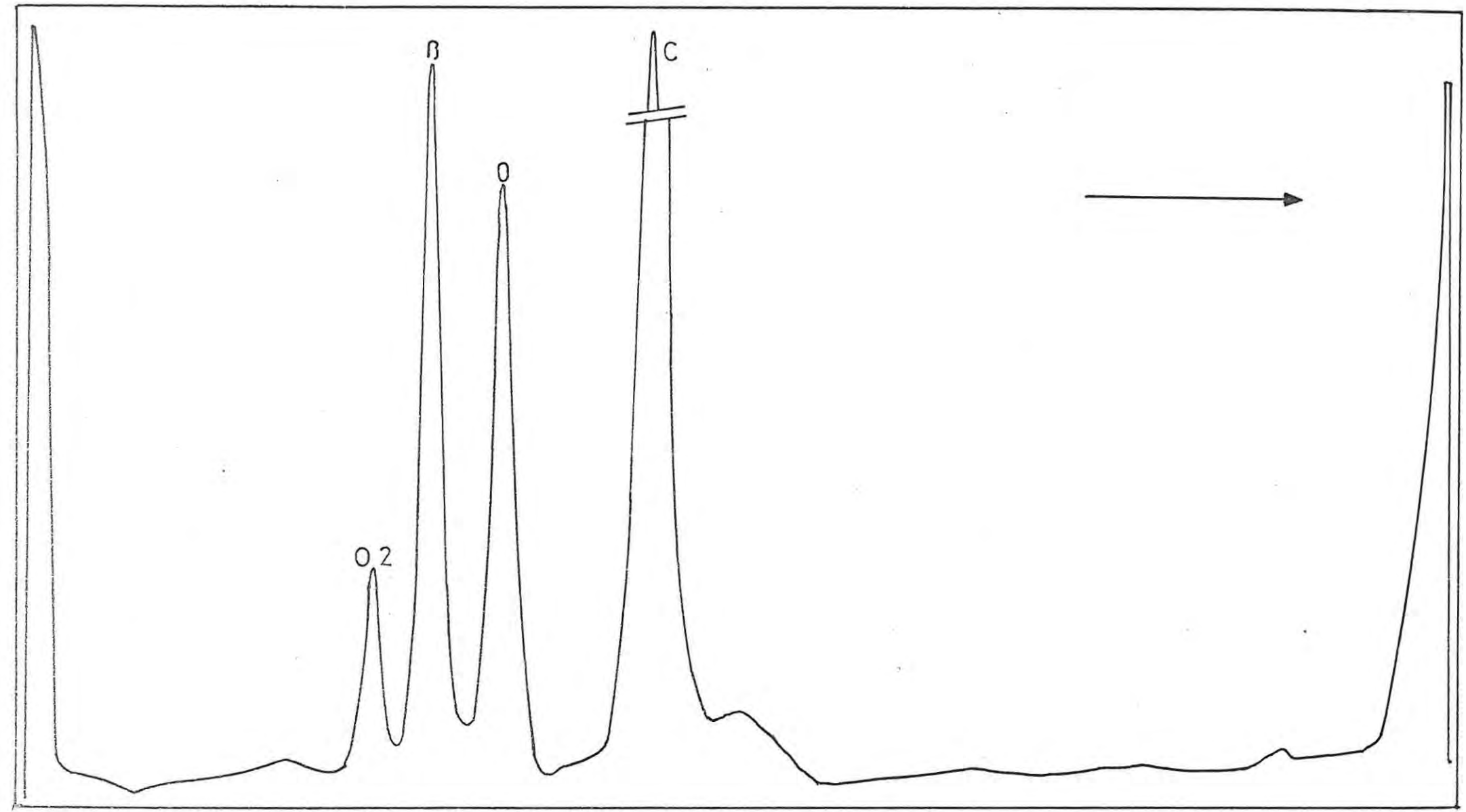


Figure 15. Densitometer trace of a gel similar to the right hand gel of Figure 14. 02 = ovalbumin dimer, β = NβV protein, 0 = ovalbumin monomer and C = cytochrome c. Arrow indicates the direction of migration. The wavelength of the densitometer was 600 nm.

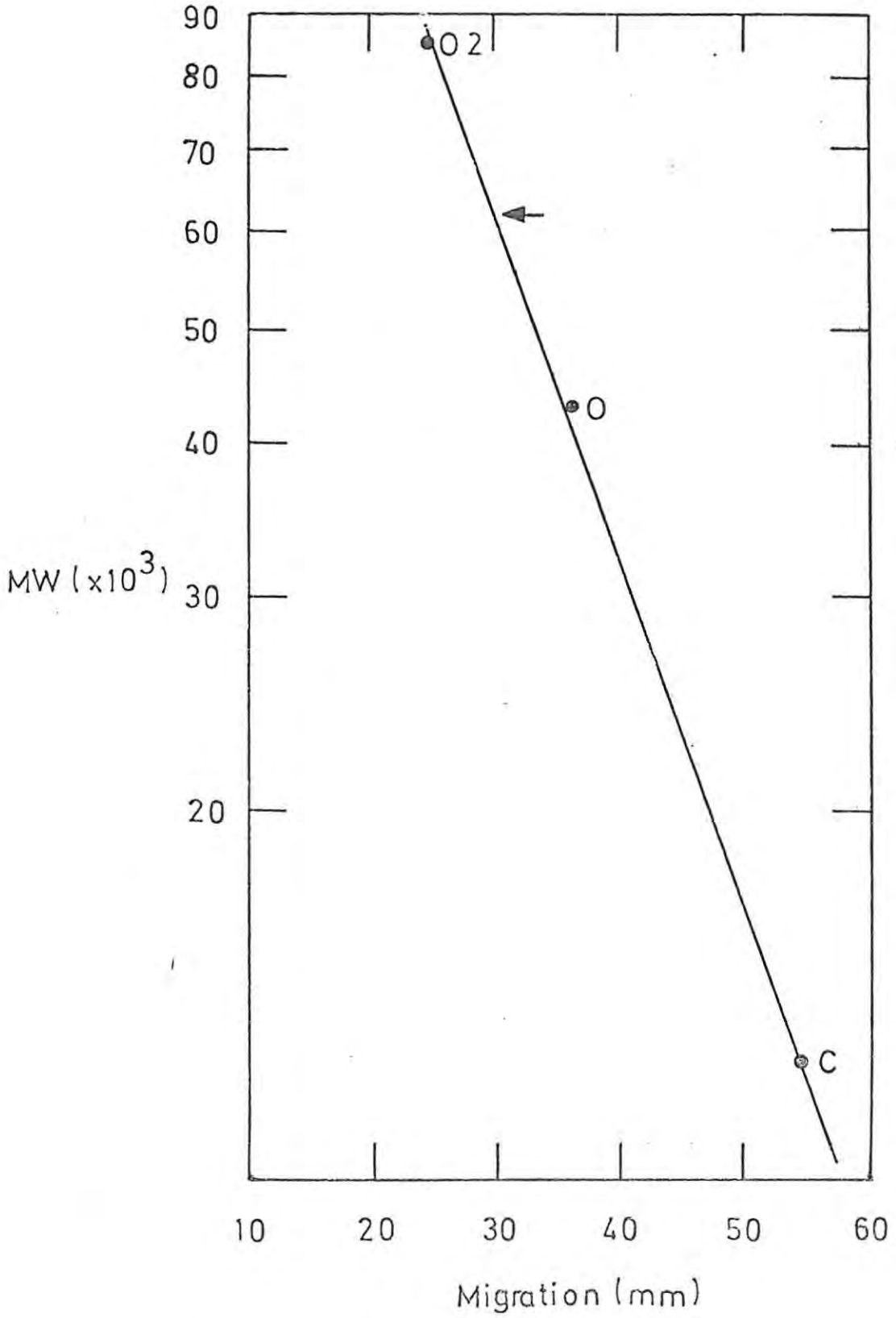


Figure 16. A calibration curve obtained from a trace of a gel similar to Figure 15. Arrow indicates the position of the NβV protein. Standards are identified as in Figure 15.

SDS-PAGE method are given in Table 5. The plot of log. R_m versus gel concentration yielded straight lines for each protein (Fig. 17), and from the calibration curve (Fig. 18), the NβV protein gave a molecular weight of 60,000. The molecular weight of the NβV protein was thus established to be between 60 and 62 x 10^3. This value is unusually high for the monomer of a small RNA virus, but on no occasion was there evidence of a smaller component being present.

The technique of SDS-PAGE was found in this laboratory to have a number of shortcomings. Firstly, accurate determinations of molecular weight were obtained only when the standards and unknown protein were electrophoresed in the same gel. This supports the findings of Verwoerd (personal communication), who found that determinations with standards and unknown lead to erroneous results when done in separate gels. The NβV protein molecular weight ranged from 30 to 70 x 10^3 when separate gels were used. However when all the proteins were included in one gel, and the calibration curve constructed from the standards in this gel, the NβV protein consistently gave a value of 62,000.

Secondly it was found that the proteins had to be boiled and used immediately. If not, results were obtained giving numerous extra bands. For example ovalbumin kept in boiling solution for 3 hours at room temperature and then boiled for 1 minute, gave rise to numerous bands, their position indicative of multimers, besides the normal monomer and dimer.

TABLE 5.

Slopes of relative mobility (R_m) versus gel concentration in SDS-PAGE.

