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Title: ISOLATION AND CHARACTERIZATION OF THE DNA FROM

NUCLEAR POLYHEDROSIS VIRUS

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The genome of the nuclear polyhedrosis virus which infects the

larvae of the tussock moth, the lepidopteran, Hemerocampa

pseudotsugata, was isolated, identified as deoxyribonucleic acid, and

characterized in terms of its physical and biological properties. An

isolation procedure for the viral DNA was established which con-

sisted of the following steps: the polyhedra were isolated by iso-

pycnic centrifugation in a sucrose density gradient; the virus bundles

were released from the polyhedra by solubilization of the polyhedron

protein at pH 11.6 and concentrated by centrifugation; the virus

bundles were disrupted by sodium dodecyl sulfate treatment to re-

lease the viral DNA genome; the viral DNA from the virus bundle

lysate was banded in a cesium chloride density gradient; and the

viral DNA fraction was recovered in a relatively pure state and

dialyzed against buffer.

The isolated viral genome was identified as DNA by its susceptibility to deoxyribonuclease, its buoyant density in cesium chloride, its ultraviolet absorption spectrum, and its melting curve. The viral DNA was found to have a buoyant density in cesium chloride of 1.710 gm/ml, a melting temperature of $92^{\circ}C$, and a guanine + cytosine base composition of 47 molar percent. The viral DNA genome was shown to exist, at times, as a circular molecule by ethidium bromide-cesium chloride density gradient analysis. The molecular weight of the molecule was found to be 96×10^6 daltons by velocity sedimentation analysis. The isolated viral DNA genome was shown to cause about 60% lethal polyhedrosis when injected into H. pseudotsugata larvae at a concentration of 25 mμg of DNA per larva.

The nuclear polyhedrosis virus of H. pseudotsugata was compared to other insect viruses and to other major groups of animal and bacterial deoxyriboviruses on the basis of the properties of the viral DNA. The nuclear polyhedrosis virus of H. pseudotsugata fits easily into the group of insect polyhedrosis viruses, but the polyhedrosis viruses, as a group, must be considered separately from other deoxyriboviruses. The nuclear polyhedrosis viruses have several features which make them a desirable model with which to study DNA replication, transcription, translation and other virus-host relationships.

Isolation and Characteristics of the DNA
from Nuclear Polyhedrosis Virus

by

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INTRODUCTION

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Nuclear Polyhedrosis Virus

Three groups of insect viruses, the granulosis, the polyhedrosis, and the sphéroidosis viruses, are unique among all other viruses in that the virus particle is occluded inside protein crystals, capsules, and membranes. Once freed of these envelopes, the virus particles resemble morphologically those affecting other types of organisms (Steinhaus, 1963; Smith, 1967a, 1967b).

The first studies of virus diseases in insects were carried out on the silkworm because of its great economic value. One of the viral diseases was called "jaundice" because of yellow spots that appeared on the infected insect in the late stages of infection. It is now known that this disease is one of those classified as "nuclear polyhedroses." A major characteristic of this disease is the presence in the tissues of large numbers of many-sided protein crystals or "polyhedra" which occlude the virus particles. Any insect virus disease in which polyhedra are present in the tissue is called

polyhedrosis.

As early as 1907, von Prowazek was able to demonstrate that material from "jaundiced" silkworms was infectious after the polyhedra were removed by filtration and centrifugation. However, the exact relationship between the polyhedra and the cause of the disease was not known. It was later suggested (Komárek and Breindl, 1924) that the causative agent of a polyhedrosis of the nun-moth caterpillar might be enclosed within the polyhedra. Bergold (1947) confirmed this by demonstrating the presence of the virus in the polyhedra by means of analytical ultracentrifugation and of the electron microscopy.

The polyhedroses are classified into two groups based on the location of the infective process: the nuclear polyhedroses and the cytoplasmic polyhedroses. Polyhedra of the cytoplasmic type are found in the cytoplasm of the cells of the midgut and not in the cell nuclei. In the nuclear type disease, the virus is found and multiplies in the cell's nucleus. In the Lepidoptera, the cell nuclei of the tracheae blood, fat bodies and epidermis are infected (Aizawa, 1963; Smith, 1967); whereas in the Hymenoptera the cell nuclei of the midgut epithelium are infected. Both RNA[1] and DNA have been found

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[1]Abbreviations used throughout text:

BPES   biphosphate, ethylenediaminetetraacetic acid, sodium chloride

to be the genetic material of different polyhedrosis viruses. Nuclear

polyhedrosis virus is a DNA virus.

Bombyx mori System

The physiology and pathology of the silkworm, Bombyx mori,

have been studied quite completely (Aizawa, 1963). Aside from its

great economic value in some parts of the world, this insect offers a

convenient system to study many of the general principles of virus

infection because the insect is a host for a number of viruses, in-

cluding an NPV. The morphology of the polyhedra of the silkworm

nuclear polyhedrosis is well known as well as the chemical composi-

tion of both the polyhedra and the virus. The silkworm (family

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CsCl	cesium chloride
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
MAK	methylated albumin kieselguhr
NaCitrate	sodium citrate
NaCl	sodium chloride
$NaClO_4$	sodium perchlorate
NaH_2PO_4	sodium phosphate (monobasic)
Na_2HPO_4	sodium phosphate (dibasic)
NaOH	sodium hydroxide
NPV	nuclear polyhedrosis virus
mRNA	messenger ribonucleic acid
$^{32}PO_4^{-3}$	radioactive phosphate ion
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TCA	trichloroacetic acid
tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
Na_2CO_3	sodium carbonate

Bombycidae) is related closely enough to the tussock moth,

Hemerocampa pseudotsugata (family Lymantriidae), so that one

would expect many similarities between the two systems. For this

reason, and since much less work on the tussock moth has been re-

ported, the research on the viral infection of the silkworm will be

reviewed in some detail.

The studies by Bergold (1947, 1963a) have defined the morphol-

ogy of the polyhedra. The polyhedra are mostly dodecahedral in

shape although variations are observed. The diameter also varies

somewhat but is generally in the range of 8-12 μ. The polyhedron

is a crystalline lattice made up of protein molecule subunits. The

virus rods are suspended in a random manner throughout the poly-

hedra and apparently do not interfere with the crystalline lattice.

The virus in the silkworm nuclear polyhedrosis appear as single

rods, although in some cases, bundles, containing as many as 19

rods, are embedded within the polyhedra. Up to nearly 100 single

rods or bundles can be found within a single polyhedron.

The virus particles are about 85-90 mμ in diameter and 330-

400 mμ long (Bergold, 1963b; Kozlov and Alexeenko, 1967). The

core of the virus contains the genetic material, DNA, and probably

some associated protein. The nucleoprotein material has been en-

visioned by Kozlov and Alexeenko (1967) as being a closed circle

twisted into a helix to appear rod shaped. The nucleoprotein core is

enclosed within two protein coats or membranes. The outermost membrane is referred to as the developmental membrane and the inner membrane is called the intimate membrane. The proteins of the polyhedra and the virus have been analyzed (Kawase, 1964).

The chemical composition of the nucleic acid of the virus has been studied by several investigators (Wyatt, 1952; Bergold, 1958; Gershenson, 1956; Aizawa and Iida, 1963). These workers were able to prepare pure DNA by conventional isolation techniques. The base composition was found to be 29.3% adenine, 28.0% thymine, 22.5% guanine and 20.2% cytosine on a mole percent basis. Based on this analysis the G+C content of the virus DNA was 42.7%.

Other investigators have studied the physical and biological properties of the DNA. Gershenson (1956) isolated protein-free DNA and DNA-free protein from the nuclear polyhedra of Antheraea pernyi and found the DNA to be infectious, but only when injected along with the polyhedron protein. Bergold (1958) working with the virus of B. mori found the isolated DNA to be infective by itself, but at a very low level of activity. Recent work with DNA from the NPV of B. mori also suggested the presence of infectivity with isolated DNA (Onodera et al., 1965).

Onodera et al. (1965) also report some of the physical properties of the isolated viral DNA of B. mori NPV. From melting curves

and formaldehyde treatment data the viral DNA was shown to be double-stranded. The A/T and G/C ratio of the bases were both equal to unity, which also suggests a double-stranded molecule. The G+C content from the base analysis agreed with the melting temperature of the DNA. The sedimentation coefficient, $S_{20,w}$, was found to be 13.1. This $S_{20,w}$ would mean a molecular weight of 1.8-2.2 x 10^6 which is much lower than would be expected from quantitative determination of the DNA content per virus particle (Allison and Burke, 1962; Luria and Darnell, 1967). Chistyakova (1967) essentially repeated Onodera's work except with a slightly different isolation procedure and found the viral DNA to be 17 S or a molecular weight of 4.6 x 10^6. Yamafuji (1967) reported the DNA to have a molecular weight of 24 x 10^6. Shvedchikova, Ulanov and Tarasevich (1968), making measurements from electronmicrographs, estimated the molecular weight to be 80 x 10^6 which is much closer to the theoretical value as determined from the DNA content per virus particle. These authors also suggested from the electronmicrographs that the viral DNA has a circular structure.

Whereas all of these investigators agree on most of the chemical and physical properties, there is very little agreement on the molecular weight of the viral genome.

The relationship of the virus to the host cell during the infective process has been studied by several workers. The virus, upon

entering a cell, first undergoes an incubation period where no new viruses are produced. The incubation period is temperature dependent and is about six days at 30°C (Smith, 1967b); it is followed by the development of lethal symptoms. The mean period of lethal infection for the H. pseudotsugata is 134 hours at 30°C (Martignoni, personal communication). Newly formed virus rods are released from the cell and infect adjacent cells. This is repeated until all of the susceptible cells are infected and the larva is essentially a bag of polyhedra.

Aizawa (1959) suggests that there are three phases making up the infective process. These phases were determined by measuring the amount of virus in the supernatant of silkworm hemolymph after virus injection. The first phase is a decrease phase. This phase is probably due to an adsorption of virus to the susceptible cells followed by replication similar to the eclipse in bacteriophage infection. The eclipse phase of NPV infection in B. mori was investigated in detail by Krieg (1958). The second phase is an increase or logarithmic phase in which the viruses are being produced and released in large numbers. The last phase is the stationary phase in which the disease has reached its terminal stage and the insect larva is moribund.

Hemorocampa pseudotsugata System

Hemerocampa pseudotsugata, the lepidopteran commonly

called Douglas-fir tussock moth, is a host to both the cytoplasmic

and nuclear types of polyhedrosis viruses. Evenden and Jost (1947)

reported observing a "wilt" disease in the larvae of the tussock moth

which was later shown to have been nuclear polyhedrosis (Steinhaus,

1951). Steinhaus and Marsh (1962) again mention positive diagnosis

of the nuclear type disease in 1956. Until recently, research has

been limited to the incidence and distribution of the disease. How-

ever, because of the economic importance of the defoliator host

(Dodge and Trostle, 1964) and with the advent of biological control

of pest insects by viruses (Steinhaus and Thompson, 1949), the

virus diseases are now being investigated more thoroughly

(Martignoni, Breillatt and Anderson, 1968; Morris, 1963b, 1968a,

1968b).

The morphology of the inclusion bodies is nearly the same as

that of the silkworm polyhedra. However, the polyhedra are much

smaller, approximately $1.5\,\mu$ in diameter (Morris, 1963a). By means

of a Coulter transducer, Martignoni and Iwai (1968) determined volume

frequency distributions of various samples of nucleopolyhedra of H.

pseudotsugata. The mean volumes of the polyhedra in the samples

were found to vary between 3.9 and 5.6 cubic microns. Hughes and

Addison (1969) have observed two "strains" of the tussock moth

nuclear polyhedrosis. The difference between the "strains" lies in

the number of virus rods enclosed in the bundles and in the length of

the rods. One "strain" appears as single rods exclusively whereas

the other may have from 1 to 28 rods per bundle and generally in the

range of 5 to 15 rods per bundle. In the bundle "strains" the rods

average 244 mμ in length, whereas the single rod "strain" average

277 mμ. The polyhedra used in this study were the "strain" which

has bundles.

Very little research concerning the method of replication of

the NPV has been done with the H. pseudotsugata infection. The pro-

cess is probably quite similar to that described for the replication of

NPV in the silkworm.

Statement of Purpose and Rationale

The present research was undertaken to study the characteris-

tics of the DNA of a NPV of Hemerocampa pseudotsugata and to in-

vestigate the properties of viral infection relative to its usefulness in

the study of in vitro DNA replication.

The reports on the silkworm polyhedrosis virus DNA suggest

that the virus of this related lepidopteran might be suited to such a

study (Bergold, 1958; Onodera et al., 1965). If the molecular weight

of the DNA were indeed small (2×10^6) it would be possible to isolate

an intact DNA molecule from an animal virus. The DNA used in in vitro replication studies should be small to avoid fragmentation during its isolation. This is necessary to insure that biological activity and physical properties of the primer and the product can be compared, and that no fragments are available for replication. The DNA primer should be double-stranded. This would provide an in vitro system for investigating the processes of initiation, strand separation, replication, and chain termination. The double-stranded nature of the viral DNA has been suggested in the literature (Onodera, et al., 1965; Chistyakova, 1967). The primer molecule should have a biological activity that can be measured easily and accurately. This can be used to check the integrity of the template molecule and the product. Biological activity was also suggested in the literature (Bergold, 1958). The source of the DNA primer and the enzyme for replication should be available in large quantities. Workers at the Forestry Sciences Laboratory, U. S. Department of Agriculture, Corvallis, Oregon, have been studying the pathology of the Douglas-fir tussock moth, H. pseudotsugata, and large numbers of larvae were available from this source. It was apparent that investigation of this insect virus might provide an in vitro system useful for direct analysis of the DNA replication process.