Protein	Numbers in Figs 17 & 18	Slope	Mol Wt	Reference
β Lactoglobulin	1	0.22	18,400	a
Ovalbumin	2	0.38	43,000	b
Ovalbumin dimer	4	0.64	86,000	b
N βV protein	3	0.48		

a = Weber and Osborne (1969)

b = Shapiro et al (1967)

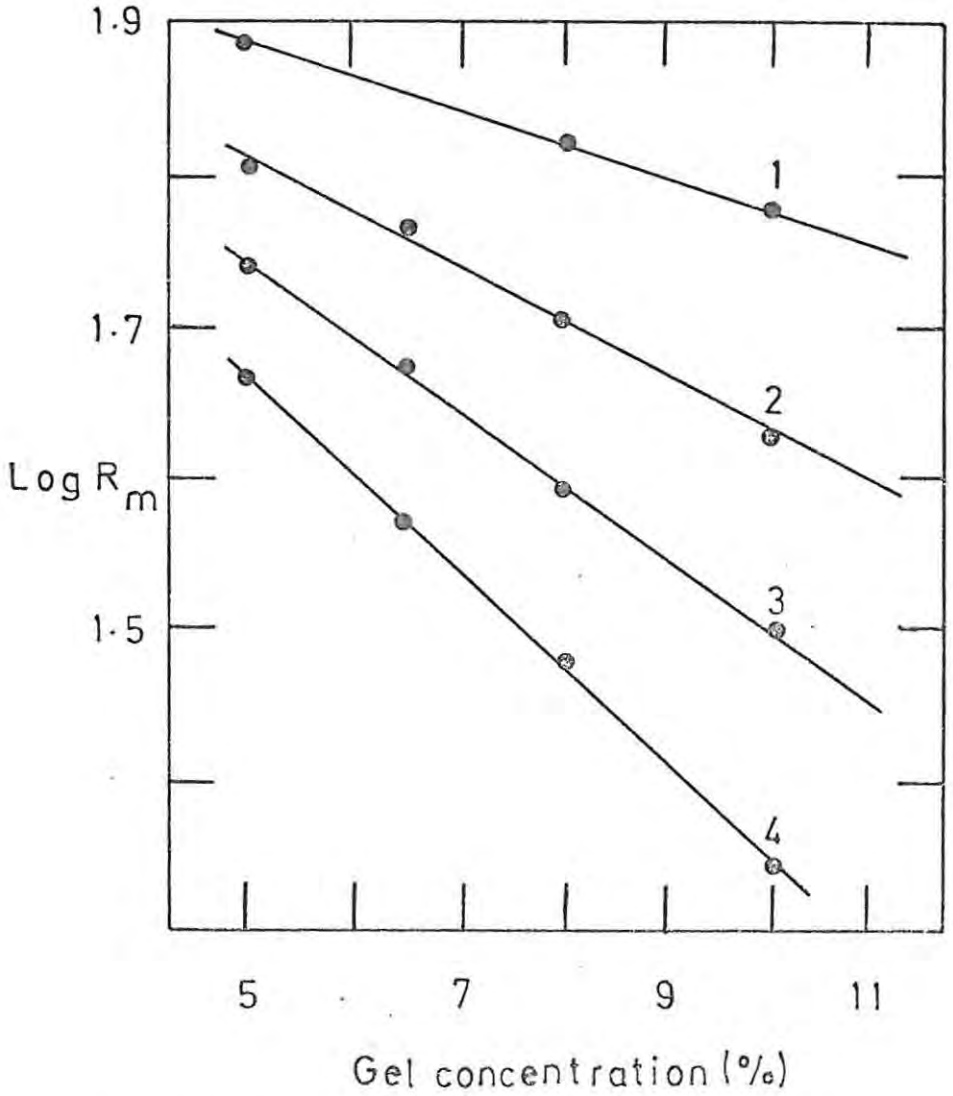


Figure 17. Relative mobilities of four proteins in polyacrylamide gels as a function of gel concentration. The negative slopes have been used to obtain the calibration curve in Figure 18. 7 = β lactoglobulin, 2 = ovalbumin monomer, 3 = N βV protein and 4 = ovalbumin dimer.

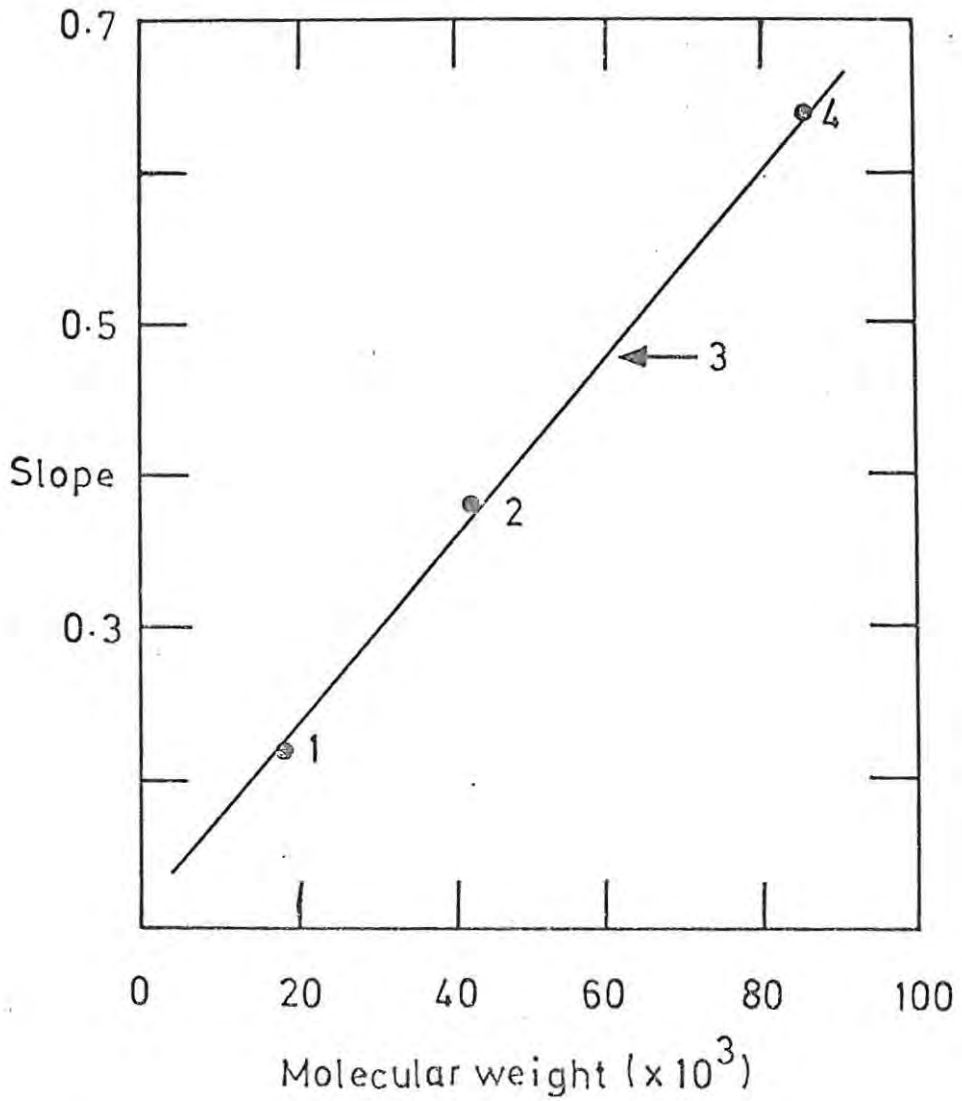


Figure 18. Slopes obtained in Figure 17 plotted as a function of molecular weight. The proteins are identified as numbers as in Figure 17. NβV protein molecular weight = 60,000.

CHAPTER V

THE NUCLEIC ACID COMPONENT OF N. CAPENSIS β VIRUS.

A. MATERIALS.

1. Identification of the nucleic acid.

Identification of the nucleic acid as being DNA or RNA was performed using whole virus or isolated nucleic acid. To 1 ml of one of the above solutions was added 4 ml diphenylamine reagent, and after mixing, this was placed in a boiling water bath for 10 minutes. A positive DNA reaction is identified by a blue colouration (Burton, 1956). For the orcinol reaction (Dische, 1955), 1 ml of virus or nucleic acid preparation was added to a solution of 0.2 ml alcohol orcinol and 2 ml acid orcinol, and boiled for 20 minutes. A green colouration indicates RNA.

2. Phosphorus analysis.

The phosphorus content of the virus was determined using the method of Allen (1940). The phosphorus content of TMV was determined simultaneously in order to act as a control. Virus samples were prepared by subjecting them to zone electrophoresis (Polson and Russel, 1967), after which the virus was concentrated by high speed centrifugation, and then resuspended in distilled water to give a concentration of 5-7 mg/ml. The virus was then dialysed against distilled water for 48 hours at $4^{\circ}C$, the water being changed every 12 hours. The NβV preparation was divided into 16 1 ml portions, and the TMV preparation into 6 1 ml portions. Half of the portions were used for dry weight determinations and half for phosphorus analysis.

Dry weights were determined in 2 x 4 cm glass crucibles with lids. These crucibles were thoroughly cleaned with chromic acid, then washed in water, heated at $160^{\circ}C$ for 1 hour, cooled to room temperature in a

dessicator and then weighed. Heating and weighing were repeated until a constant weight was obtained. Dry weights of virus aliquots were then obtained by the same heating procedure.

For the determination of phosphorus, a standard curve of weight phosphorus versus absorbance at 600 nm was obtained. A solution containing 0.4839 grams KH_2PO_4/litre (100 µg P/ml) was prepared and dilutions were made to give a range of 0-100 µg/ml. To 1 ml aliquots of these dilutions was added 2 ml 60% perchloric acid, and the mixture was then washed into a 25 ml measuring cylinder with distilled water. 1 ml 8.3% w/v ammonium molybdate was added, followed, after mixing, by 2 ml amidol reagent. The solution was made up to 25 ml with distilled water and mixed well. After 5 minutes the absorbance was read at 600 nm. The readings were plotted against µg phosphorus and the standard curve obtained. (Fig. 12) Virus samples were treated in the same way, except that after addition of perchloric acid, they were incubated at 160°C for 1 hour, after which time the samples were brown in colour. The samples were cleared by the addition of 0.1 ml 30% H_2O_2, and a further 15 minutes incubation at 160°C, before addition of the final two reagents.

3. Isolation of nucleic acid.

(a) Method 1. Initially the nucleic acid of NβV was isolated according to the phenol method described by Knight (1963). Virus at a concentration of 5 mg/ml or less was added to an equal volume of water saturated phenol containing 0.01% hydroxyquinolene. Mixing was performed at 33 rev/min on a turntable sloping at an angle of 30°. After 15 minutes the emulsion was broken by centrifugation at 2,500 rev/min on an MSE bench centrifuge. The dense cloudy aqueous layer was removed and had to be subjected to 4-5 further phenol extractions to produce a clear aqueous layer. Residual phenol was then removed by ether extractions.

(b) Method 2. SDS/hot phenol extraction. This procedure was developed due to the low yields and poor quality of the RNA obtained using method 1 (see Results). This entire procedure was performed at 60°C. The nucleoprotein was first incubated at this temperature for 30 minutes in the presence of 1% SDS, 1% 2-Me and 0.05% polyvinyl sulphate (PVS), during which period the virus opalescence disappeared. Protein was then removed by the addition of one volume of water saturated phenol, and, following emulsification, the phases were allowed to separate. The clear aqueous layer was removed, and subjected to ether extractions at room temperatures.

Concentrations of RNA were determined spectrophotometrically using an extinction coefficient ($E^{1\%}_{260\ nm}$) of 250.

For gel electrophoresis studies, the RNA preparations were cooled to 4°C and to this was added 2 volumes of ice cold 95% ethanol and a few drops of 3M-sodium acetate, pH 5.0. The precipitate was collected by low speed centrifugation after 4 hours at -12°C. The pellet of RNA was dried in a dessicator *in vacuo* and then resuspended in the electrophoretic buffer to give a concentration of 1-2 mg/ml. For base ratio studies the pellet obtained after centrifugation was resuspended in 10 ml distilled water, dialysed for 24 hours against distilled water and lypholysed. The RNA was then resuspended in 0.4N-NaOH to give a concentration of 20 mg/ml. The RNA used in density gradient ultracentrifugation and Tm experiments was diluted to 50 µg/ml and dialysed against the appropriate buffer for 24 hours at 4°C.

In the isolation of NβV RNA, method 2 was superior to method 1, regarding both quality and quantity of the RNA. The yield using method 1 varied between 20 and 30% of the total theoretical yield, while with method 2, yields of between 65 and 85% of the total yield were common. The ultraviolet absorption spectra of the RNA obtained by both methods were identical (Fig. 20). The 260/280 ratio varied between 2.0 and 2.1,

but method 1 always yielded a more degraded product than method 2 (see Results).

The RNA of TMV and TYMV were isolated by method 2, or, as in the case of BMV, by the phenol-detergent method described by Knight (1963).

All glassware and buffers used in these isolation procedures were autoclaved.

4. Determination of RNA base ratios.

The method described by Knight (1963) using paper electrophoresis was employed. The RNA was dissolved in 0.4N-NaOH to give a concentration of 20 mg/ml, and hydrolysed for 24 hours at 37°C. Whatman 3M paper was cut to fit the Shandon electrophoresis tank, soaked in electrophoresis buffer (0.05M-formate, pH 3.5:6.4 grams ammonium formate and 10.3 grams 88% formic acid in 6 litres), and excess buffer removed by pressing the paper between sheets of Whatman No. I. 15-20 µl quantities of hydrolysate were applied in 2 cm transverse streaks at the cathode end of the paper. Electrophoresis was performed at 160 volts (10 mA) for 9-12 hours at room temperature. The paper was then removed, dried, and ultraviolet absorbing zones were detected by means of an ultraviolet lamp. The zones were cut out and eluted for 12 hours in 5 ml portions of 0.01N-HCl, together with the appropriate blanks. The absorbancies of the nucleotides were measured in a spectrophotometer, and their concentrations determined using their extinction coefficients (Wyatt, 1955).

5. Melting profile of the RNA.

The melting profile of the NβV RNA in 0.1M-phosphate, pH 7.2 was determined using the SP800 A ultraviolet spectrophotometer equipped with the SP 876 Temperature Programme Controller and heating stage. The wavelength was constant at 260 nm and the samples were heated at a rate of 1°C/minute. The increase in absorbance was expressed as the ratio:

$$\frac{\text{Absorbance at } T^{\circ}C}{\text{Absorbance at } 30^{\circ}C}$$

(Murant et al, 1972).

6. Sucrose density gradient centrifugation.

The sedimentation patterns of NβV RNA were examined on linear-log sucrose density gradients (Brakke and Van Pelt, 1970a), following the procedure of Gumpf (1971) for the Spinco SW 50.1 rotor, or, of Gonsalves and Shepherd (1972) for the Spinco SW 25.1 rotor. After centrifugation, ultraviolet absorbing zones were detected as described in Chapter III B2.

The sedimentation coefficient of the RNA was determined using TMV RNA as a standard. In these experiments, one tube of the rotor contained TMV RNA, the other two NβV RNA. The depths of the peaks were measured on the recorded patterns as the distance from the maximum ordinate of the peaks to the centre of the initial sampling zone. The point corresponding to the log. of the depth of TMV RNA and the log. of 31.1 (S_{20W} of TMV RNA = 31.15; Brakke and Van Pelt, 1970b) was positioned on a graph and a line of slope 0.8 was drawn through it. The log. of the S value of NβV was read from this line. BMV RNA was also examined on linear-log gradients to indicate the resolution of the technique.

7. Polyacrylamide gel electrophoresis.

Viral nucleic acid was examined for heterogeneity by electrophoresis in 2.5% polyacrylamide gels. Gels were prepared using the buffer systems of Loening (1967) or Peacock and Dingman (1967). Electrophoresis was performed in 8 x 0.8 cm glass tubes. Cyanogum 41 was dissolved in 25 ml buffer to give a concentration of 5% and degassed for 30 minutes. 10 ml were withdrawn, 0.1 ml 5% ammonium persulphate and 0.03 ml DMAP were added and then 1 ml quantities of this mixture were poured into the tubes which had been sealed at the bottom with Parafilm. This 5% acrylamide was allowed to gel for 15 minutes and functioned as a plug to support the weaker 2.5% gel above it. The remaining 15 ml of Cyanogum solution was added to 15 ml buffer, degassed for a further

15 minutes, and to the 30 ml volume was added 0.3 ml 5% ammonium persulphate and 0.1 ml DMAP. After mixing, the solution was poured into the glass tubes to a height of 6 cm. A few drops of buffer were overlayed on each gel to produce a flat starting surface. The gels polymerised in 15-20 minutes. The tank buffer was diluted to produce a voltage of 100-150 V at 10 mA/tube. 20-50 µl aliquots of RNA preparations at 1-2 mg/ml and containing 10% glycerol or sucrose were applied to each gel, and electrophoresed at room temperature or 4°C for 1-2 hours. After this period, the gels were removed by squirting water between the walls of the tube and the gel, and stained according to the method of Koenig (1971).

B. RESULTS AND DISCUSSION.

1. Identification of the nucleic acid.

Preparations of virus and isolated nucleic acid gave positive orcinol and negative diphenylamine reactions, identifying the nucleic acid as RNA.

2. Phosphorus analysis and base ratio determinations.

The standard curve obtained for the phosphorus analysis is shown in Fig. 19. The results obtained with the virus preparations are given in Table 6. The phosphorus content of 0.46% obtained with TMV agrees well with the value of 0.45% reported by Knight and Woody (1958).

The results of the base ratio determination are given in Table 7. From this ratio the phosphorus content of the RNA was calculated to be 9.42%, using the known phosphorus contents of the individual nucleotides. Using the value of 16.3×10^6 for the molecular weight of the virus (Polson et al, 1970), and the calculated viral phosphorus content of 1.03%, the molecular weight of the RNA was calculated to be 1.8×10^6, and the nucleic acid content of the virus to be 11%.

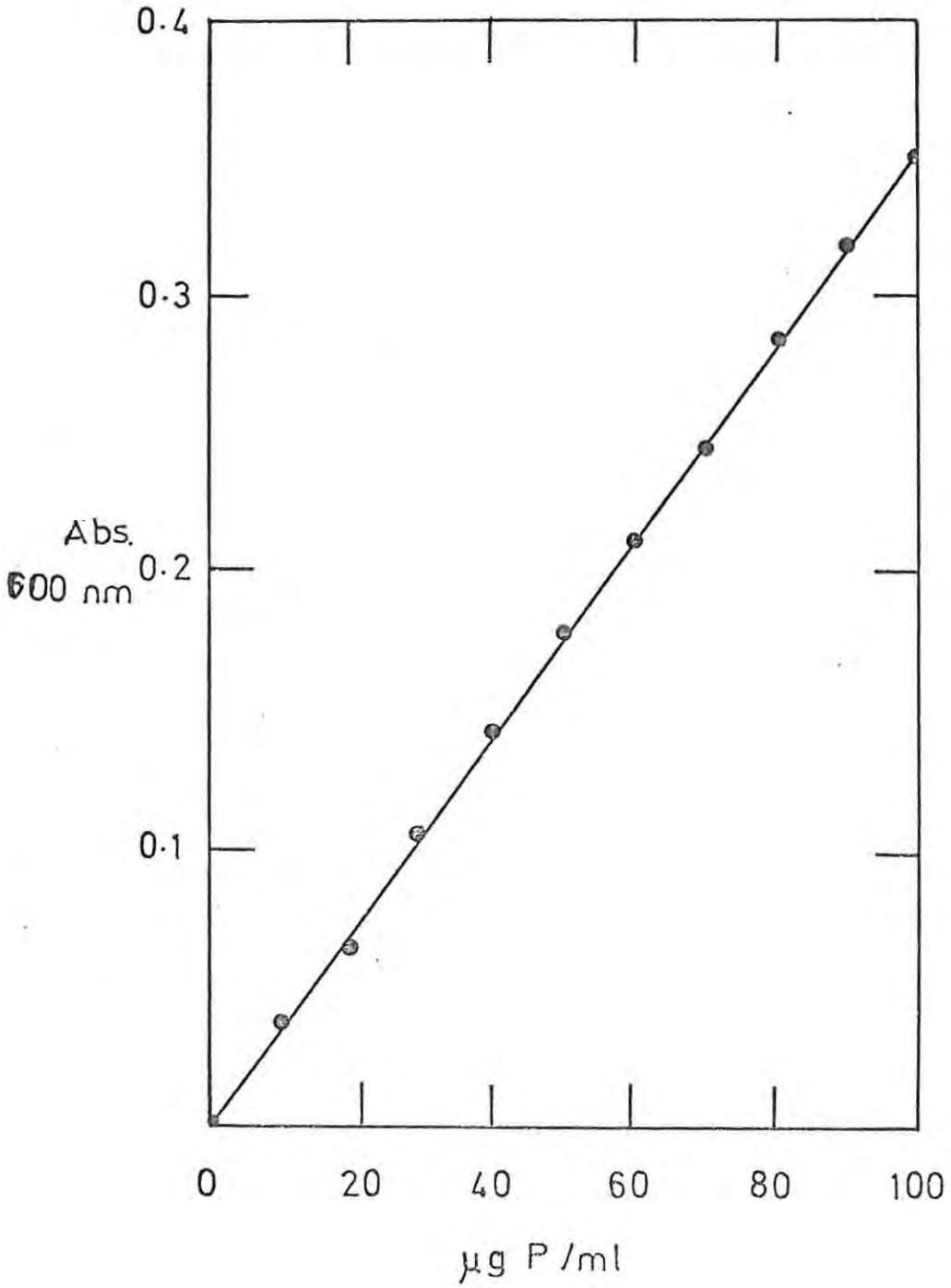


Figure 19. Plot of µg phosphorus/ml versus absorbance at 600 nm, obtained by treating dilutions of a standard solution of KH_2PO_4 according to the method of Allen (1940).

TABLE 6.

Details of phosphorus analysis of NβV and TMV.

Virus	NβV	TMV
No. determinations	8	3
Dry wt (µg)	7064 ± 70	5460 ± 60
Abs. 600 nm	0.258 ± 0.008	0.08 ± 0.006
Wt. phosphorus (µg)	73	25
% phosphorus	1.03	0.46

TABLE 7.

Nucleotide ratios obtained with alkaline hydrolysis of *N. capensis* β virus RNA.

Trial	Moles per 100 moles of nucleotides.[a]			
	Ap	Gp	Cp	Up
1	24.3	27.1	25.9	22.7
2	23.8	27.7	26.3	22.1
3	24.1	27.7	25.8	22.2
4	25.0	28.4	24.3	22.2
5	23.1	27.9	25.1	23.8
Average	24.1 ± 1.0	27.8 ± 0.7	25.5 ± 1.2	22.6 ± 1.2

a = Each value given as an average of four replications.