The NPV system may also be used for the study of transcription and translation. The virus is quite complex and the viral genome

probably codes for a number of viral specific proteins. Nevertheless, several of these viral specific proteins are produced in relatively large quantities and can be easily identified and isolated. The presence of the viral specific proteins could be detected immunologically. In vivo studies could be performed to demonstrate the timing and sequence of viral protein appearance in the host following infection with the virus. In vitro studies could provide information about the mRNA and the translation mechanism. Ultimately, data of this type could lead to a better understanding of the virus replication process in vivo.

Stemming directly from the above information also would be ideas concerning the pathogenic effects on the host. Questions concerning the relationship of the viral DNA to the host DNA might be investigated. The control of the functions of infected cells are apparently taken over by the viral DNA. Is the viral DNA in the host nucleus present as episomal material and replicated in a manner similar to bacteriophage systems? Or is the host DNA synthesis completely destroyed? What comparisons and implications might this have to carcinogenesis in general?

The NPV of H. pseudotsugata is presently being studied at the Forestry Sciences Laboratory, Corvallis, for use as a biological control agent of the host larvae which defoliate Douglas firs. Any information concerning the method of infection by the virus would be

of value to these studies. Basic information about the molecular structure and components of the virus would be important to those studying both the virus and its infective efficiency.

The publications of Onodera et al. (1965) and Chistyakova (1967) were used as the initial guide for viral DNA isolation. We set out to isolate the viral DNA in relatively large quantities and in as pure a form as possible. We then proposed to demonstrate conclusively that the material so isolated was, indeed, the viral genome and made of DNA. The viral DNA would then be characterized physically and biologically so that it could be used in the studies mentioned above.

METHODS

Cesium Chloride Gradients

Cesium chloride gradients were used for preparative and analytical work (Vinograd, 1963). In both cases high purity grade CsCl, 100% - Minus 40 mesh, obtained from Penn Rare Metals Division of Kawecki Chemical Company, Revere, Pennsylvania, was used. A saturated solution of CsCl was prepared in 0.01 M tris buffer at pH 7.0. The solution was filtered through a cellulose acetate filter (B - 6, Schleicher and Scheull Co., Keene, New Hampshire) to remove insoluble material and to provide suitable optical grade CsCl solution.

In preparative runs 8.0 ml of saturated CsCl was mixed with DNA solublized in 0.01 M tris buffer, pH 7.0, to give a density of 1.690 gm/ml as determined by refractive index at $20^{\circ}C$. The volume was approximately 10 ml total. The DNA-CsCl mixture was centrifuged in nitrocellulose tubes with a mineral oil overlay. Centrifugation was carried out in the Spinco fixed-angle 40 or 60 rotor at 102,000 x g for 48 hours at $20^{\circ}C$ (Fisher, Cline and Anderson, 1964; Flamm, Bond and Burr, 1966). The DNA fractions were recovered by puncturing the bottom of the tubes and collecting drops (0.3 ml for the 40 and 60 rotors, 0.2 ml for the SW 50 rotor). The refractive index was read on every fifth tube to determine the density

of the fractions and the absorbances at 260 mμ and 280 mμ were measured on each tube to determine the amount of nucleic acid in each fraction.

Analytical runs were carried out using the Spinco SW 50 rotor and with the Spinco Analytical Ultracentrifuge Model E. For the SW 50 runs the total DNA-CsCl volume was decreased to one-half, otherwise, the procedure was identical to the preparative runs. For the Model E runs the An-D rotor was used with a 12 mm Kel-F, 2° single sector cell. A standard quartz window was used on the bottom of the cell and a -1° quartz wedge window on top. The centrifuge was run at 44,000 rpm at 25°C for 20 hours. The sample was prepared by mixing 0.8 ml saturated CsCl with 0.2 ml DNA and placing 0.6 ml of this into the cell. The instrument was equipped with an electronic scanner connected to a Hewlett-Packard X-Y recorder which plotted the scans directly onto graph paper. Scans were made at 266 and 280 mμ. The band position of the test DNA was determined in relation to the band of a known marker DNA as described by Vinograd (1963) and Szybalski (n.d.).

Cesium Chloride - Ethidium Bromide Gradients

The intercalative dye, ethidium bromide, was used in conjunction with CsCl gradients to determine circularity of the DNA molecule (Waring, 1965; Radloff, Bauer and Vinograd, 1967; Bauer and

Vinograd, 1968). It has been shown that per unit length of double-stranded DNA, less dye will bind to a circular molecule than to a linear molecule. This binding changes the densities of linear and circular molecules so that they may be separated on a CsCl gradient. Circular DNA bands at a higher density than linear DNA in the presence of ethidium bromide.

CsCl-ethidium bromide gradients were runs in the Spinco fixed-angle 40 and SW 50 rotors. For the 40 rotor, a mixture of 6.4 ml saturated CsCl, ethidium bromide (100 μg/ml final concentration), and DNA solublized in 0.01 M tris, pH 7.0, was prepared in a total volume of 10 ml. The average density, as determined by refractive index at 20°C, was 1.55 gm/ml. For runs in the SW 50 rotor, the mixture was decreased to one-half volume at the same average density. In both cases the centrifuge was run at 204,000 x g at 20°C for 48 hours. Fractions (0.3 ml for the 40 rotor, 0.2 ml for the SW 50 rotor) were collected from the bottom of the tubes and the refractive index read on every fifth fraction. . Fractions in the peak region were precipitated with 10% TCA, collected on cellulose acetate filters washed with 2.5% TCA, dryed, and counted in a liquid scintillation counter. If no label was present or if absorbance was needed in addition to counts, the absorbance at 260 mμ was determined on each tube. The samples were diluted to 1 ml with 0.01 M tris buffer, pH 7.0, and the absorbances measured prior to

precipitation with TCA.

Sucrose Density Gradients

Sucrose density gradients were used throughout the study for preparative and analytical work. In either case the procedure was essentially the same. Sucrose solutions of 5% and 18. 7% (w/w) were prepared containing 0. 003 M EDTA and 0. 01 M tris, pH 7. 0. The 5-18. 7% (w/w) linear gradients were pre-formed at 4°C directly in the centrifuge tubes with a simple two-chamber, gravity flow gradient-maker. The sample material was layered gently on top of the sucrose immediately before making the run. Gradients of 5 ml and 30 ml were used in the Spinco SW 50 and SW 25. 1 rotors, respectively. In the SW 50 rotor the maximum sample size was 0. 7 ml and in the SW 25. 1 rotor the maximum was 2. 0 ml. The SW 50 rotor was run at 0. 5°C at 50, 000 rpm and the SW 25. 1 at 25, 000 rpm, both in the Spinco Model L-2 ultracentrifuge. The length of runs varied depending on the material in the sample. Fractions of 0. 2 ml and 1. 0 ml in the SW 50 and SW 25. 1 rotors, respectively, were collected dropwise by puncturing the bottom of the tube with a hypodermic needle. Absorbance and/or radioactivity was determined on each fraction to locate the sample band.

Methylated Albumin Kieselguhr Column Chromatography

The methylated albumin kiselguhr (MAK) column was prepared and used according to the procedure of Mandel and Hershey (1960). Twenty ml of 0. 1 M NaCl in 0. 05 M phosphate buffer, pH 6. 7, was added to 4 g of washed kieselguhr (Celite 503, Johns-Manville, New York, New York), and 10 ml of 0. 1 M NaCl in the same buffer was added to 1 gm kieselguhr in separate beakers. The two mixtures were then boiled to expel air and cooled. One ml 1% methylated albumin (Bovine albumin, The Armor Laboratories, Kankakee, Illinois) was added to the 4 g mixture and stirred for 5-10 minutes. About one-half of the 1 g mixture was poured onto the bottom disc of the column (1 x 20 cm) and the 4 g mixture added on top followed by the remainder of the 1 g mixture. The column was washed with 75 ml of 0. 1 M NaCl in the phosphate buffer.

The DNA sample was equilibrated with 0. 05 M phosphate buffer at pH 6. 7 by dialysis. The DNA sample was added to the top of the column and washed with 50 ml of 0.25 M NaCl in the pH 6. 7, 0. 05 M phosphate buffer. The sample was eluted with a linear gradient from 0. 25 M to 1. 0 M NaCl in the phosphate buffer. Three ml fractions were collected and the absorbance at 260 mμ and refractive index read directly. If radioactive labeled material was being chromatographed, the DNA was precipitated with 10% TCA, collected

on cellulose acetate filters and the radioactivity detected in a liquid scintillation counter.

Sedimentation Coefficient Determination

The sedimentation coefficient of the viral DNA was determined using the Spinco Model E analytical ultracentrifuge (Burge and Hershey, 1963; Crothers and Zimm, 1965). The instrument was equipped with an electronic scanner which automatically took an absorbance scan of the sample chamber from the outer edge of the rotor toward the center edge and corrected for the absorbance due to the solvent. The data was printed out directly on a Hewlett-Packard X-Y chart recorder. The sedimentation velocity was studied using the moving boundary technique. A Spinco An D rotor equipped with a 12 mm al-Epon, $2\text{-}1/2^{\circ}$ double sector cell fitted with quartz windows with wide aperature holders was used. A scanner counterweight with reference holes was used in the second hole of the rotor. The runs were made at 12,000 rpm and between $23^{\circ}C$ and $25^{\circ}C$. Absorbance scans of the moving boundary were taken at 266 mμ, recording five to seven scans per run. The time factor was recorded in terms of $\omega^2 t$ on a Beckman $\omega^2 t$ integrator attached to the centrifuge.

The DNA sample was dialyzed to equilibrium in BPES (0.006 M Na_2HPO_4, 0.002 M NaH_2PO_4, 0.001 M EDTA, 0.179 M NaCl).

To avoid shearing the sample, the chamber was filled from the open

end with the cell partially disassembled. The BPES solvent was

placed in the reference chamber. The actual DNA concentration in

the cell was read directly off the X-Y chart recorder. Separate

runs were made at several DNA concentrations. The scans were

interpreted and the sedimentation coefficient determined as described

in the Results section.

Absorbance - Milligram Conversion Factor Determination

The conversion factor, between absorbance of the viral DNA

in solution and the actual mg of DNA in solution was determined by

measuring the absorbance at 266 mμ (A$_{266}$) and determining the

DNA content quantitatively. The diphenylamine reaction for colori-

metric estimation of DNA as described by Burton (1965) was used.

The diphenylamine reagent was prepared by dissolving 1.5 g

recrystallized diphenylamine (C$_6$H$_5$)$_2$ NH, in 100 ml glacial acetic

acid. To this was added 1.5 ml concentrated sulfuric acid, the

solution mixed well, and stored in the cold. Viral DNA in water

solution was tested directly without precipitation since the samples

were relatively pure. Two ml of the diphenylamine reagent were

added to 1 ml DNA solution, whose A$_{266}$ had previously been deter-

mined. After 16 hours incubation at 37°C the A$_{600}$ was determined

and the DNA concentration read from the standard curve. Calf

thymus DNA was used to establish the standard curve.

Ultraviolet Spectral Analysis

The viral DNA was studied by ultraviolet (UV) spectral analysis. These studies were performed on a Beckman spectrophotometer with a Gilford sample changer. Cuvettes with a 1 mm path length were used with a DNA concentration which gave an absorbance of approximately 0.5 A_{260}/ml. The absorbance from 225 mμ to 290 mμ was determined and plotted.

Melting Curve Analysis

The melting curve analysis was carried out on the Cary Model 14 spectrophotometer. The change in absorbance as a function of temperature was recorded on a Hewlett-Packard X-Y recorder. The absorbance was monitored at 260 mμ. The temperature was determined by a thermocouple probe placed beside the sample cuvette. The thermocouple was calibrated so that the millivolts of resistance sensed by the probe could be converted directly to degrees centigrade. The temperature of the sample chamber was controlled by a Brinkmann Lauda K - 2/R pump. The chamber with the sample in place was allowed to equilibrate at approximately 50°C for one hour and the temperature then increased at a rate of 15°C per hour up to 110°C.

The viral DNA sample was dialyzed against SSC buffer (0.15 M NaCl, 0.015 M NaCitrate, pH 7.2) and used at a concentration of 0.3 A_{260} units (15 μg) per ml. The SSC buffer was used in the blank cuvette during the melting temperature determination.

Biological Assay Procedure

The infectivity of the purified viral DNA was determined by biological assay. These tests were performed with 75 mg H. pseudotsugata larvae in the fourth instar. Injections of DNA were made directly into the hemocoel of larvae under general ether anesthesia using a BD 30 gauge needle and an Agla micrometer syringe outfit from Burroughs Wellcome and Company, London, England. The 5 μl inoculum contained the NPV viral DNA, 0.012 μg streptomycin, 0.012 units penicillin, 0.5 μg methylated albumin and 15% (w/w) sucrose. After inoculation the insects were kept in an incubator at 30°C and at death they were diagnosed microscopically for the presence of polyhedral bodies in the tissue.

Insect Rearing and Polyhedron Production

The basic procedure for rearing the Douglas-fir tussock moth was developed by Lyon and Flake (1966). The procedure varies only slightly from that described by these workers.