3. Isolation of the nucleic acid.

The ultraviolet absorption spectrum of purified RNA is shown in Fig. 20. This curve is typical of the spectra obtained with RNA preparations isolated from NβV, as well as those obtained with RNA isolated from the standard viruses. Initially, method 1 was used to isolate nucleic acid from the purified virus. The RNA prepared by this method showed a high degree of degradation; when sedimented on linear-log sucrose density gradients, the material sedimented only a short distance from the meniscus (Fig. 21a). It was thought that the addition of phenol could result in the rupture of RNA: protein bonds, thus producing degraded RNA. Method 2 was thus developed in order to solubilise the protein before the addition of phenol. The sedimentation pattern of the RNA prepared by method 2 is shown in Fig. 21b and indicates that the RNA is much less degraded than that prepared by method 1.

The degradation found in the cases of the RNA prepared by method 1 did not appear to be the result of enzymatic degradation, either prior to or after release of the RNA from the virus. Virus incubated at room temperature with 10 μg pancreatic ribonuclease/ml for 30 minutes and then treated following method 2, yielded RNA which sedimented as in Fig. 21b. Furthermore, if the virus was incubated at room temperature in the presence of 1% SDS, 1% 2-Me and 0.05% PVS (conditions which would inhibit or denature nucleases, but which did not cause loss of virus opalescence) and the RNA was extracted following method 1, highly degraded RNA was produced.

The isolation of high molecular weight RNA required the degradation of the virus with SDS, prior to partition of the components with phenol. The procedure developed (method 2) is very similar to one developed by Clark and Lister (1971) who found that, during isolation, extensive degradation of tobacco streak virus RNA occurred using the standard

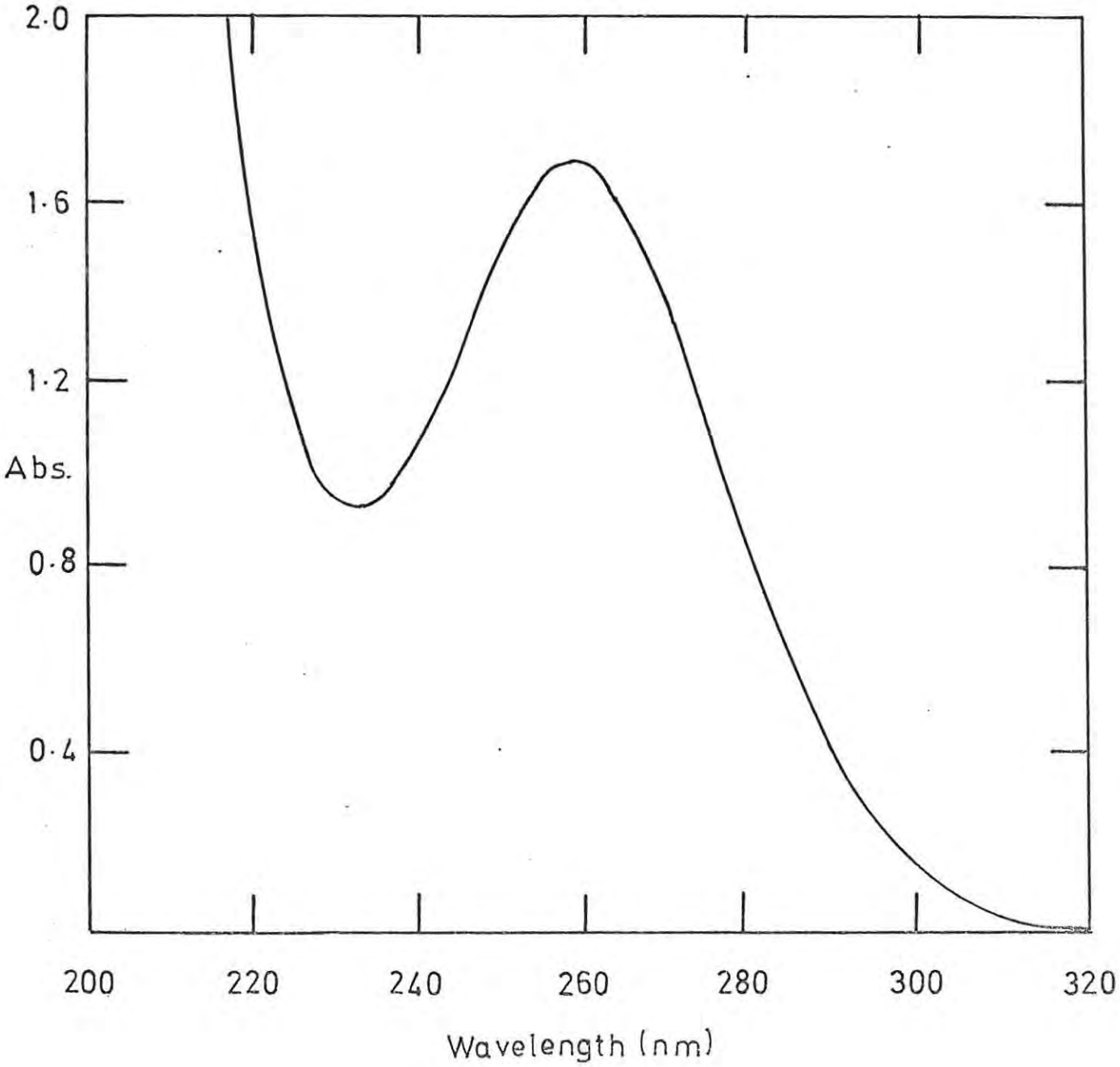


Figure 20. Ultraviolet absorption spectrum of NβV RNA.

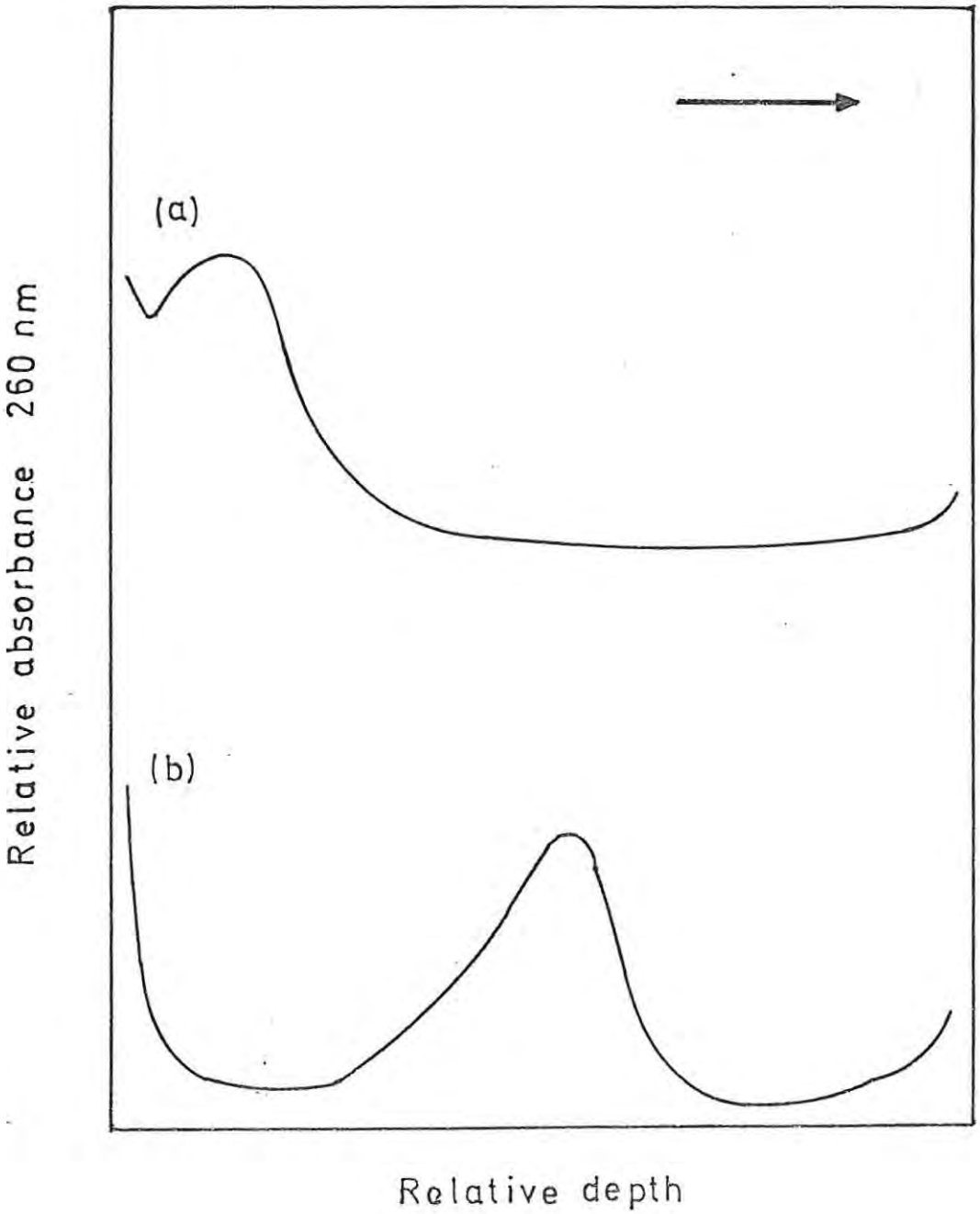


Figure 21. Optical density profiles of NβV RNA after gradient centrifugation. (a) = nucleic acid prepared by method 1 and (b) = nucleic acid prepared by method 2. Centrifugation was for 3.5 hours at 45,000 rev/min in a Spinco SW 50.1 rotor. Arrow indicates direction of sedimentation.

phenol procedure. High molecular weight RNA was obtained only by prior incubation of TSV at 40°C for 30 minutes in the presence of 1% SDS and 3 mg/ml bentonite, prior to partition with phenol. They proposed that this degradation occurring during isolation was due to the protein and RNA being separated too vigorously. Thus, in the case of NβV, it is possible that the stability of the virus is due in part to extensive RNA : protein bonding and that sudden rupture of the viral structure, while these bonds are intact, leads to breakage of the RNA.

It was always necessary when isolating RNA from NβV to use initial virus concentrations of 5 mg/ml or less. At higher concentrations, the yield of RNA obtained with method 2 was substantially lower. When preparations of 10-20 mg/ml were used (see Chapter V B5), the yields of RNA were of the order of 50 µg/ml, instead of the theoretically possible yield of 1-2 mg/ml. This may have been due to co-precipitation of the RNA with the protein upon addition of phenol reagent. When such high concentrations of virus were used, large aggregates of precipitated protein developed, whereas with more dilute virus preparations all the protein appeared to solubilise. H. Huismans (personal communication) states that in the isolation of 2RNA from bluetongue virus, the presence of such a protein precipitate is an indication of low quality RNA being extracted.