Egg masses were collected from the field and stored at -20°C.

After a sufficient diapause period the egg masses were removed from storage and surface sterilized to destroy any naturally occurring virus contamination. This was done by placing not more than four egg masses (about 800 eggs) in 300 ml Clorox and stirring for 30 minutes. The eggs were rinsed in distilled water to remove the Clorox.

Upon hatching from the sterilized eggs, the insects were placed in petri dishes and fed synthetic medium. The following is a list of ingredients for medium "No. 64" (Thompson, 1966).

Dry ingredients	in gms
Casein, vitamin free	42
Sucrose	42
Wheat germ	36
Wesson's salts	12
Alphacel	6
Ascorbic acid	4.8
Aureomycin	0.28

Liquid ingredients	in mls
Distilled water	236
Choline chloride (10% in water)	12
Methyl parahydroxybenzoate (15% in 95% Ethanol)	8
Potassium hydroxide (4M)	6
Sorbic acid (10% in 95% Ethanol)	3.6
*Vitamin stock	2
Linolenic acid (55%)	5
Agar solution	
agar	10 gm
distilled water	400 ml

*Vitamin stock	in mg
Niacin	600
Calcium pantothenate	600
Riboflavin	300

*Vitamin stock	in mg
Thiamin hydrochloride	150
Pyridoxin hydrochloride	150
Folic acid	150
Biotin	12
Vitamin B_{12}	1.2
Distilled water	100 ml

The synthetic medium was mixed in the proportions given and prepared in larger quantities to be stored in the refrigerator for several days. Larvae were allowed to grow in petri dishes with the medium being replaced every week.

Insects to be used for polyhedron production were removed for inoculation during the 5th instar. Inoculations were made with a BD 30 gauge needle directly into the hemocoel of anesthetized larvae. A 10 μl volume per insect was used. The inoculum, consisting of free tussock moth NPV sufficient to cause approximately 100% death in the 5th-instar larvae at ten days post-inoculation at room temperature, was obtained from Dr. M. E. Martignoni of the Forestry Sciences Laboratory, Pacific Northwest Forest and Range Experiment Station, Corvallis, Oregon. The larvae were kept at room temperature after inoculation and collected immediately after death. The dead larvae were stored at -20°C until needed.

Polyhedron Isolation

The nuclear polyhedra were isolated from virus infected insects

which had been raised, inoculated and harvested as explained above.
The basis techniques of polyhedron isolation were described by
Bergold (1947). The procedure used in this investigation is a refine-
ment of Bergold's technique (Martignoni, 1967). The basic proce-
dure for this isolation did not change, although the centrifugation
technique was altered to allow larger amounts of material to be pro-
cessed at one time. The frozen larval tissue was homogenized in a
large blendor keeping the homogenate as cool as possible. Approxi-
mately 800 gm of larvae were homogenized in 0.01 M tris buffer,
pH 7.0, for five minutes, cooled, and again homogenized for five
minutes. The homogenate was then strained through two layers of
cheese cloth to remove debris. The homogenate was diluted 1:1
with 66% w/w Sucrose made up in 0.01 M tris pH 7.0. The nuclear
polyhedra were then separated from the cytoplasmic polyhedra, if
any, and purified by isopycnic centrifugation as described by
Martignoni (1967), in a horizontal swinging-bucket rotor such as the
Spinco SW 25.2. The nuclear polyderon band was located between
54% and 59% (w/w) sucrose. Two stages of centrifugation were used.
In the first, three layers of sucrose were placed into the 60 ml tubes
by pipette. (All sucrose solutions were w/w percent, in 0.01 M tris,
pH 7.0). These layers consisted of a 10 ml underlay of 66% sucrose,
followed by 10 ml 60%, and 15 ml 53% gently placed in the tube
followed by 28.5 ml of the homogenate diluted with 66% sucrose to

approximately 33%, placed on top. The tubes were then centrifuged at 63,000 x g for one hour. The polyhedron layer could easily be detected, since it appeared as a chalky white band between the 53% and the 60% sucrose layers. The polyhedron band was removed with a pipette, diluted 1:1 with 0.01 M tris, pH 7.0, and centrifuged at 12,000 x g for ten minutes to concentrate the polyhedra. The polyhedra were resuspended in the tris buffer and adjusted with 66% sucrose to approximately 33% sucrose in a volume of 23.5 ml for the second stage of purification.

In the second stage, bands of 10 ml 66%, 5 ml 59%, 5 ml 58%, 5 ml 57% and 10 ml 55% sucrose were placed in the tube with the 23.5 ml polydedron suspension layered on top. Centrifugation conditions were as before and the polyhedron band which appeared between 57% and 58% sucrose was again removed by pipette. The polyhedra were then washed several times with the tris buffer, pelleted at 12,000 x g for ten minutes and the supernatant discarded each time.

This technique was satisfactory for small quantities, but as larger amounts were needed it became impractical to use the small volume swinging-bucket tubes. The zonal centrifuge rotor was used to increase the amount of material that could be handled at one time (Anderson et al., 1966, 1967). The technique is essentially the same as described above using isopycnic banding in tubes, except that much more homogenate can be processed (Martignoni, et al., 1968).

The separation was performed in a Spinco Model L preparative ultracentrifuge with a B-XXIII Aluminum rotor (Anderson et al., 1968).. This rotor was provided by Dr. N. G. Anderson, director of the Molecular Anatomy Program.

Operation of the rotor was essentially the same as described by Anderson et al. (1968) and Martignoni, Breillatt and Anderson (1968). With the rotor running at 1800 rpm, it was filled completely with 66% (w/w) sucrose. Approximately 1450 ml of sucrose was required to completely fill the rotor. The step-gradient was formed by pumping the layers into the center port, displacing an equal volume of underlay at the edge of the rotor. The pumping rate was approximately 1200 ml/hour, although higher rates could be used. Fifty ml of 60% followed by 50 ml of 50% sucrose was used to form the gradient. A volume of 400 ml homogenate was then layered onto the top of the gradient through the center port. The homogenate was made as described above. The loading seal was removed and the rotor turned up to 20,000 rpm for three hours. The instrument was then decelerated to 1800 rpm and unloaded by pumping distilled water into the center port, forcing the sample out the edge port. A volume of 300 ml was removed before collecting 25 ml fractions. The polyhedron band was pooled, diluted 1:1 with tris buffer, and centrifuged at 12,000 x g for ten minutes. The polyhedra were again suspended in a 33% sucrose solution in preparation for a second

banding.

In the second banding the sucrose layers were changed so that a shallower gradient resulted. With the rotor full of 66% sucrose underlay, the following layers of sucrose were pumped into the center port: 100 ml 60%, 100 ml 57.0%, 50 ml 56.1%, 50 ml 55.6%, 50 ml 54.3%, 50 ml 53.6%, 50 ml 52.0% and 750 ml polyhedra suspension. The centrifuge run was made as before. Finally, the collected polyhedra were washed several times with 0.01 M tris buffer, pH 7.0, pelleted at 12,000 x g for ten minutes and the supernatant discarded each time as was done with the swinging-bucket technique.

A third procedure of polyhedron purification, which was able to process a larger volume of homogenate, utilized the K-X centrifuge developed by Dr. N. G. Anderson et al. (1969). The rotor used has a volume capacity of 6.7 liters and the sample is processed in a continuous flow manner. Two separate runs were made with the first designed to remove heavy particles, but not polyhedra, and the second to collect polyhedra in the sucrose gradient (Breillatt et al., 1969).

The rotor was filled in a static position. Two liters of 60% sucrose followed by 4.7 liters 47% sucrose were layered into the rotor. The centrifuge was then accelerated at 4 rpm/second up to 3500 rpm which relocates the gradient against the rotor wall. The

sample (made up as before, except in 43% sucrose to a total volume of 25 liter) was then pumped through the rotor at a rate of 42-48 liters/hour. The effluent as collected was free of large debris and retained 95% of the polyhedra. The effluent became the sample for the second run.

The following sucrose gradient was pumped into the rotor in a static position for the second run: 100 ml 47%, 1000 ml 49.8%, 1300 ml 55%, 1200 ml 56.8%, 1300 ml 58%, 1000 ml 61.7%. The rotor was accelerated to 3500 rpm to relocate the gradient. The sample was then pumped through the rotor at a rate of 25 liters/hour and the rotor slowly accelerated to 30,000 rpm as the flow continued. The higher speed of the rotor and the lower pumping rate allowed the polyhedra to be pulled out of the sample and start to move toward equilibrium in the sucrose gradient. The effluent contained approximately 10% of the input polyhedra. After the entire sample had been pumped through the rotor, the centrifuge was accelerated to 35,000 rpm for 70 minutes to isopycnically band the polyhedra. To collect the polyhedron band the centrifuge was slowed to a stop through a carefully controlled deceleration. The deceleration re-located the gradient in the bottom of the rotor. Fractions of 100 ml were collected from the bottom of the rotor, the polyhedron band re-moved and washed as described in the other two procedures. A total of 1.193 kg wet weight of tissue were processed in this manner with

the final concentration of polyhedra approximately $1.5 \times 10^{10}/\text{ml}$ or 3.4×10^{12} total.

Preparation of ^{32}P-labeled Polyhedra

^{32}P-labeled polyhedra could be obtained using essentially the same techniques described for preparation of unlabeled polyhedra (Carnegie and Beaudreau, 1969). Approximately 150 fifth instar larvae were inoculated with 5 µl of virus suspension directly into the hemocoel. The inoculum had an approximate LD_{50} for this size insect at 30°C of one week. It was found earlier that maximum virus DNA production occurred on or about the fourth day post-inoculation and continued at a high rate until death. On the fifth day after virus inoculation, the insects were injected with 125 µc $^{32}PO_4^{-3}$ per insect, also directly into the hemocoel, and the incubation at 30°C continued ($^{32}PO_4^{-3}$ obtained from International Chemical and Nuclear Corporation, City of Industry, California, in the form of phosphoric acid ^{32}P, carrier free in 0.1 N HCl). The labeled material was adjusted to pH 7.4 with 0.1 N NaOH and diluted with 0.1 M tris buffer, pH 7.4. At the end of one week the insects were harvested and the tissue frozen at -70°C. Polyhedra were isolated from the labeled tissue as described previously using sucrose gradients in the Spinco SW 25.1 rotor.

Insect DNA Isolation

DNA from the host insect was isolated from the larval stage. Preparations were made to obtain both ^{32}P-labeled DNA and unlabeled DNA.

To prepare unlabeled DNA the procedure outlined by Firshein, Berry and Swindlehurst (1967) was followed. The insects (25 healthy fifth instar larvae of about 12 gm total live weight) were homogenized in 20 ml SSC (0.015 M NaCitrate - 0.15 M NaCl), at pH 8.0, in a VirTis homogenizer, slowly at first and then on high speed for 15 seconds. The homogenate was diluted to 65 ml with the pH 8.0 SSC buffer and centrifuged at 5100 x g for 20 minutes. The pellet was washed twice in 20 ml SSC and centrifuged each time at 6500 rpm for 20 minutes. The final pellet was suspended in 20 ml SSC and 20% SDS added to a final concentration of 1.5%. The mixture was allowed to stand at room temperature for 15 minutes and then at 4°C for one hour with agitation. After warming to room temperature insoluble material was sedimented at 12,000 x g for ten minutes and discarded. To the 22 ml of supernatant was added 5 M NaClO$_4$ to a final concentration of 1M. To this an equal volume (26.5 ml) of chloroform-isoamyl alcohol (24:1) solution was added and the mixture agitated at room temperature for 20 minutes. Centrifugation at 5100 x g for ten minutes separated the aqueous from the organic phase. The aqueous

phase was removed and extracted twice more with the chloroform-isoamyl alcohol solution. Three volumes (68 ml) cold 95% ethanol were added to the final aqueous phase. The precipitated DNA was slowly wound out on a glass stirring rod. The DNA was then redissolved in 10 ml 0.15 M NaCl. RNase in 0.2 M tris, pH 7.0 was added to a final concentration of 50 μg/ml, and the mixture incubated at 37°C for 30 minutes. Protein was removed again by making the solution 5M $NaClO_4$ and extracting with equal volumes chloroform-isoamyl alcohol solution. The aqueous layer was removed after centrifugation at 5100 x g for ten minutes, and the DNA again precipitated with cold 95% ethanol. The DNA was wound out on the glass stirring rod and was redissolved in 2 ml SSC buffer. The DNA solution was then stored at 4°C until used.

^{32}P-labeled DNA was isolated from healthy fifth instar larvae in the same manner. Twenty larvae were injected with 100 μc $^{32}PO_4^{-3}$ per insect. The injections were made directly into the hemocoel in a 5 μl volume. Following injection with ^{32}P, the insects were incubated for 24 hours at 37°C and then sacrificed by freezing at -70°C. ^{32}P-labeled DNA was isolated from these insects using the procedure described above for unlabeled DNA.

RESULTS

Isolation of Viral DNA

The development of a suitable isolation procedure for viral DNA was a major part of this research program. The NPV bundles were liberated from the isolated polyhedra by alkaline hydrolysis (Martignoni, personal communication). Polyhedra (3-4 gm wet weight) were suspended in the following solution: 0.01 M tris pH 7.0, 0.9% NaCl and 0.00075% sodium dioctyl sulfosuccinate. Not more than an equal volume of freshly prepared 0.5 M Na_2CO_3 was added to the mixture and 0.2 M NaOH added to bring the mixture to a final pH of 11.6. The mixture was then centrifuged at 6,700 x g for ten minutes which removed all large particulate material and non-hydrolyzed polyhedra. The pellet was washed several times with 0.01 M tris buffer and Na_2CO_3 at pH 11.6. The combined supernatants were then centrifuged at 63,500 x g for 30 minutes to pellet the liberated virus particles.

First attempts to free the DNA from the virus particles were made using SDS to dissolve the virus particle coat. The purified viruses were suspended in 0.5 ml distilled water and 20% SDS added to a final concentration of 0.4%. The mixture was allowed to stand for one hour at 4°C. The lysate was then placed directly onto a 5-18.5% (w/w) sucrose density gradient and centrifuged at 63,000 x g

for 14 hours. A typical run is shown in Figure 1. The fractions were recovered by puncturing the bottom of the tube with a needle and collecting drops. However, this technique was hindered greatly by large amounts of gelatinous material near the bottom of the tube. This gelatinous material was thought to be nucleic acid complexed with protein which had not been completely dissociated by the SDS. The 260/280 mμ absorbance ratio of about 1.0 in the pellet suggested that this material might be nucleoprotein.

Various changes were made in the procedure in an attempt to completely dissociate the remaining protein that might be complexed with the DNA and to disrupt any DNA aggregates. The following changes were tried:

1) A mixture of Triton X-100 (1.0%) in 0.14 M NaCl at pH 8.0 and pronase at 100 μg/ml was substituted for SDS in an attempt to dissolve the virus more completely (Chistyakova, 1967).

2) EDTA (0.003 M) was added to all buffers and gradients to help prevent aggregation.

3) The viral lysate was extracted with phenol before the sucrose gradient to remove protein.

4) MAK column chromatography was attempted on the DNA after the sucrose gradient (Onodera et al., 1965). This failed because of the presence of detergent in the DNA sample which disrupted the column packing.
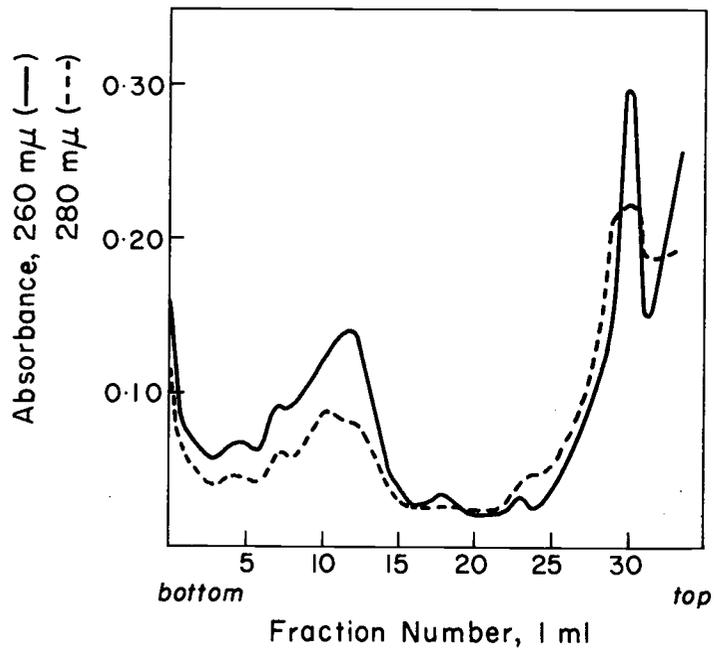
Figure 1. Sucrose density gradient sedimentation analysis of NPV lysed with SDS. The 5 - 18. 5% (w/w) sucrose gradient was run in the Spinco SW 25. 1 rotor at 63. 500 x g for 14 hrs.
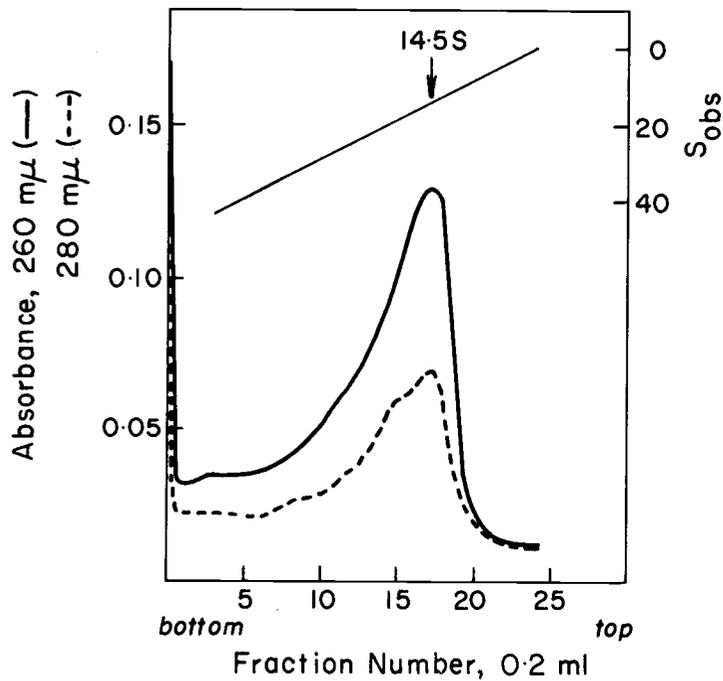
Figure 2. Sucrose density gradient sedimentation analysis of NPV DNA. This is a typical pattern observed after several different purification procedures were carried out on the DNA. The 5 - 18. 5% (w/w) sucrose gradient was run in the Spinco SW 50 rotor at 204,000 x g for 3 hrs.

5) Smaller amounts of material were applied to sucrose and glycerol density gradients to avoid overloading the gradients.

6) A CsCl density gradient was used before the sucrose gradient to band the DNA and to dissociate and separate the protein from the DNA.

None of these changes had any effect on the gelatinous pellet. The sucrose density gradients were all quite similar. Figure 2 shows a typical sucrose density gradient. Figure 2 differs from Figure 1 only in that the smaller Spinco SW 50 rotor was used for the gradient in Figure 2. The large amount of material in the pellet was still present but the peak at about 14.5 S was encouraging since Onodera et al. (1965) and Chistyakova (1967) had reported the DNA of the B. mori NPV to be in this size range. However, the presence of the gelatinous material in the pellet was still a problem to be considered.

Luria's comment (Luria and Darnell, 1967) raised some doubt as to the accuracy of the 14.5 S estimate of the sedimentation coefficient. He stated that the size of the DNA in the B. mori NPV, determined by quantitative measurements of the amount of DNA per virus particle, should be close to 10^8 daltons in molecular weight. It was found that, by shortening the centrifugation time for the sucrose gradients, the pelleted material from the preparations of Figure 2, could be banded near the bottom of the tube with an

observed sedimentation coefficient (S_{obs}) of about 50 S (Figure 3) which corresponds to nearly 10^8 daltons. The material that banded in the 50 S peak was shown to be primarily nucleic acid by absorbance studies and was considered to be the true genome fraction.

The final NPV DNA isolation procedure was established as shown below:

1) Purification of polyhedra

2) Hydrolysis of polyhedra

3) Concentration of virus

4) Lysis of virus with SDS

5) CsCl density gradient

6) Dialysis

The final isolation procedure was arrived at after trials and failures with other methods as mentioned above. The purification of free virus was unchanged throughout the study and was found to be acceptable as described above. The free virus from 3-4 gm polyhedra (wet weight) were suspended in 2 ml distilled water. The suspension was made 0.003 M in EDTA and 0.6% SDS and incubated for two hours at 37°C. The suspension generally became clear after this period of time and was extremely viscous. The suspension was then mixed with CsCl (saturated solution in 0.01 M tris buffer, pH 7.0) and centrifuged to equilibrium. Eight ml of CsCl were mixed with the 2 ml of viral lysate and EDTA was added to 0.003 M. The

tubes were centrifuged at 102,000 x g at 22°C for 48 hours in the

Spinco fixed-angle 65 rotor. Fractions of 0.3 ml were collected

drop-wise from the bottom of the tube. Figure 4 shows at typical

preparative CsCl gradient. The peak tubes were pooled and dialyzed

against 500 ml of 0.001 M tris buffer, pH 7.0, 0.003 M EDTA, with

three changes of one hour each. The resulting DNA solution was

stored at 4°C for use as needed.

DNA Synthesis in Larvae Infected with Virus

Throughout this study both in developing a satisfactory isola-

tion procedure and in characterizing the viral DNA, ^{32}P-labeled

DNA was used in many of the procedures. In order to obtain DNA of

reasonably high specific activity it was of advantage to add the radio-

active label to the growing larva during a period of maximal viral

DNA synthesis. The time study of DNA synthesis in larvae inocu-

lated with virus was undertaken to determine the point of maximal

synthesis.

It was established that the viral and cellular DNA's had dif-

ferent buoyant densities and could be resolved in a CsCl buoyant

density gradient. The peak fraction of the cellular DNA has a den-

sity of 1.695 gm/ml and the peak fraction of the viral DNA has a

density of 1.710 gm/ml. DNA from the uninfected fifth-instar H.

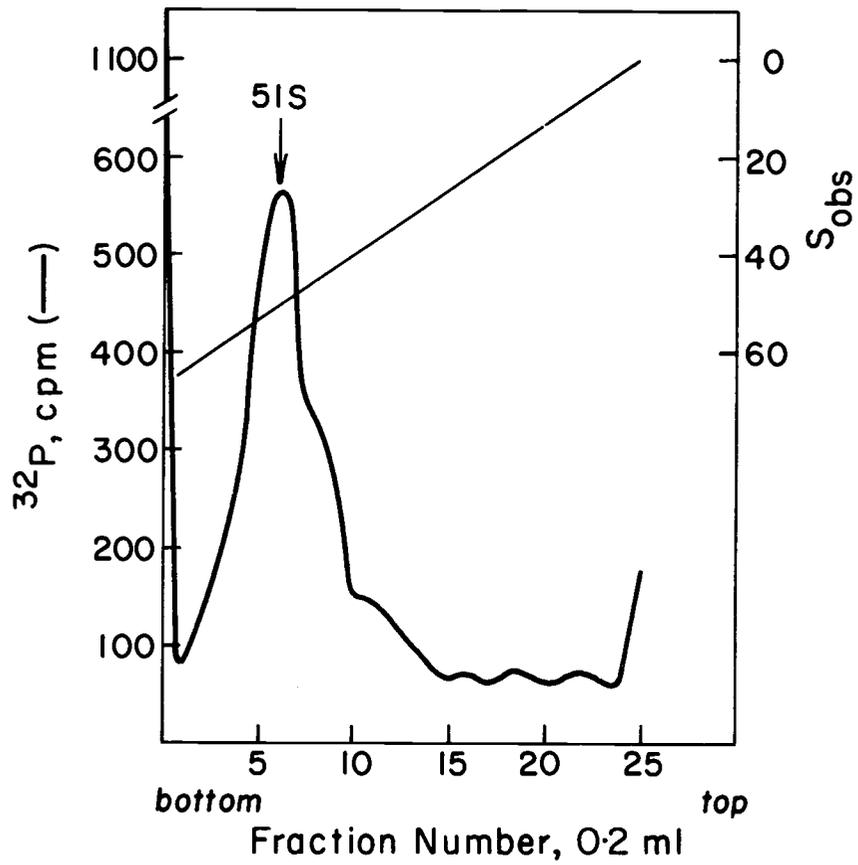pseudotsugata larvae was labeled by intrahemocoelic injection of 100

Figure 3. Sucrose density gradient analysis of NPV ^{32}P-DNA.
The 5 - 18.5% (w/w) sucrose gradient was run in the
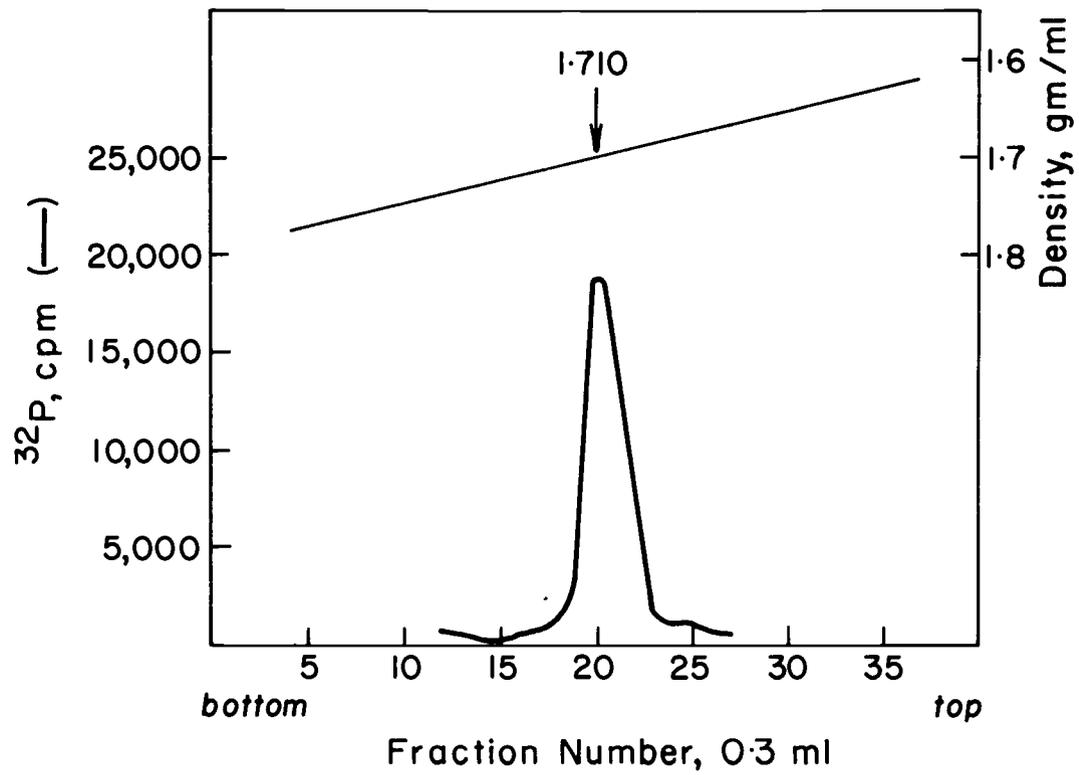Spinco SW 50 rotor at 204,000 x g for 2 hrs.