4. Sedimentation coefficient and melting profile of NβV RNA.

The sedimentation profile of the RNA of NβV prepared by method 2 is shown in Fig. 21b. Using TMV RNA as a standard, a calibration curve as described by Brakke and Van Pelt (1970b) was constructed, using a value of 31.1S for the sedimentation coefficient of TMV RNA (Brakke and Van Pelt, 1970b). The S of NβV RNA was calculated to be 32.2 ± 1S (average of 4 determinations).

The resolution of this technique was illustrated by the sedimentation

behaviour of BMV RNA on linear-log sucrose density gradients (Fig. 22). BMV RNA clearly separated into the three components of 25, 20 and 15S.

The melting profile of the NβV RNA in 0.1M-phosphate, pH 7.2, showed a gradual increase in absorbance at 260 nm and no marked Tm, typical of IRNA. Increase was maximal at 90°C, and was 20% in the case of RNA prepared by method 2 and 19% for that prepared by method 1. The melting profile of the RNA prepared by method 2 is shown in Fig. 23, and has an approximate Tm of 56°C. This is a fairly high Tm for 1RNA. The Tm of 1RNA is related to the G:C content of the RNA. The G:C content of NβV RNA (mole fraction) is 0.53 (see Table 7). Gesteland and Boedtker (1964) have shown that the RNA of the phage R17 has a Tm of 58°C and a G:C content of 0.52 (mole fraction), whereas TMV and E.coli RNA have Tm values of 51 and 54°C, and G:C ratios of 0.41 and 0.50 respectively.

As the RNA prepared by method 1 showed a characteristic melting profile of 1RNA, the number of helical regions in this RNA must have been similar to that in the less degraded RNA prepared by method 2. Cox (1966) has shown that 1RNA consists of short helical regions within which the number of base pairs is between 4 and 17. It is possible that the breakage occurring in the NβV RNA prepared by method 1 was largely confined to unpaired regions. Thus the short helical regions would have remained intact, giving this RNA a melting profile similar to that of the less degraded preparations.

5. Polyacrylamide gel electrophoresis.

This technique was not developed to a satisfactory extent in this work. Results obtained using the RNA prepared by method 2 were very variable, and on only 2 out of 12 occasions were bands obtained with NβV RNA (Fig. 24). These gels showed a pattern comparable to that obtained with the sedimentation profile on linear-log gradients, indicating a high concentration of high molecular weight material, as

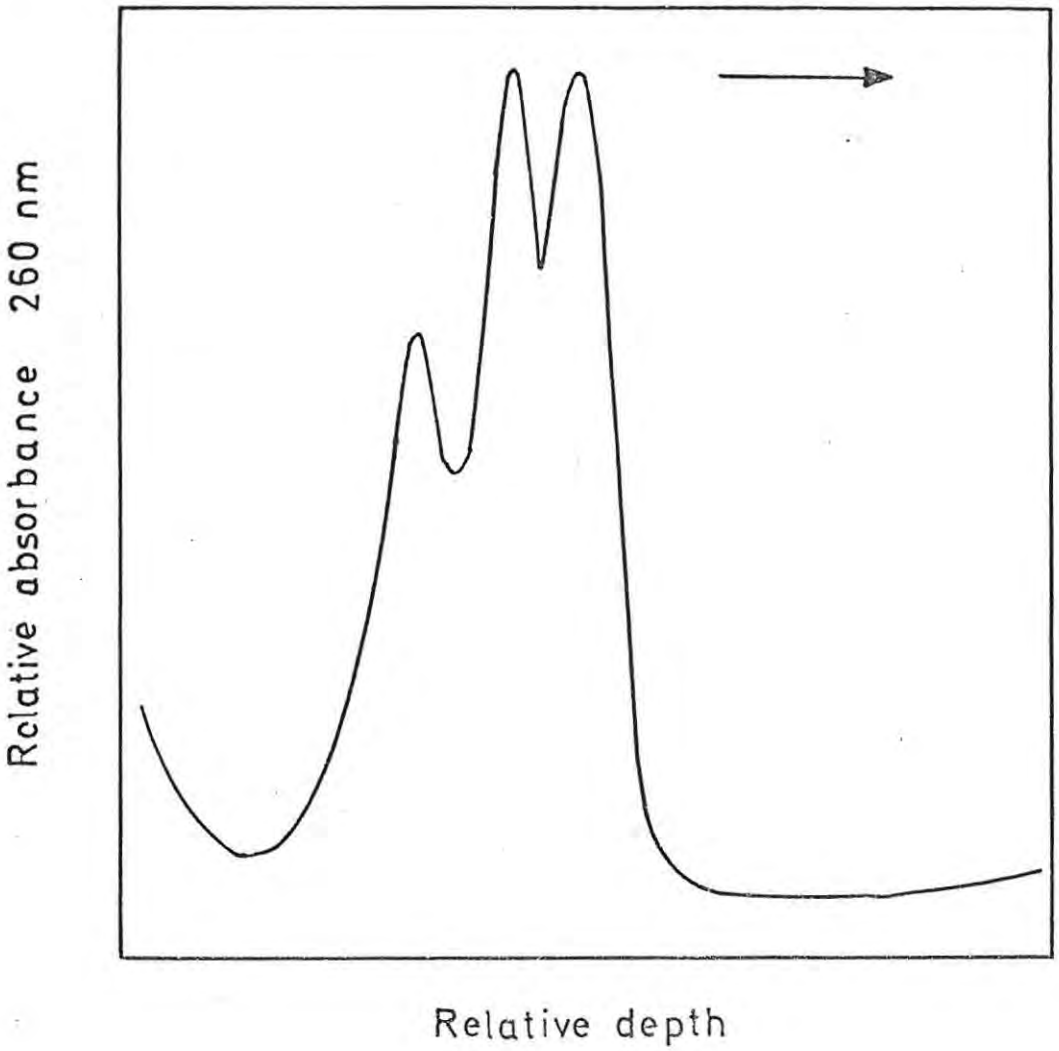


Figure 22. Optical density profile of BMV RNA after gradient centrifugation. Centrifugation was for 15 hours at 23,000 rev/min in a Spinco SW 25.1 rotor. Arrow indicates direction of sedimentation.

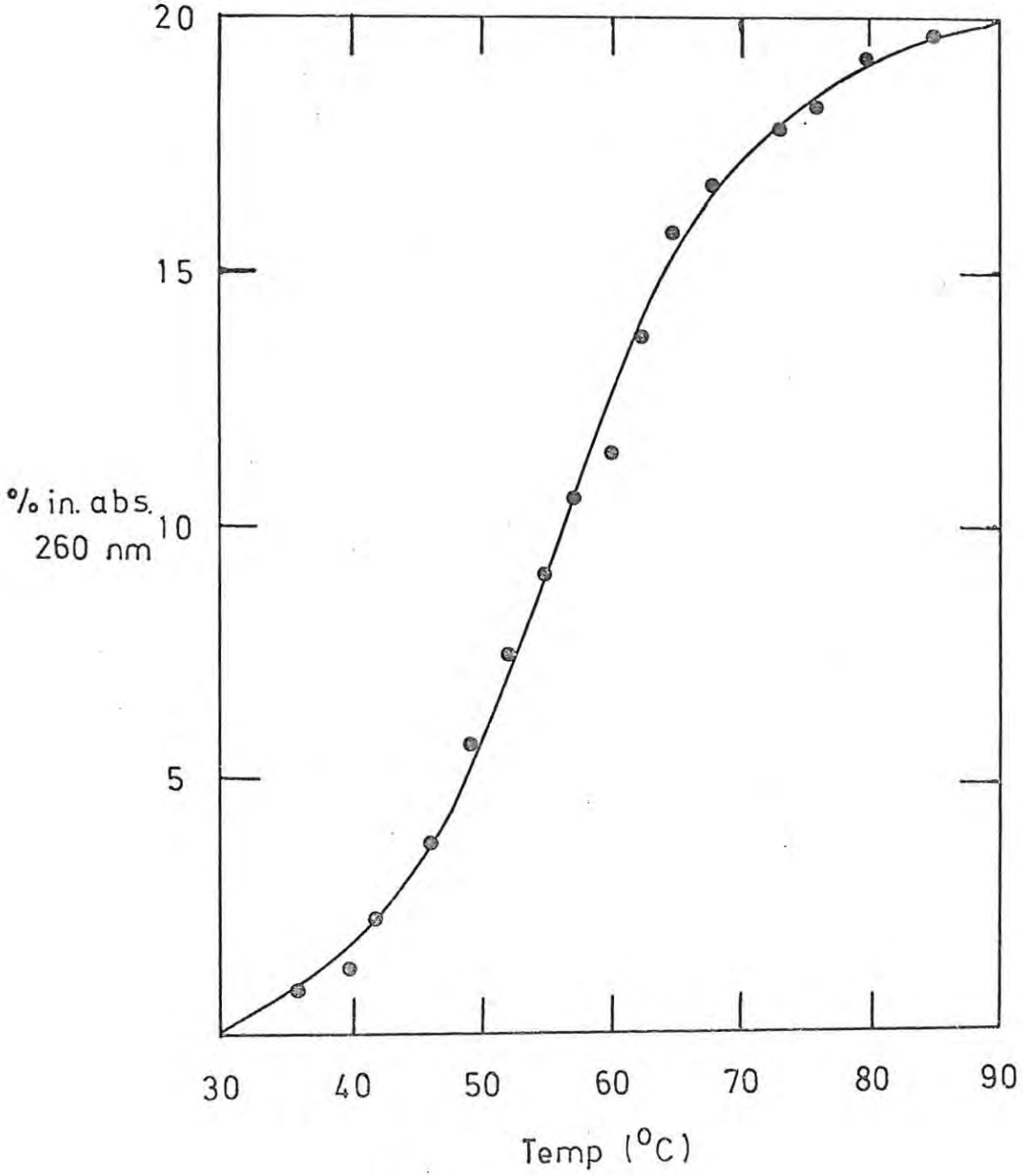


Figure 23. Melting profile of NβV RNA in 0.1M-phosphate, pH 7.2.

well as a certain amount of smaller material, as indicated by the broadness of the band.

Alternative procedures were tested in order to reduce the number of steps required to prepare the RNA. Firstly, RNA was extracted from very concentrated virus as described in Chapter V B3, but this resulted in the isolation of very low concentrations of RNA which would not have been detected by the staining procedures. This isolation procedure worked well with BMV RNA, when the phenol-detergent method (Knight, 1963) was employed, as yields of 0.5 to 2 mg RNA/ml could be obtained from concentrated preparations of this virus. When subjected to PAGE, this RNA not only resolved into its 3 components, but on occasions, the largest component also resolved into its two fractions of molecular weights 1.09×10^6 and 0.99×10^6 (Lane and Kaesberg, 1971) (Fig. 25a); the plot of the log. of molecular weight versus migration also gave a straight line (Fig. 25b). NβV was also dissociated by a similar method to that described by Hull (1972).

Solutions containing 10-20 mg/ml virus, 1% SDS, 1% 2-Me, 1 M urea and 5% glycerol were heated at 50°C for 30 minutes, during which period the opalescence disappeared. When this mixture was applied directly to gels, the only band obtained was that of the SDS artefact (Fig. 26) as described by Adesnik (1971). Although Hull (1972) used this method successfully, the lack of bands obtained in this work with NβV RNA, and that of the standards, supports the conclusion of Loening (1967) that thorough deproteinisation of RNA preparations was required to prevent the RNA from sticking to the gel surface. Pre-electrophoresis of the gels for periods up to 45 minutes, as well as varying the buffer systems, had no effect on the inability to obtain bands.

Although the staining procedure used (Koenig, 1971) appeared to work well with BMV RNA (Fig. 25a), it would have been useful to have

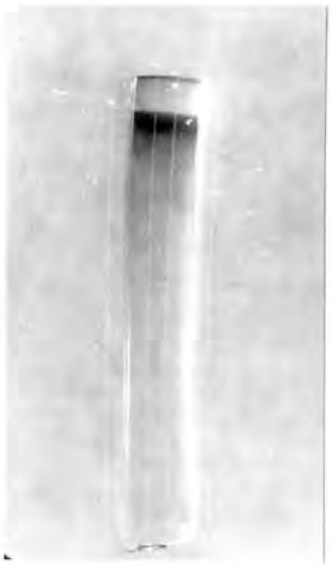


Figure 24. Electrophoretic pattern of NβV RNA prepared by method 2, concentrated by ethanol precipitation and electrophoresed in a 2.5% gel. Migration is from top to bottom.

(a)

(b)

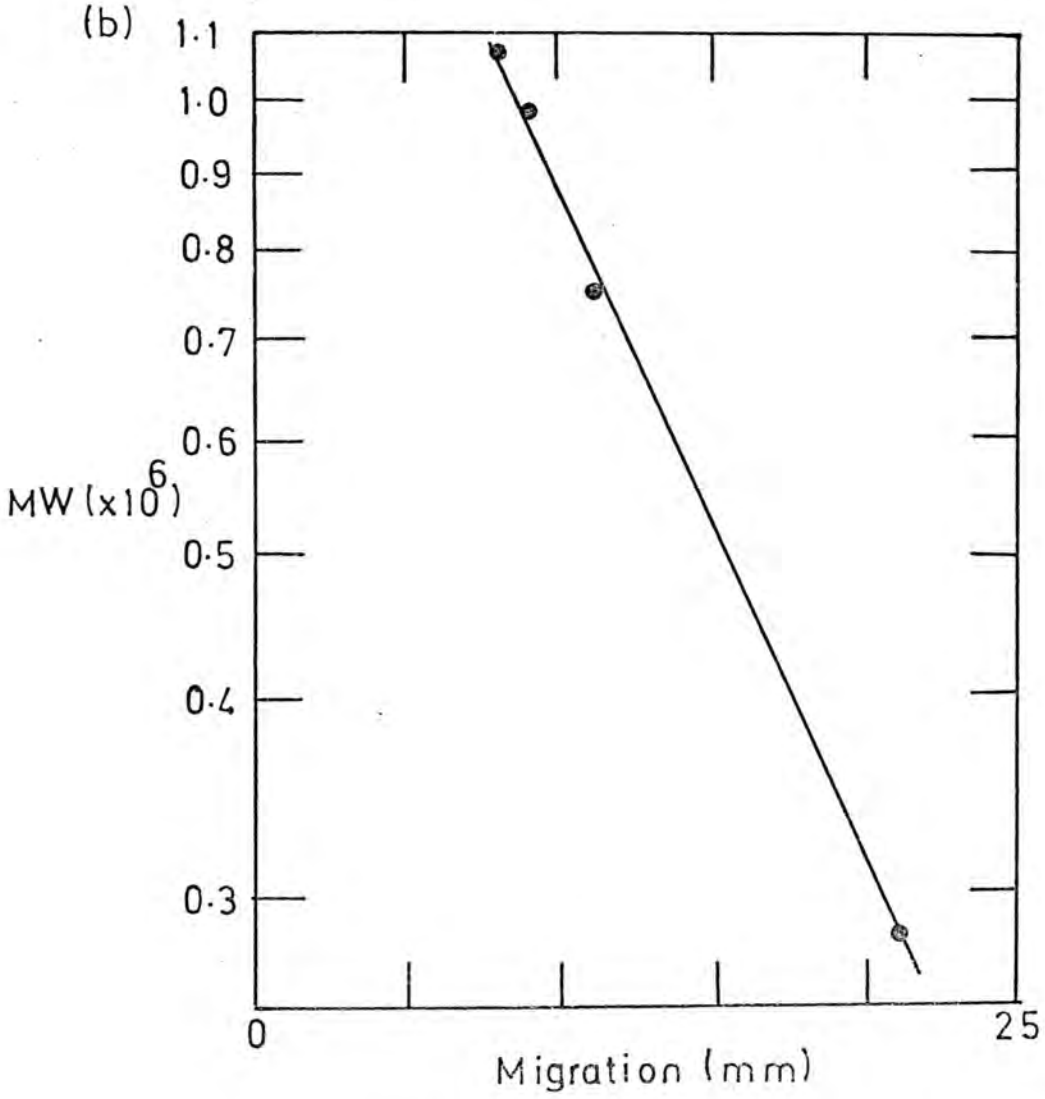


Figure 25. (a) Electrophoretic pattern of BMV RNA after electrophoresis in a 2.5% gel. Migration is from top to bottom. (b) Plot of migration off densitometer trace of above gel versus molecular weight.

Figure 26. SDS-artefact obtained by electrophoresing a sample containing SDS in a gel which does not contain SDS.

had the use of an ultraviolet scanning densitometer, which would have enabled the assay of very small concentrations of RNA in the gels.

This technique thus appears to have many idiosyncrasies which are not found in the PAGE of proteins. The lack of bands with NβV RNA did not appear to be due to degradation, as analysis of RNA preparations which had been applied to gels, by density gradient centrifugation showed the RNA sedimented as in Fig. 21b.

CHAPTER VI

GENERAL DISCUSSION.

From the physico-chemical data obtained, it is possible to propose a structure for NβV. Using the value of 11% for the nucleic acid content, and a value of 61,000 for the molecular weight of the subunit, the number of subunits per virus particle can be calculated. Using the value of 16.3×10^6 for the molecular weight of the virus (Polson et al, 1970), the number of protein subunits is calculated to be 236. The virus molecular weight of 17.4×10^6 obtained in this work by the Yphantis "high speed" sedimentation equilibrium technique would give 253 subunits. However, the number of subunits in a virus particle is always a multiple of 60 (Caspar and Klug, 1962). The nearest multiple of 60 between 236 and 252 is 240. Since the number of subunits is equal to 60T, where T represents the triangulation number, this would assign to NβV a T = 4 structure. This suggested the arrangement of the subunits into 42 capsomeres, assuming hexamer-pentamer clustering, as shown in Fig. 27. This diagram indicates that the subunits could be grouped into 12 pentamers on the vertices and 30 hexamers along the edges of the icosahedron, giving a total of 240 subunits.

Examination of NβV by electron microscopy showed no evidence of large morphological units representing a 42 capsomere structure. The only distinct features were the areas of staining, radiating from apparent 5-fold symmetry axes (Fig. 28), suggesting that these edges were devoid of structural components, and that the subunits were confined to the triangular faces of the icosahedron.

A sample of NβV was sent to Dr J.T. Finch, MRC Laboratory of Molecular Biology, Cambridge. Finch examined the virus by electron microscopy, and from these micrographs obtained a 3-dimensional reconstruction of the virus by Fourier Synthesis (Crowther et al, 1970). This method

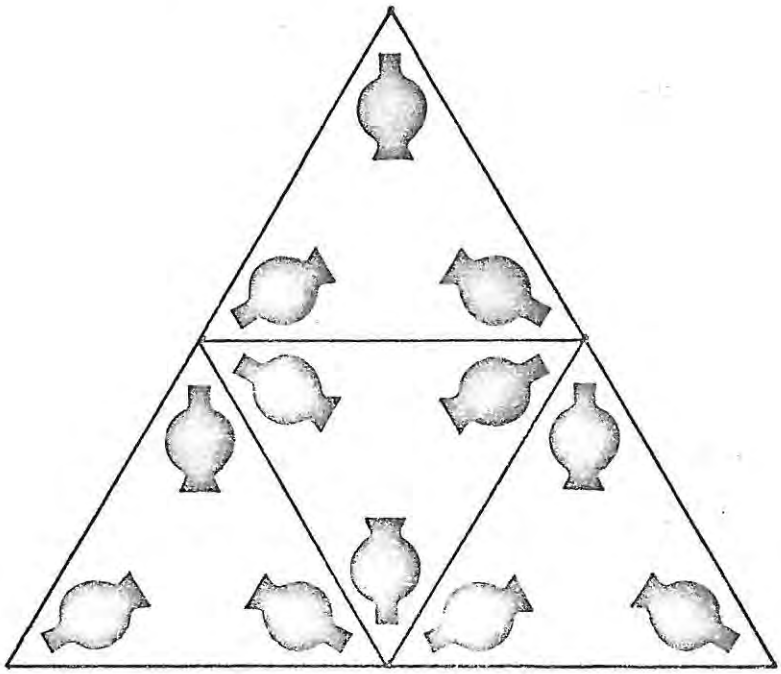


Figure 27. The possible arrangement of 12 subunits on a single face of an icosahedron, showing pentamer and hexamer clustering, assuming a T = 4 structure.

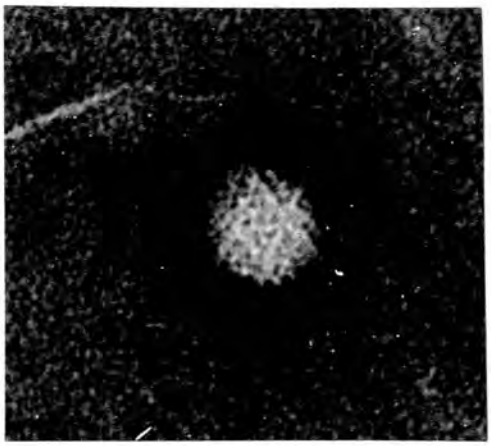


Figure 28. Electron micrograph of an NβV particle showing the areas of staining radiating from apparent 5-fold symmetry axes.

involves the examination of particles from as many views as possible. The area of interest on a micrograph is converted to an array of optical density values by means of a computer-controlled film scanner. The next operations are performed by a digital computer. The digitised densities are transformed by computation into a set of Fourier amplitudes and phases. The Fourier was initially computed to a resolution of 20 Å, and the reconstruction showed units grouped into Y shaped trimers at the trimer position of the T = 4 lattice, the arms of the Y pointing to the lattice points. The protein was shown to be confined to fairly planar triangles, bounded by deep channels connecting the 5-fold axes. These channels correspond to the lines of high density stain seen radiating from the 5-fold axes in electron micrographs.