Figure 4. CsCl preparative gradient of NPV ^{32}P-DNA. The gradient was run in a Spinco fixed-angle 65 rotor at 102,000 x g and 22° C for 48 hrs.

μc of $^{32}PO_4^{-3}$ per larva and the DNA isolated as described in the

Methods section. Unlabeled DNA was obtained from nucleopolyhedra

using the technique described in the previous section. Approximately

10 μg (34,000 cpm) ^{32}P-labeled cellular DNA was mixed with 87 μg

NPV DNA and centrifuged to equilibrium. Buoyant density profiles

for the two DNA's are illustrated in Figure 5.

This difference in density on CsCl gradients was utilized to

study the DNA synthesis in larvae after infection with NPV. Fifth-

instar larvae were infected with NPV by intrahemocoelic injection.

After 24 hours, two infected insects received 125 μc of $^{32}PO_4^{-3}$,

also by intrahemocoelic injection. At the end of a two hour incubation

period the insects were frozen at -70°C. Each day, two of the infect-

ed insects were pulsed with $^{32}PO_4^{-3}$ in a similar manner throughout

a five day period. All insects were incubated at 30°C during the

study. The DNA was then isolated separately from each group.

After thawing and homogenizing, the larvae were treated with 1%

SDS, extracted two times with chloroform-isoamyl alcohol mixture

(24:1) in the presence of 5 M $NaClO_4$, and the DNA precipitated with

two volumes of cold ethanol. The DNA was then redissolved in 0.01

M tris buffer, pH 7.0, and centrifuged to equilibrium in CsCl. The

results are shown in Figure 6. Incorporation of $^{32}PO_4^{-3}$ during days

1, 2 and 3 occurred in cellular DNA having a density of 1.695 gm/ml.

At days 4 and 5, however, a majority of the ^{32}P-label was located at
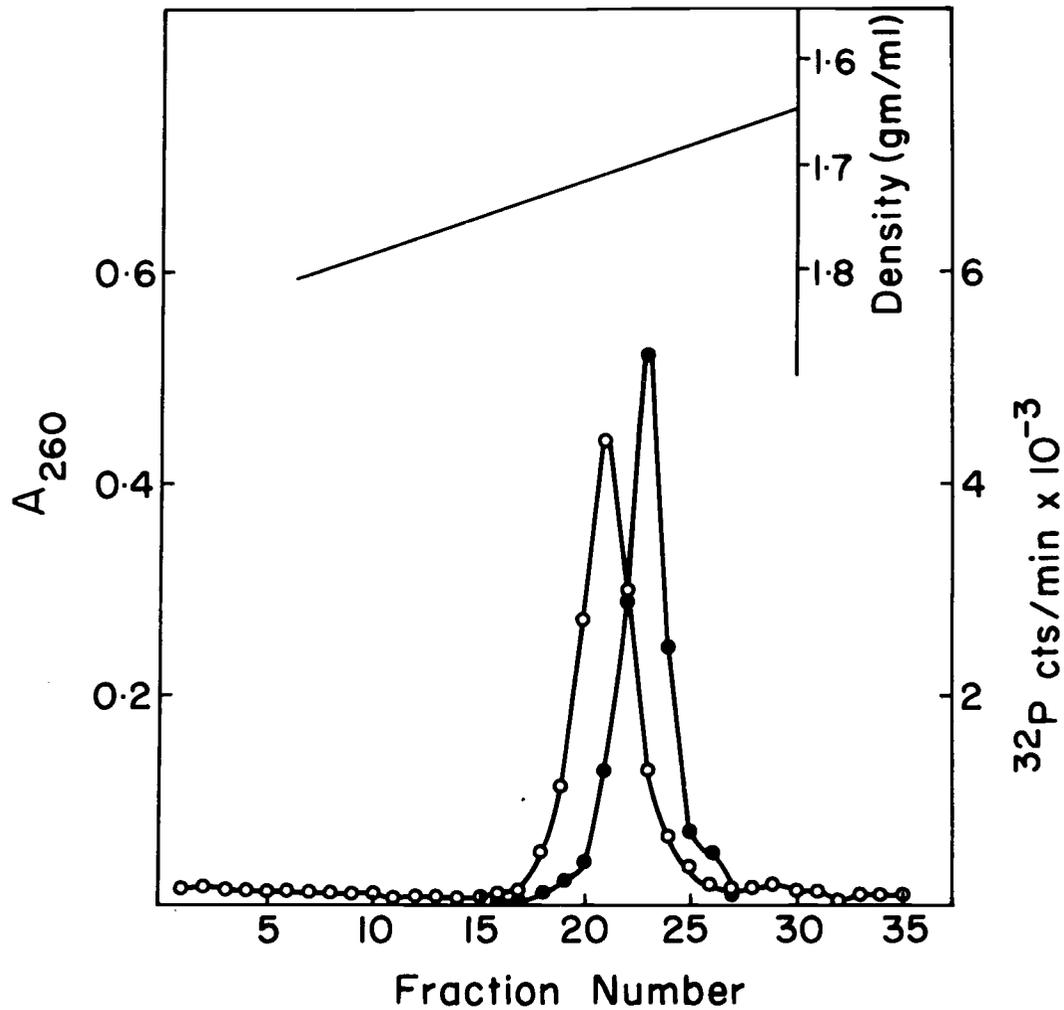
Figure 5. CsCl gradient of a mixture of H pseudotsugata larval
DNA and NPV DNA. A mixture of 10 μg of 32p -
labeled H pseudotsugata larval DNA (●) and 87 μg
of NPV DNA (o) was centrifuged in a Spinco fixed-
angle 65 rotor at 102,000 x g and 22° C for 48 hrs.
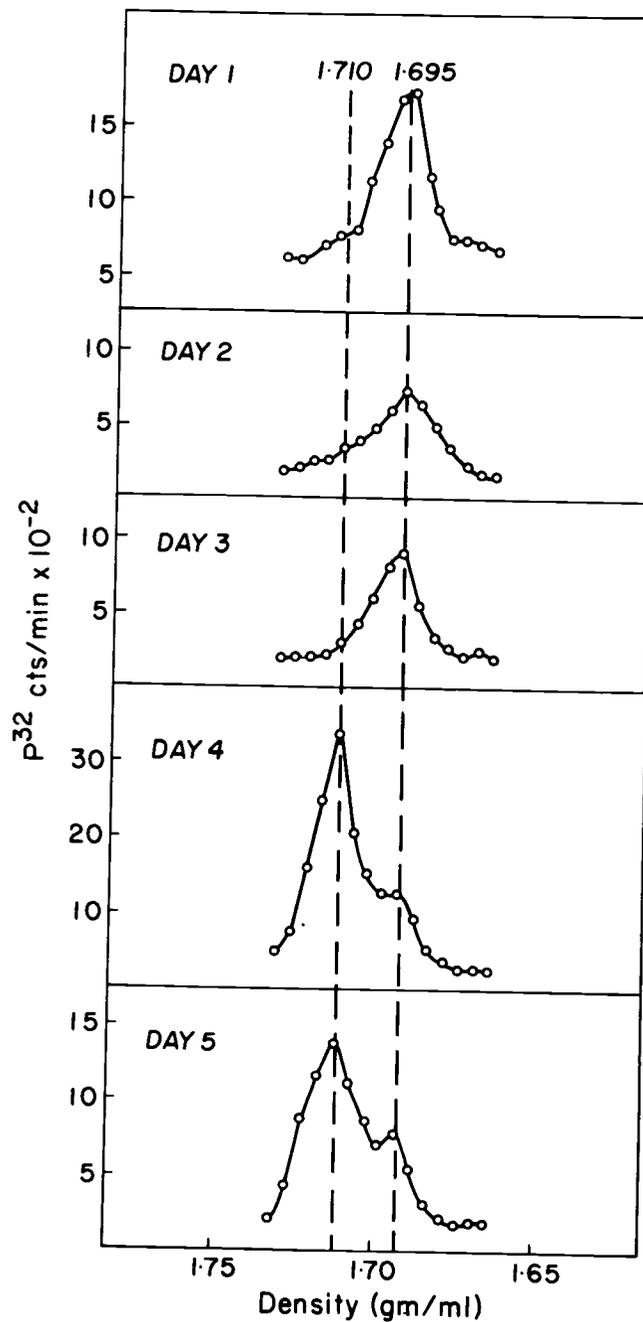
Figure 6. A series of CsCl gradients of ^{32}P - labeled DNA synthesized in vivo after infection with NPV. DNA was synthesized in vivo during a 2 - hr period, 1 to 5 days after infection of H. pseudotsugata larvae with NPV. The gradients were run in a Spinco fixed-angle 65 rotor at 102,000 x g and 22° C for 48 hrs.

a density of 1.710 gm/ml where the DNA from the virus was shown to

band. Sometime between 72 hours and 96 hours after infection, at

$30^{o}C$, a large burst of viral DNA synthesis takes place and appears to

continue through the fifth day. The mean period of lethal infection at

$30^{o}C$ is 134 hours following intrahemocoelic inoculation (Martignoni,

personal communication). Knowing the time of maximal viral

DNA synthesis has allowed us to obtain isotope labeled nucleopolyhe-

dra from which DNA can be isolated with specific activities of 10^{5}

cpm/μg.

Characteristics of Viral DNA

Deoxyribonuclease Treatment

The material which was isolated from NPV was analyzed by a

series of tests designed to show that the material as isolated was DNA.

The DNA was then characterized both physically and biologically in

order to completely identify the molecule in terms of size, conforma-

tion, chemical composition and infective properties.

The sucrose density gradient was used to demonstrate the effect

of deoxyribonuclease (DNase) on the isolated material. ^{32}P-labeled

DNA which had been isolated as described above was incubated at

room temperature for 30 minutes in the presence of 0.005 M $MgCl_2$

and 50 μg/ml DNase (Pancreatic endonuclease, Sigma Chemical

Company, St. Louis, Missouri). The incubation mixture was then

layered directly onto a sucrose density gradient. The results are

shown in Figure 7. The only peak of ^{32}P activity is in the region of

3 S. DNase is known to degrade DNA to oligonucleotides (Mahler and

Cordes, 1966) which would band down into the sucrose density grad-

ient slightly. Comparison with Figure 3 shows that no activity is

found in the 50 S region, the region in the gradient where the intact

DNA molecule bands. These results indicate that the isolated

material was degraded by a catabolic enzyme that is specific for

DNA, and is strong evidence for defining the isolated viral material

as DNA.