In a second reconstruction, Finch obtained a contour plot at 7 Å intervals, and two reconstructions are shown in Figs 29 and 30. Fig. 29 is a view somewhere between the vertical 5-fold axis and 2-fold axis, the subunit position being indicated by the markers. Fig. 30 is a view between a 5-fold and a 3-fold axis showing one near face of the icosahedron, and also the deep groove at the top along one of the edges of the icosahedron.

Thus from the chemical and physical studies performed on this virus, as well as the electron microscope work of Finch, it appears that NβV consists of 240 subunits, arranged on a T = 4 lattice. This result is very interesting in that NβV is the first example of a T = 4 structure which has been conclusively established. Another interesting feature is the lack of hexamer-pentemer clustering of the subunits, thereby accounting for the lack of distinct morphological features in electron micrographs (Fig. 28). This evidence finally refutes the proposal of Tripconey (1970), that NβV consisted of 162 capsomeres.

The other small RNA-containing insect viruses which have been extensively studied are those of G.podocarpi (Longworth et al, 1973),

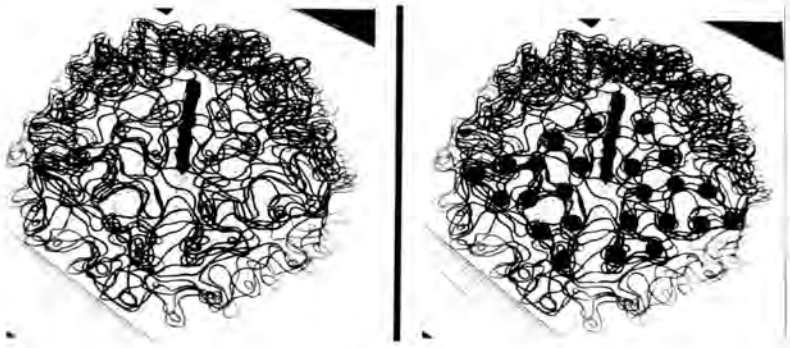


Figure 29. Contour map of the reconstruction of N∩V, of a view somewhere between the vertical 5-fold axis and 2-fold axis. The subunit positions are indicated in the right hand photograph by the dots.

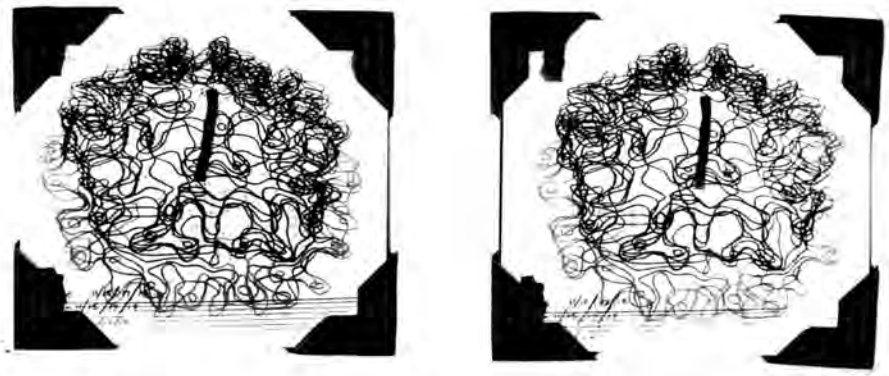


Figure 30. Contour map of the reconstruction of NβV, of a view between a 5-fold and 3-fold axis, showing one near face of the icosahedron. This photograph can be removed and examined under a stereomicroscope.

and recently, sacbrood and acute paralysis of the honey bee A.mellifera (Newman et al, 1973 a). Both the A.mellifera viruses were shown to have S_{20W} values of about 160S, and diameters of 28 nm, to contain 1RNA with a G:C mole content of 0.37, and to have buoyant densities of 1.33 gm/ml in caesium chloride. From their reported 260/280 ratios it is possible to calculate that both these viruses contained greater than 20% nucleic acid, using the method of Warburg and Christian (1946). NβV has very little in common with the viruses from G.podocarpi and from A.mellifera, apart from containing 1RNA and being of similar size. The percentage RNA of NβV (11%) is far lower than that of both G.podocarpi virus (37%; Longworth et al, 1973) and also the two bee viruses (over 20%). The S_{20W} value (220S) of NβV is greater, and its density (1.298 gm/ml) for lower, than that of the G.podocarpi virus (180S; 1.35 gm/ml) as well as the two bee viruses (160S, 1.33 gm/ml). NβV is also distinct from the virus of G.podocarpi in that it contains a single polypeptide in its capsid, as opposed to the 5 polypeptides in the capsid of G.podocarpi virus.

The problem arises as to how the nonoccluded RNA insect viruses should be classified. The animal picornaviruses are described as having diameters of 15-30 nm, 1RNA and no essential lipid envelope (Rueckert, 1971). Longworth et al (1973) stated the virus of G.podocarpi has many properties in common with the enterovirus group. However its size and S_{20W} value reported by them are somewhat high for the picornaviruses. McFerran et al (1971) state that the accepted size of the picornaviruses does not include the reported size of a significant number of animal enteroviruses. Newman et al (1973 a) state that although the two bee viruses have several physico-chemical properties similar to those of the mammalian picornaviruses, they are not unequivocal members of any of the subgroups proposed by Newman et al (1973 b). Acute paralysis virus is a typical enterovirus, except for an increase in buoyant density above pH 7 (Newman et al, 1973 a).

It appears that firstly, the size range of the picornaviruses will have to be extended from 15-30 nm to 15-40 nm, to embrace many other small RNA viruses. Secondly, attempts to class the picornaviruses of the invertebrates into the same subgroup with those of mammals would seem to be unwise.

It is thus proposed that the picornaviruses of the invertebrates be classed as a completely separate subgroup of the picornaviruses, and then further subdivided on the basis of their individual physico-chemical, serological and host-type characteristics.

CHAPTER VII

SUMMARY.

NβV was successfully isolated from the diseased larvae of the pine emperor moth, N.cytherea capensis, and some physico-chemical characteristics were determined.

The molecular weight of the virus was re-examined using the "high speed" sedimentation method of Yphantis (1964). From the results obtained in this work and the values obtained by Polson et al (1970), it was concluded that the molecular weight of NβV was between 16-17 million.

A method of producing soluble protein, by treating the virus with formic acid, was developed. Some characteristics of this protein above and below its isoelectric point were determined. The capsid of NβV was shown, by SDS-PAGE, to consist of a single species of molecular weight 60-62,000.

The virus was shown to contain 11% 1RNA. The phosphorus content was determined to be 1.03%. A method of isolating relatively undergraded RNA was developed; the S_{20W} value of this RNA was calculated to be 32.2S and the base ratio found to be 28% guanylic acid, 23% uridylic acid, 25% cytidylic acid and 24% adenylic acid.

From the chemical data obtained, the number of subunits was calculated to be 240, suggesting a T = 4 structural arrangement. This was confirmed by electron microscope studies by J.T. Finch. NβV is thus the first established example of a virus with a T = 4 structure.

The cryptogram of NβV is R/1 ; 1.8/11; S/S; I/O.

ADDENDUM 1.

COMPUTER PROGRAMME.