Methylated Albumin Kieselguhr Column Chromatography

The MAK column was also used to identify the isolated material

as DNA. ^{32}P-labeled material as isolated above was applied to the

column and eluted as shown in the profile of Figure 8. The ^{32}P-

radioactivity elutes sharply at 0. 7 M NaCl which is characteristic of

DNA on the MAK column, as is shown by comparison with E. coli

DNA. This is additional evidence for classifying the isolated viral

genome as DNA. Onodera et al. (1965) found B. mori NPV DNA to

elute from the MAK column at 0. 7 M NaCl. The two DNA's in this

experiment were analyzed on separate MAK columns and plotted in

the same figure for comparison.
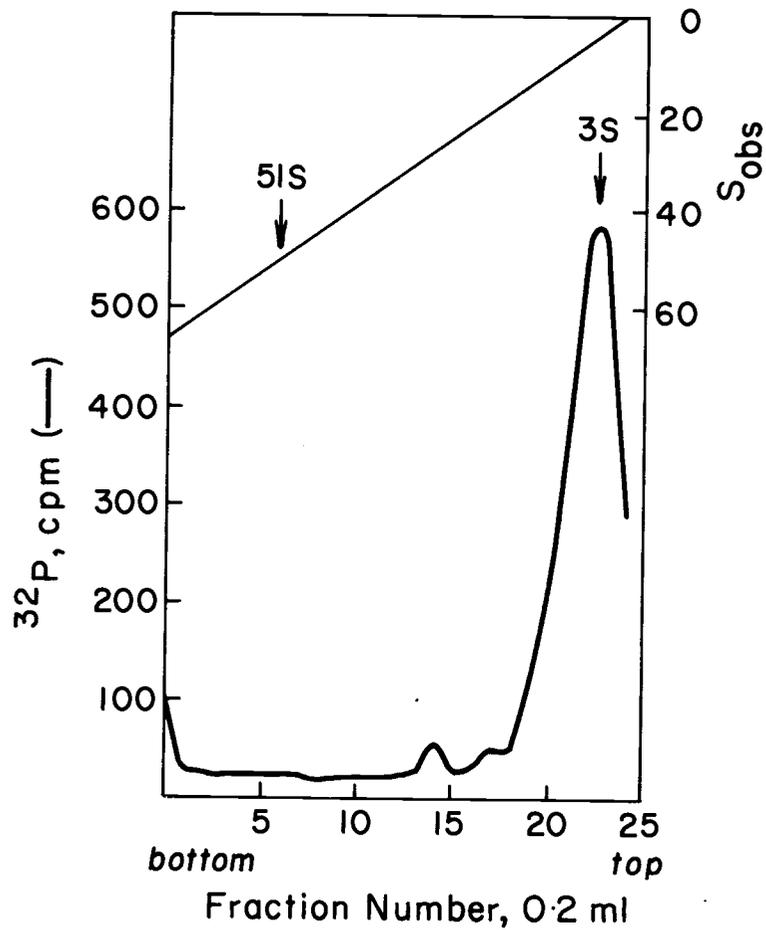
Figure 7. Sucrose density gradient of NPV ^{32}P-DNA treated with
DNase. 50 μg/ml DNase in the presence of 0.005 M $MgCl_2$
was incubated with NPV DNA at room temperature for
30 min. The 5 - 18.5% (w/w) sucrose gradient was run in
the Spinco SW 50 rotor at 204,000 x g for 2 hrs.
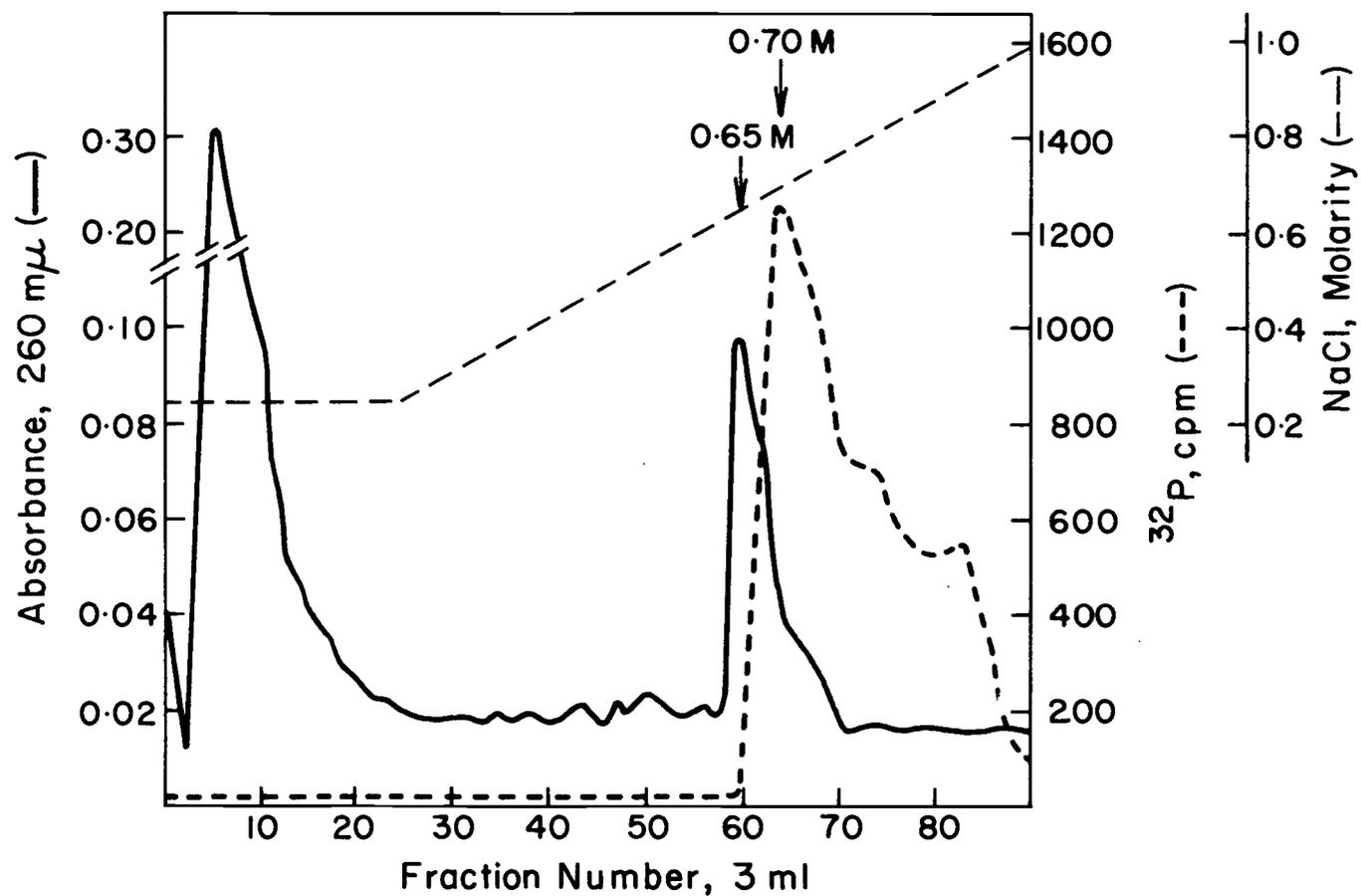
Figure 8. Composite of NPV ^{32}P-DNA and E. coli DNA on MAK columns. NPV ^{32}P-DNA (---) was chromatographed and eluted as shown with NaCl. E. coli DNA (——) was chromatographed separately on another column and also eluted with NaCl.
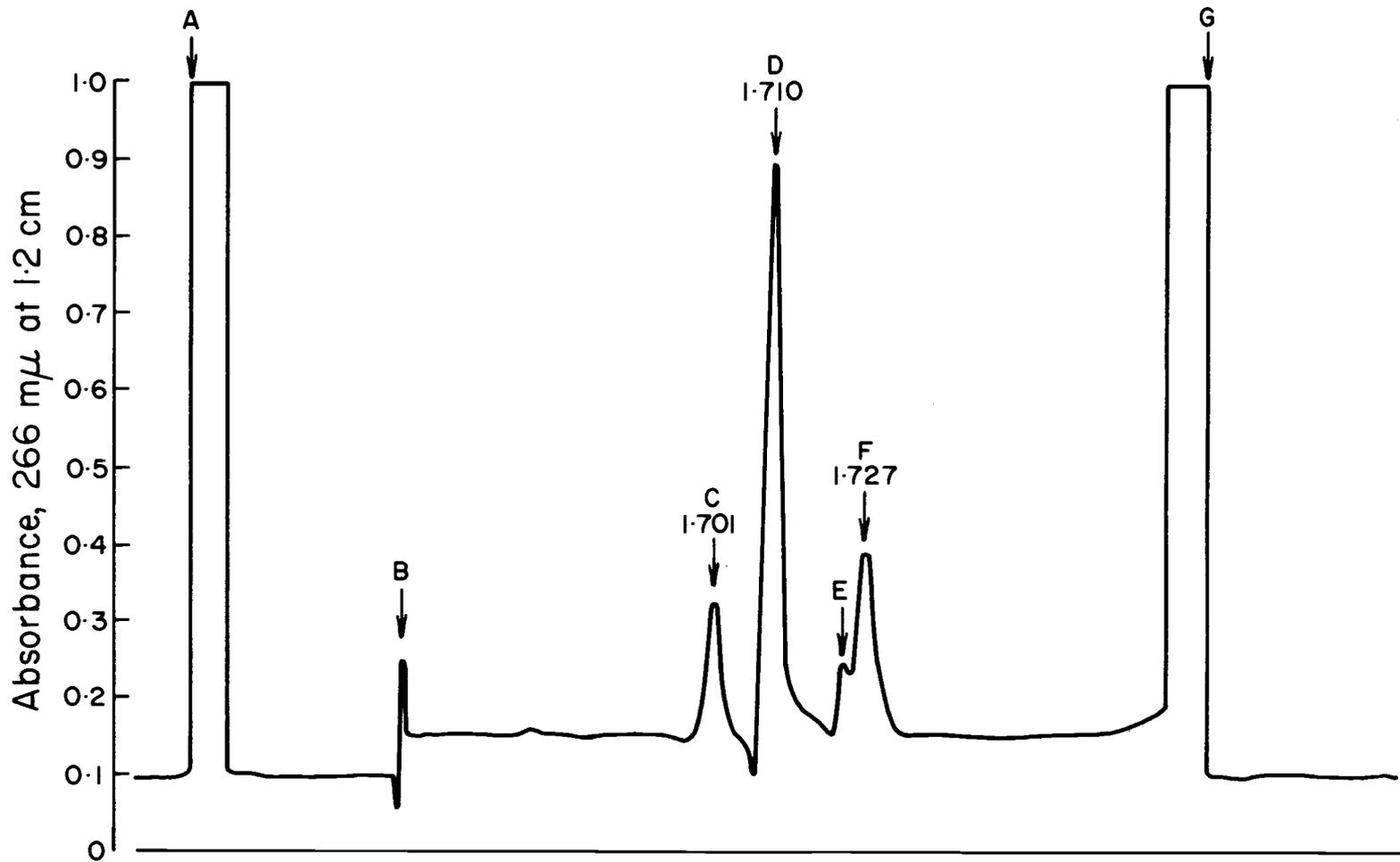
Figure 9. CsCl analytical gradient of NPV DNA. NPV DNA was mixed with M. lysodiektus
DNA as a density marker. Figure represents a cross section view of Spinco
Model E An-D rotor cell. A, inside reference hole; B, meniscus; C and D, bands
from NPV DNA preparation; E and F, bands from M. lysodiektus DNA marker;
G, outside reference hole. The gradient was run on the Spinco Model E at
44,000 rpm and 25° C for 20 hrs.
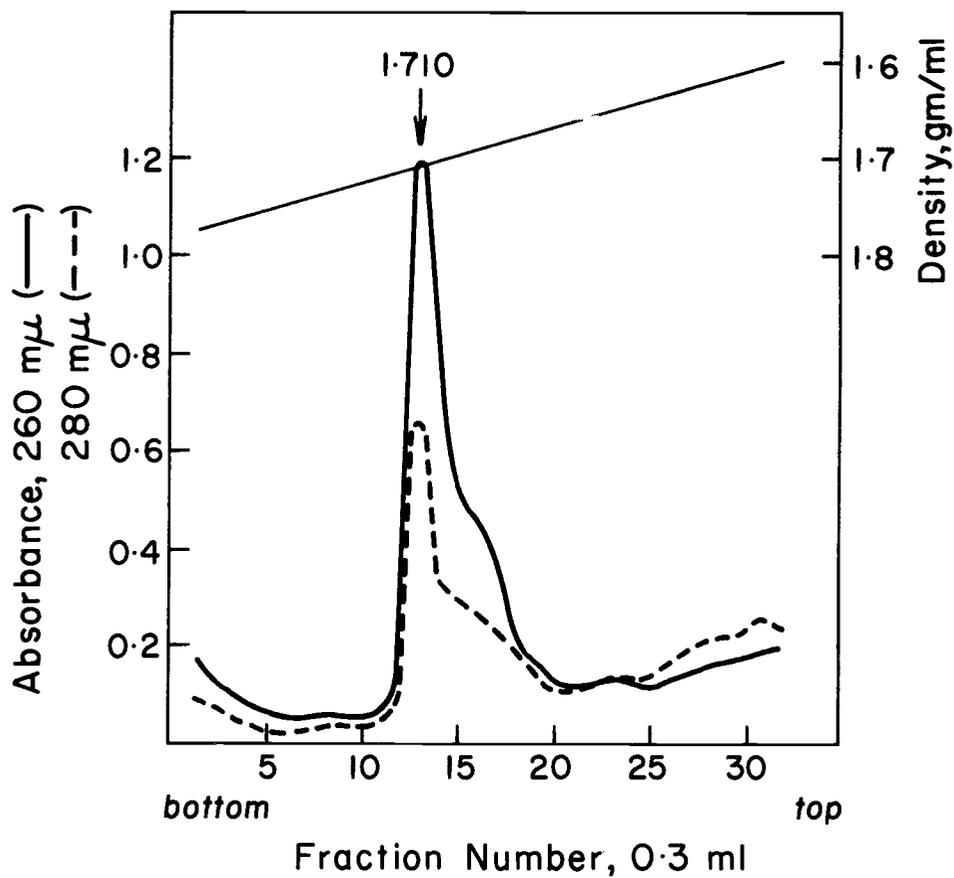
Figure 10. CsCl preparative gradient of NPV DNA. The gradient was run on the Spinco fixed-angle 65 rotor at 102,000 x g and 22° C for 48 hrs.
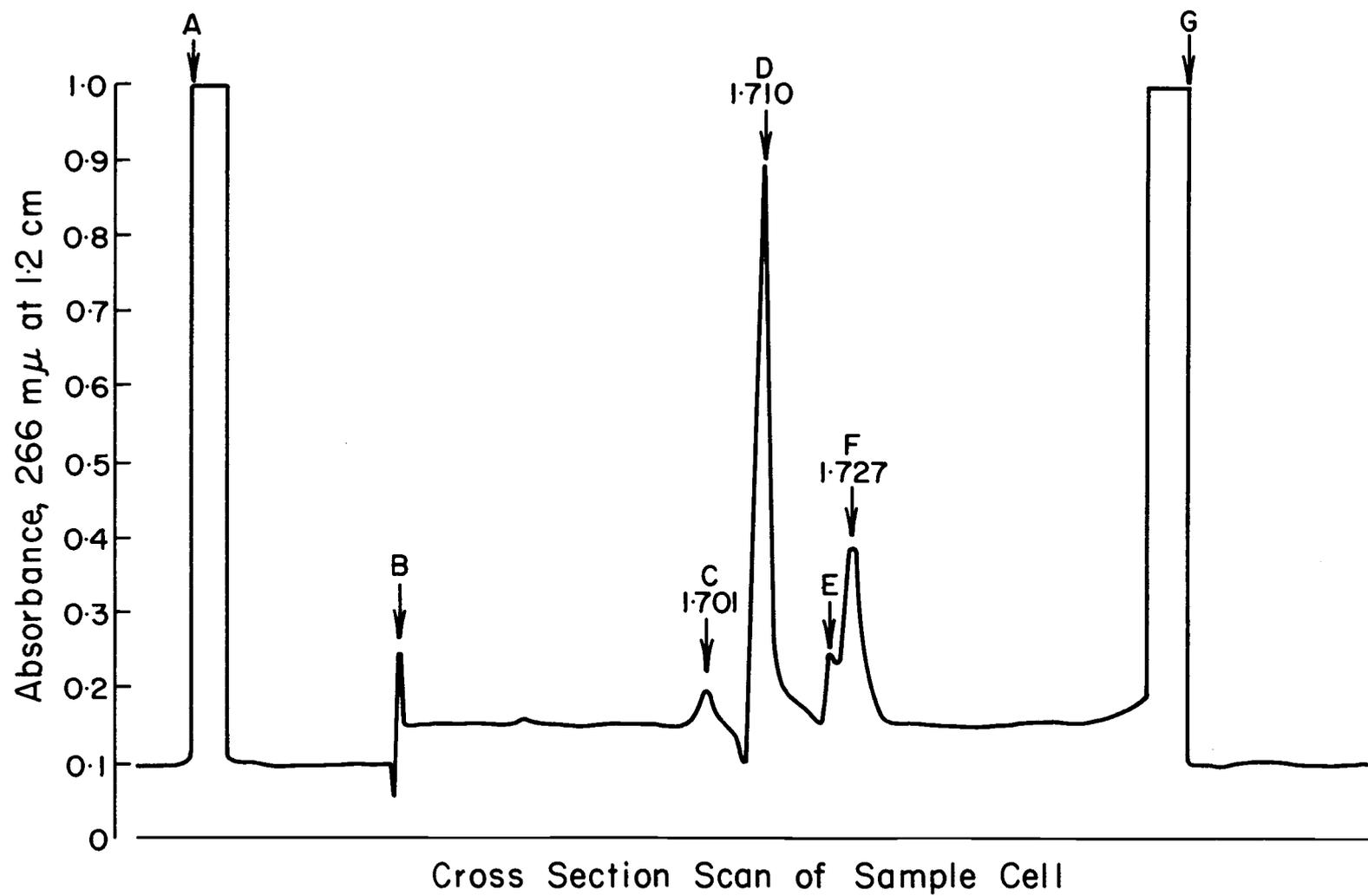
Figure 11. CsCl analytical gradient of NPV DNA after further purification. NPV DNA was recovered from the heavy side of CsCl preparative gradient (Figure 10), mixed with M. lysodiektus DNA as a marker and centrifuged as in Figure 9.

Cesium Chloride Gradients

The CsCl buoyant density gradient was used to identify the material as DNA and to illustrate several other points. Figure 9 shows the viral DNA banded isopycnically with <u>Micrococcus</u> <u>lysodiek-</u><u>tus</u> as a marker in the Model E analytical ultracentrifuge. The fact that the isolated material bands within the 1.65-1.75 gm/ml range is good evidence that it was DNA. The exact buoyant density of the molecule in CsCl is 1.710 gm/ml with the marker DNA banding at 1.727 gm/ml. The <u>M</u>. <u>lysodiektus</u> DNA marker contained the minor unknown contaminant shown, but did not affect the density of the major marker peak.

Secondly, as shown in the figure, there was a third peak that was shown to be primarily nucleoprotein by absorbance studies. This suggests that the preparation as isolated was not pure unless only the heavy portion of the peak in the preparative CsCl gradients is used. This encouraged us to use the preparative CsCl gradients as a purification step, collecting only the heavy side of the DNA peak. Figure 10 shows a typical preparative CsCl gradient. Fractions 12, 13, and 14 only were collected from this gradient and analyzed on the Spinco Model E. Figure 11 shows the analysis after removal of a large portion of the contaminating nucleoprotein observed in Figure 9.

Thirdly, as illustrated in Figure 5, the isolated material was significantly different from the cellular DNA fraction in terms of their buoyant densities.

Finally, the CsCl gradient shows that no RNA was present in the purified DNA preparation. RNA is known to band near the bottom of the tube in these gradients, a region where no UV absorbing material was present.

Absorbance - Milligram Conversion Factor

It was often desirable to know the amount of DNA present in the solution with which we were working. For instance, it was necessary to know the exact concentration of the solution of DNA used in the sedimentation coefficient determinations.

In order to use the absorbance of the DNA solution as a precise indicator of the milligram concentration, it was necessary to determine a conversion factor from absorbance to milligrams. Since the spectroscopic characteristics differ from one DNA to another, the conversion factor had to be determined specifically for the viral DNA. This was done by simply measuring the absorbance of a solution of known concentration of viral DNA. The concentration of a standard solution of viral DNA was determined using the diphenylamine method for determination of DNA as explained in Methods. The absorbance at 266 mμ was then read on the standard solution. Two hundred

sixty-six mμ was used because the Model E ultracentrifuge determination of sedimentation coefficient was performed at this wavelength (see Molecular Weight below).

Table 1 shows the results of the conversion factor determination. Calf thymus DNA was used as the standard with a conversion factor of 18.7 A_{260}/mg (Mahler and Cordes, 1966). After establishing the A_{266} versus mg DNA standard curve with calf thymus DNA, the A_{266} for the viral DNA was read directly from the curve and the conversion factor, A_{266} to mg DNA, calculated. This value was calculated to be 24.45 A_{266}/mg DNA for NPV DNA.

Table 1. Determination of Conversion Factor Between Absorbance and Milligram Concentration for Nuclear Polyhedrosis Virus DNA.

Tube no.	A_{260}	A_{266}	A_{600}*	mg DNA/ml	factor
1 thymus DNA	0.849	0.778	0.237	0.04540	18.7 A_{260}/mg**
2 "	0.835	0.768	0.240	0.04565	"
3 "	0.811	0.746	0.227	0.04337	"
4 "	0.196	0.179	0.061	0.01048	"
5 "	0.179	0.162	0.053	0.00957	"
6 "	0.196	0.178	0.053	0.01048	"
7 viral DNA	0.112	0.109	0.024	0.00440	24.77 A_{266}/mg***
8 "	0.113	0.111	0.026	0.00460	24.13 "

*A_{600} read after diphenylamine reaction.
**Conversion factor used to establish standard curve. Taken from Mahler and Cordes (1966).
***Conversion factor calculated from standard curve. Average value is 24.45 A_{266}/mg DNA.

Molecular Conformation

Having demonstrated that the isolated viral genome was DNA, we set out to study the properties of the molecule. The conformation of the DNA molecule is of interest since some viral DNA's are known to be circular structures. Shvedchikova, et al. (1968) have published electromicrographs which suggest a cyclic structure of the DNA of the granulosis virus of the Siberian bombyx (Dendrolimus sibiricum) which is closely related to the polyhedrosis virus of H. pseudotsugata.

The possibilities of circularity were investigated using the intercalating dye, ethidium bromide. The dye molecule binds to the DNA, giving the nucleic acid a lighter buoyant density. More dye binds, per unit length, to the linear form than to the circular form. Therefore, the circular-DNA-dye complex is more dense than the linear-DNA-dye complex and the two conformational types will band at different positions on a CsCl buoyant density gradient.

During the isolation of one preparation of ^{32}P-labeled DNA, the sample was split prior to the CsCl gradient step in the procedure. One sample was put on the normal CsCl gradient and one was mixed with the ethidium bromide dye and centrifuged to equilibrium in CsCl. The results are shown in Figure 12. According to Radloff et al. (1967), the dye will shift the density of the dye-bound DNA to a range
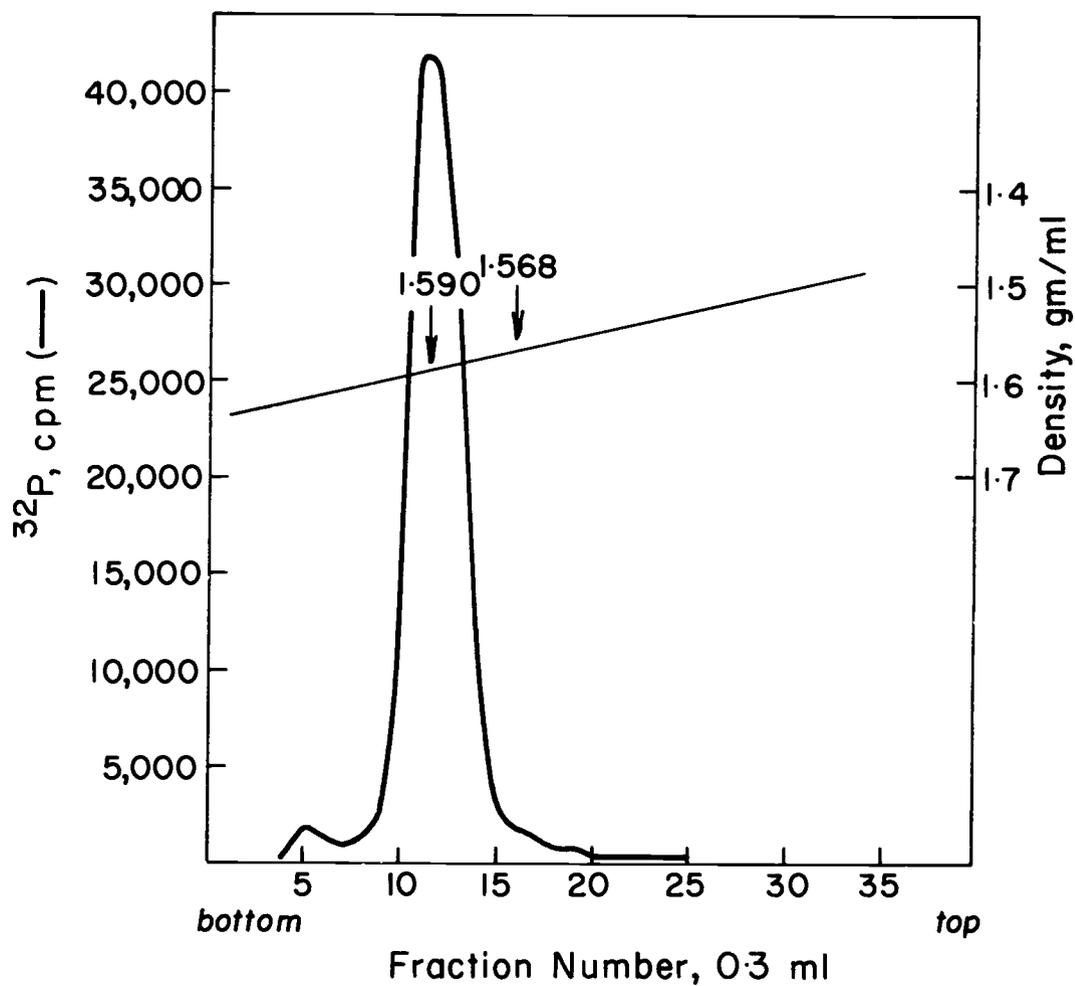
Figure 12. CsCl gradient of NPV ^{32}P-DNA mixed with ethidium bromide dye. The DNA was mixed with 100 μg/ml ethidium bromide and centrifuged in a Spinco fixed-angle 65 rotor at 102, 000 x g and 22° C for 48 hrs.

between 1.57 and 1.62 gm/ml. As shown in Figure 12, the dye-bound viral DNA bands at 1.600 gm/ml. It was not known, however, whether this density represented the location of circular dye-bound or linear dye-bound DNA molecules. Radloff et al. (1967) suggest a difference of approximately 0.04 gm/ml between the two dye-bound forms. To determine the significance of the peak at 1.600 gm/ml, a viral DNA solution, part of which was shown to band at 1.600 gm/ml when mixed with the dye, was sonicated at $0^{\circ}C$ for one minute at a maximum setting using a Biosonik II sonicator, made by Bronwill Scientific, Rochester, New York. The sample was then split, one part put directly on CsCl and the other mixed with the ethidium bromide dye and put on a CsCl gradient.

Figure 13 shows that the sonicated viral DNA still bands at 1.710 gm/ml on CsCl in the absence of the dye. Figure 14 shows the sonicated viral DNA in the presence of the dye. Two peaks are now present, one at 1.600 gm/ml as before, and another at 1.568 gm/ml. The peak at 1.568 gm/ml must represent the portion of the sample converted to the linear dye-bound form by sonication. The 1.600 gm/ml peak is 0.032 gm/ml heavier and represents the portion still in the circular conformation. Figures 13 and 14 are evidence that the material observed in Figure 12 banding at 1.600 gm/ml must have a circular conformation.