The following computer programme was designed for the ICL 1901A computer, in order to calculate the results of data obtained in sedimentation equilibrium experiments (Chapter III C). For each set of data, the initial values of Y1 to Y5 are punched on a card. On each succeding card are punched the values of X obs. and the corresponding values of Y1 to Y5. For each value of X obs., the programme calculates the values of X squared (cm), and the values of ln c. The programme also calculates the slope, which can be substituted into the sedimentation equilibrium equation.

```
DOC JOB PRINT        [XKEI     30/08/73

          JOB BOYP,1, JAKE
          FORTEST JAKE
     !    IN ED(PROGRAM COMP)
     !    LOAD #XFAF
     !    OUT LP0(COMPILATION)
     &    IN /JD,/0
     !    ENTER 1
     !    AT DELETED FI,GO TO 9F01
     !    98 DISPLAY 'RUN FAILED'
     !    END
     !    9F01
     !    IN ED(PROGRAM COMP)
     !    LOAD #
     !    CORE 13000
     !    RESUME
     !    AT DELETED FI,GO TO 9F04
     !    GOTO 98
     !    9F04 IN ED(PROGRAM DUMP)
     !    LOAD #JAKE
     !    9F02 IN /0
     !    OUT LP0(EXECUTION)
     !    ENTER 0
          ****

DOC COMPILATION      [BOYP     30/08/73


FORTRAN COMPILATION BY #XFAE IK 4D     DATE  30/08/77

     LIST
     RUN
     DUMP ON(PROGRAM DUMP)
     WORK (ICLF-DEFAULT)

     PROGRAM(JAKE)
     INPUT5=CR0
     OUTPUT6=LP0
     TRACE2
     END
```

```
      MASTER YPHANT
C
C     THIS PROGRAM CALCULATES THE LEAST SQUARES SLOPE OF THE CURVE RESULTI
C     ING FROM THE YPHANTAS HIGH SPEED SEDIMENTATION EQUILIBRIUM.
C
C     THE DATA REQUIRED BY THE PROGRAM IS, FOR EACH SET OF READINGS:-
C     CARD 1:     AN INDICATOR IN THE 1 ST COL.
C              0 IF THERE IS A SET OF DATA.
C              9 IF THERE IS NO MORE DATA.
C     CARD 2:     A COMMENT CARD THAT WILL BE USED TO IDENTIFY ANY RESULTIN
C              NG PRINTOUT.
C     CARD 3:     A CARD CONTAINING THE INITIAL VALUES OF Y1,Y2,Y3,Y4,Y5, T
C              THESE ARE PUNCHED IN THR FORM:
C              DDD.DD DDD.DD DDD.DD DDD.DD DDD.DD
C              ON ONE CARD. NOTE THAT NO SPACES APART FROM THE ONE SEPARATI
C              G INDIVIDUAL DATA ITEMS. AND THAT A DECIMAL POINT ( NOT COMM
C              A( MUST BE USED.
C
C     CARD 4: THIS AND SUBSEQUENT CARDS ARE THE DATA CARDS, IN THE FORM:
C              DDD.D DDD.DD DDD.DD DDD.DD DDD.DD DDD.DD
C              THIS CARD CONTAINS XOBS,Y1,Y2,Y3,Y4,Y5 IN THAT ORDER.
C     THE FINAL CARD OF THE DATA MUST HAVE 999.99 PUNCHED ON IT.
C
C     AS MANY SETS OF DATA AS ARE REQUIRED MAY BE PREPARED (IN THE SAME FO
C     RM AS  ABOVE. THE FINAL CARD OF ALL THE DATA MUST HAVE A 9 PUNCHED O
C     N IT.
C
      REAL TITLE(10), XSQ(30), MFACT,MEAN, MLOG
      MFACT=2.185
C
C     THE MAGNIFICATION FACTOR MAY BE ALTERED BY CHANGING THE ABOVE CARD.
C
1     READ(5,2)IND
2     FORMAT(I1)
C
C THE COMPUTER HAS JUST READ IN THE 1 ST CARD.
C
      IF(IND.EQ.9) GO TO 14
C
C     CHECK WHETHER LAST SET OF DATA OR NOT.
C
      READ(5,3)TITLE
3     FORMAT(10A8)
C
C     READ IN THE COMMENT CARD.
C
      WRITE(6,4)TITLE
4     FORMAT(1H1,20X,10A8)
C
C     PRINT OUT HEADING AT TOP OF NEW PAGE.
C
      WRITE(6,5)
5     FORMAT(1H ,33X,54HYPHANTIS HIGH SPEED SEDIMENTATION EQULIBRIUM RES
     1ULTS.,//)
      READ(5,6)YI1,YI2,YI3,YI4,YI5
6     FORMAT(5(F6.2,1X))
C
C     READ IN INITIAL VALUES OF THE Y'S.
C
      WRITE(6,7)
```

```
7       FORMAT(1H ,18X,4HXOBS,2X,3HXSQ,3X,2HY1,5X,2HY2,5X,2HY3,5X,2HY4,5X,
     1 3HDY1,2X,3HDY2,2X,3HDY3,2X,3HDY4,2X,3HDY5,2X,4HMEAN,2X,5HLOG C,/)
        WRITE(6,8)YI1,YI2,YI3,YI4,YI5
8       FORMAT(1H ,20X,12X,5(F6.2,1X))
C
C    READ IN INITIAL VALUES OF Y. PRINTED OT HEADINGS AND INITIAL Y.
C
        AXY=0
        AX=0
        AY=0
        AXS=0
        I=1
9       READ(5,10)XOBS,Y1,Y2,Y3,Y4,Y5
10      FORMAT(F5.1,1X,5(F6.2,1X))
C
C    READ IN A CARD OF DATA.
C
        IF(XOBS.GT.999.0) GO TO 12
C
C   CHECK THAT ALL THE DATA HAS BEEN READ IN. IF IT HAS, GO TO SECTION TH
C   AT CALCULATES THE SLOPE.
C
        XSQ(I)=((XOBS/MFACT)**2)/10Q0
C
C    CALCULATE X SQUARED.
C
        DY1=Y1-YI1
        DY2=Y2-YI2
        DY3=Y3-YI3
        DY4=Y4-YI4
        DY5=Y5-YI5
        MEAN=(DY1+DY2+DY3+DY4+DY5)/5.0
        MLOG=ALOG(MEAN)
C
C    CALCULATE ALL RELEVANT PARAMETERS.
C
        WRITE(6,11)XOBS,XSQ(I),Y1,Y2,Y3,Y4,Y5,DY1,DY2,DY3,DY4,DY5,MEAN
     1    ,MLOG
11      FORMAT(1H ,18X,F5.1,1X,F5.2,1X,5(F6.2,1X),5(F4.2,1X),F5.3,1X,
     1    F5.3)
C
C    PRINT OUT ALL THE PARAMETERS THAT HAVE BEEN CALCULATED AND THOSE TH
C    T HAVE BEEN READ IN.
C
C    THE FOLLOWING SECTION ACCUMULATES THE DATA FOR CALCULATION OF THE SL
C    PE AND INTERCEPT
C
        AXY=AXY+XSQ(I)*MLOG
        AX=AX+XSQ(I)
        AY=AY+MLOG
        AXS=AXS+XSQ(I)**2
        I=I+1
C
C    GO TO READ IN NEXT DATA CARD.
C
        GO TO 9
12      J=I-1
        A=(AXS*AY-AX*AXY)/(J*AXS-AX**2)
C
C     CALCULATES INTERCEPT.
```

```
C
      B=(J*AXY-AX*AY)/(J*AXS-AX**2)
C
C    CALCULATES THE SLOPE.
C
      WRITE (6,13)A,B
13    FORMAT(1H ,///,41X,12HINTERCEPT = ,F7.3,/,41X,8HSLOPE = ,F7.3)
      IF(IND.EQ.0) GO TO 1
C
C    CHECK IF THE FINAL DATA SET HAS BEEN FINISHED.
C
14    STOP
      END

END OF SEGMENT, LENGTH   339, NAME  YPHANT
```

```
        FINISH

END OF COMPILATION -  NO ERRORS

S/C SUBFILE:      12 BUCKETS USED

DOC BOYP              [BOYP     30/08/73


CONSOLIDATED BY XPCK 11E     DATE     30/08/73

PROGRAM JAKE
COMPACT DATA (15AM)
COMPACT PROGRAM (DBM)
CORE                 4352

                     SEG      YPHANT
                     SEG      ALOG
                     ENT      FTRAP
                     ENT      FRESET

LAST BUCKET USED OF PROGRAM DUMP(4095) IS      29

DOC EXECUTION       [BOYP     30/08/73
```

ADDENDUM 2.

TISSUE CULTURE.

Attempts were made to culture the haemocytes of B.mori in order to establish a cell line for virus studies. Kurtti and Brooks (1970) found that the prohaemocytes of Lepidoptera larvae showed abundant mitoses, and that these were the most ideal cells for tissue culture studies.

The only medium tested in these studies was that described by Mitsukashi (1967). Generally 10 larvae were used in an isolation procedure, and haemolymph was collected as described by Kurtti and Brooks (1970). To 1 ml of haemolymph was added 5 ml of tissue culture medium and this mixture was transferred to a FALCON 30 ml tissue culture flask. The liquid was left stationary for 30 minutes, to allow the cells to adhere to the surface, after which it was changed and the flasks were incubated at 25°C. Although initial starting titres were of the order of 10^5 cells/ml, only cells of plasmatocyte morphology adhered to the flasks, and after 1-2 weeks had only increased in number up to groups of 5-10 cells. No further division was observed, even after repeated changes of the medium. The medium used did not appear to be successful.

In order to become aquainted with tissue culture methods, the tissue culture of rat and mouse kidney cells was performed, using the methods of Negroni (1964).

4-5 day old mice or rats up to 4 weeks old were used. The animals were anaesthetised with chloroform, and the kidneys removed and placed in a sterile petri dish with a few ml of phosphate buffered saline (PBS). The kidneys were then torn extensively with sterile knives. To 1 ml of torn tissue in PBS was added 5 ml of 1:10 Bacto Trypsin (1:250 Difco Bacto Trypsin solution diluted 1:10 with PBS), and the mixture was incubated at 37°C for 30 minutes. The tissue was then pelleted by centrifugation

at 500 rev/min for 5 minutes on an MSE bench centrifuge, washed and resuspended in TCM (Addendum 3), at a concentration of 10^5 cells/ml. 5 ml aliquots were then added to a FALCON tissue culture flask, and incubated at 37°C. The medium was changed 30 minutes later, and then every 4 days afterwards.

Within 1 week, growth was confluent, and cells were subcultured. This was done by removing the medium, and adding 5 ml 1/10 Bacto Trypsin to each flask. 30 minutes later the Trypsin mixture containing the cells was removed, the cells were washed, and resuspended in fresh TCM. Both rat and mouse cells were subcultured on 4 occasions.

Figs 31 and 32 are photomicrographs of rat and mouse kidney cells respectively, obtained in this study.

Figure 31. Photomicrograph of rat kidney cell monolayer.

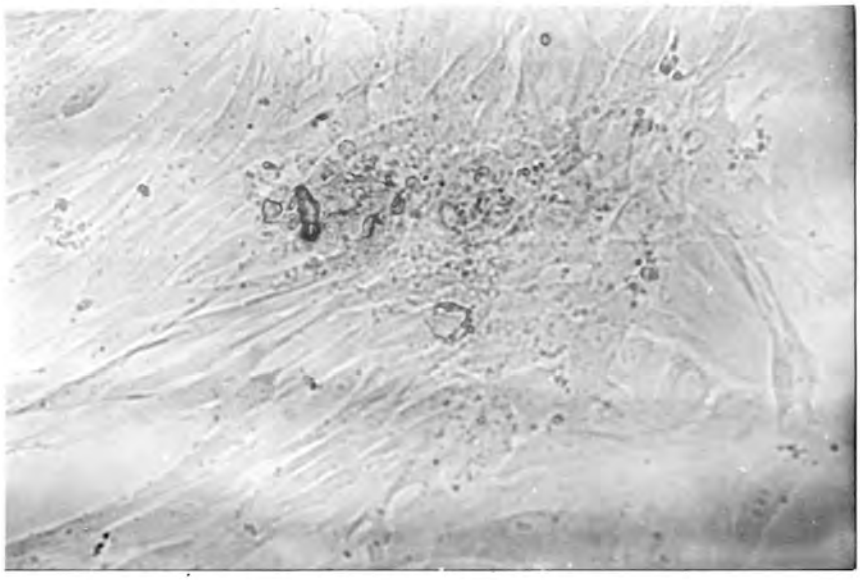


Figure 32. Photomicrograph of mouse kidney cell monolayer.

ADDENDUM 3.

MATERIALS.

Amidol Reagent: 2 grams amidol and 40 grams $Na_2S_2O_3$ added to 200 ml water. The solids were dissolved by placing the mixture on a magnetic stirrer for 1 hour.

Undissolved material was then removed by filtration through cotton wool. The reagent was stored at $4^{\circ}C$ and discarded after 10 days.

Phenol Reagent: 500 grams phenol crystals
200 grams water
0.7 grams 2-hydroxyquinolene

Stored at $37^{\circ}C$ for 24 hours, then in a brown bottle at room temperature.

Phosphate-buffered saline: 8.0 grams NaCl
0.2 grams KCl
1.15 grams $Na_2H\ PO_4$
0.2 grams $KH_2\ PO_4$
Water to 800 ml. Autoclaved.

Tissue Culture Medium TCM: 90 ml TC 199
10 ml calf foetal serum
5 mg Streptomycin sulphate

Sterilized by Seitz filtration, pH adjusted to 7.2 by bubbling with sterile CO_2.

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