The circular conformation could be demonstrated only in the
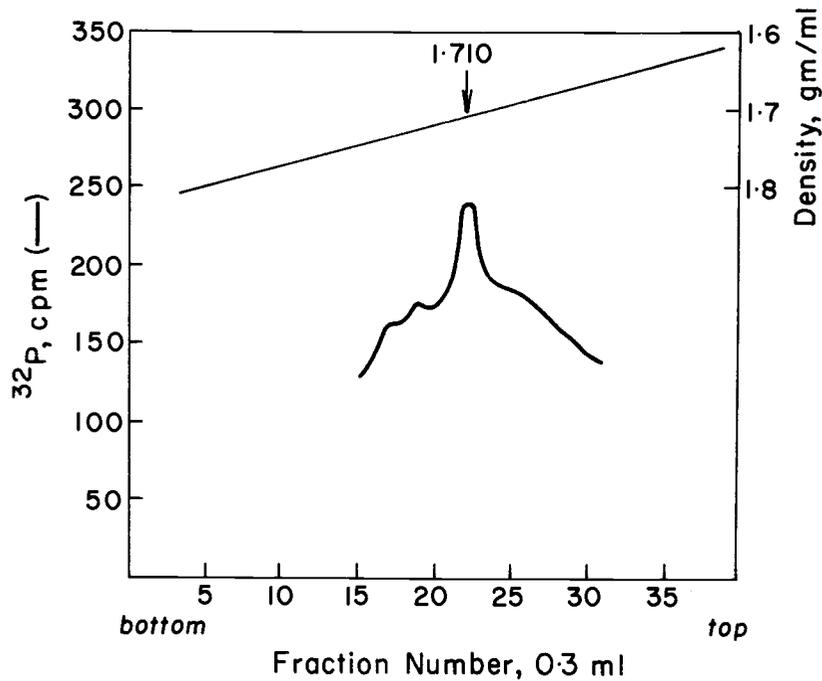
Figure 13. CsCl gradient of sonicated NPV ^{32}P-DNA preparation
was subjected to 1 min. sonication of 0° C at maximum
setting on Biosonik II sonicator. The gradient was run
in the Spinco fixed-angle 65 rotor at 102,000 x g and
22° C for 48 hrs.
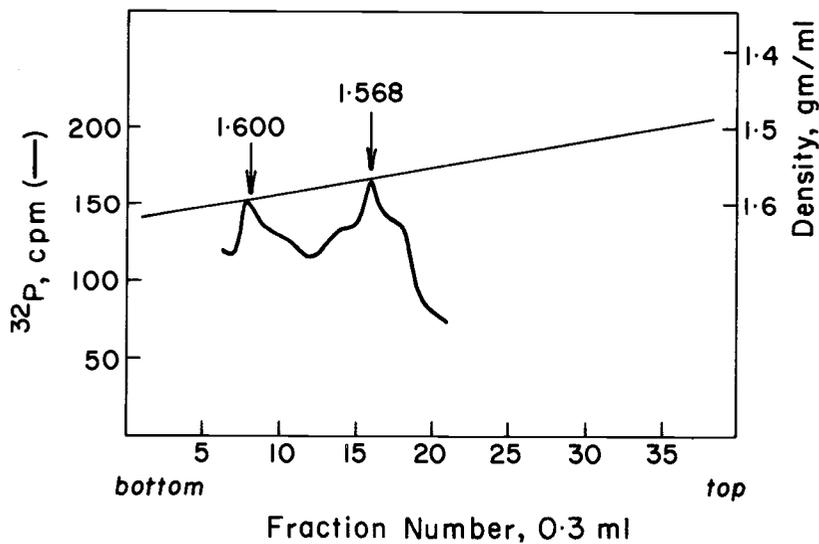
Figure 14. CsCl gradient of sonicated NPV ^{32}P-DNA mixed with
ethidium bromide dye. NPV ^{32}P-DNA preparation
was part of the preparation in Figure 13. This
preparation was split and mixed with 100 μg/ml
ethidium bromide. The gradient was run as in
Figure 13.

relatively crude preparations. Apparently the circularity is extremely unstable and can be converted to the linear form by the manipulations required by additional purification procedures. Figure 15, for example, shows the DNA used in the molecular weight determination to be linear, banding at a density of 1.560 in the presence of dye, after only one additional purification step. The presence of circularity should be investigated further.

Molecular Weight

Sucrose density gradient velocity sedimentation centrifugation showed the viral DNA to have an observed sedimentation coefficient (S_{obs}) of about 50 S (Figure 3). However, it was advisable to determine a more accurate sedimentation coefficient and, hence, molecular weight, using the more precise Spinco Model E analytical ultracentrifuge.

Velocity sedimentation gradients were run on the Model E as described above in Methods. The data obtained in a typical run are presented in Figure 16. From these data one can calculate the observed sedimentation value, S_{obs}.

$$S_{obs} = \frac{2.303 \, (\Delta \log_{10} r)}{\Delta \omega^2 t} \tag{1}$$

where r is the radius from the center of rotation to the sedimenting
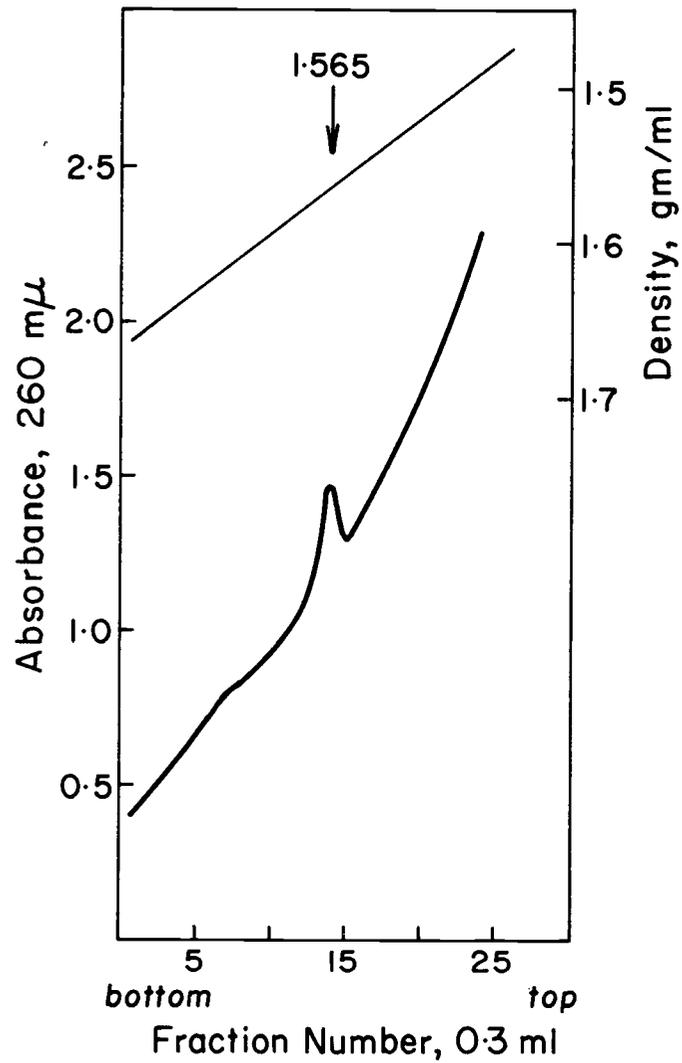
Figure 15. CsCl gradient of NPV DNA used in sedimentation co-
efficient determination mixed with ethidium bromide
dye. NPV DNA was mixed with 100 μg/ml ethidium
bromide and centrifuged in a Spinco fixed-angle 65
rotor at 102,000 x g and 22° C for 48 hrs.
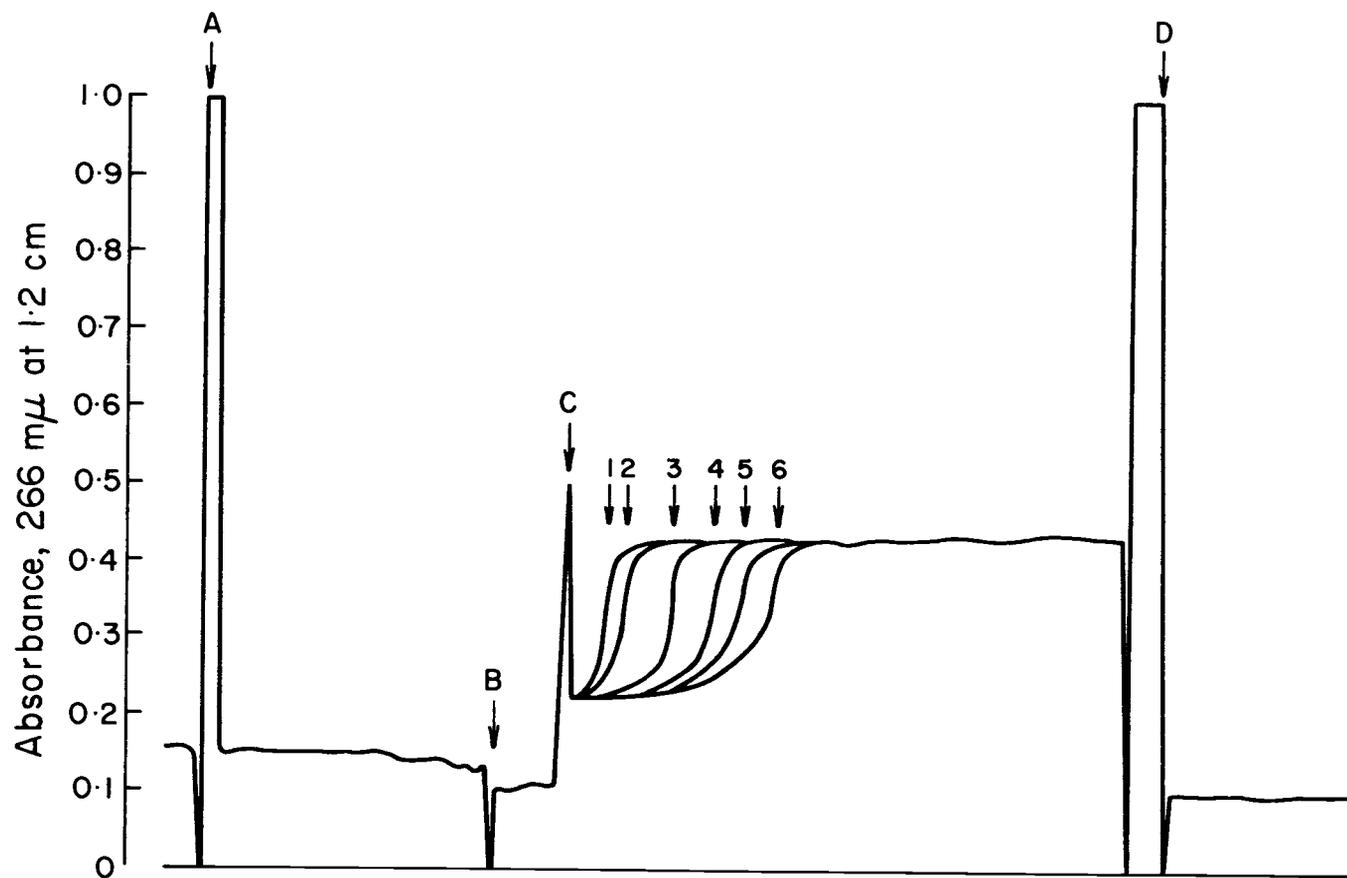
Figure 16. Sedimentation patterns for determination of NPV DNA in Model E analytical ultra-
centrifuge. NPV DNA sample was placed in one side of the double-sector cell and
buffer as a blank in the other side. The gradient was run at 12,000 rpm and about
24° C. A, inside reference hole; B, meniscus of buffer; C, meniscus of DNA
sample solution; D, outside reference hole; 1 - 6, sedimenting boundary of DNA
molecules observed at different times as the DNA boundary moved toward the
bottom of the cell.

boundary at time t. The actual dimensions of the rotor and sample cell are known. By measuring the plotted distance between reference holes A and D (Figure 16) the magnification factor of the X-Y recorder can be determined. The distance from A to the boundary position as measured from the X-Y plot can then be converted to the actual distance that the boundary has moved. The $\omega^2 t$ value, where ω is velocity in radians per second and t is time in seconds, is measured directly as described above. In Figure 16 six scans were made and the $\omega^2 t$ recorded as the scanner passed the boundary position. The set of r and $\omega^2 t$ values obtained were then plotted as shown in Figure 17. The $\log_{10} r$ versus $\omega^2 t$ plot results in a straight line, the slope of which is equal to $\Delta \log_{10} r / \Delta \omega^2 t$ and can be multiplied directly by the constant, 2.303 in equation 1, to obtain the S_{obs}. The slope of the line in Figure 17 was determined by applying the least-squares fit test to the six experimental values. The S_{obs} for the example was 48.65 S.

Having determined an observed value from the data, it must then be converted to the sedimentation value based on sedimentation in water at $20^{\circ}C$, $S_{20,w}$.

$$S_{20,w} = S_{obs} \left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta}{\eta_0}\right)\left(\frac{1 - \bar{v}\, \rho_{20,w}}{1 - \bar{v}\, \rho_t}\right) \qquad (2)$$

where S_{obs} is observed sedimentation value as determined above,

$$S_{obs} = \frac{(2 \cdot 303)(\Delta \log_{10} r)}{(\Delta \omega^2 t)}$$

$$S_{obs} = \frac{(2 \cdot 303)(18 \cdot 6 \times 10^{-3})}{9 \times 10^9} = 47 \cdot 60 \times 10^{13}$$
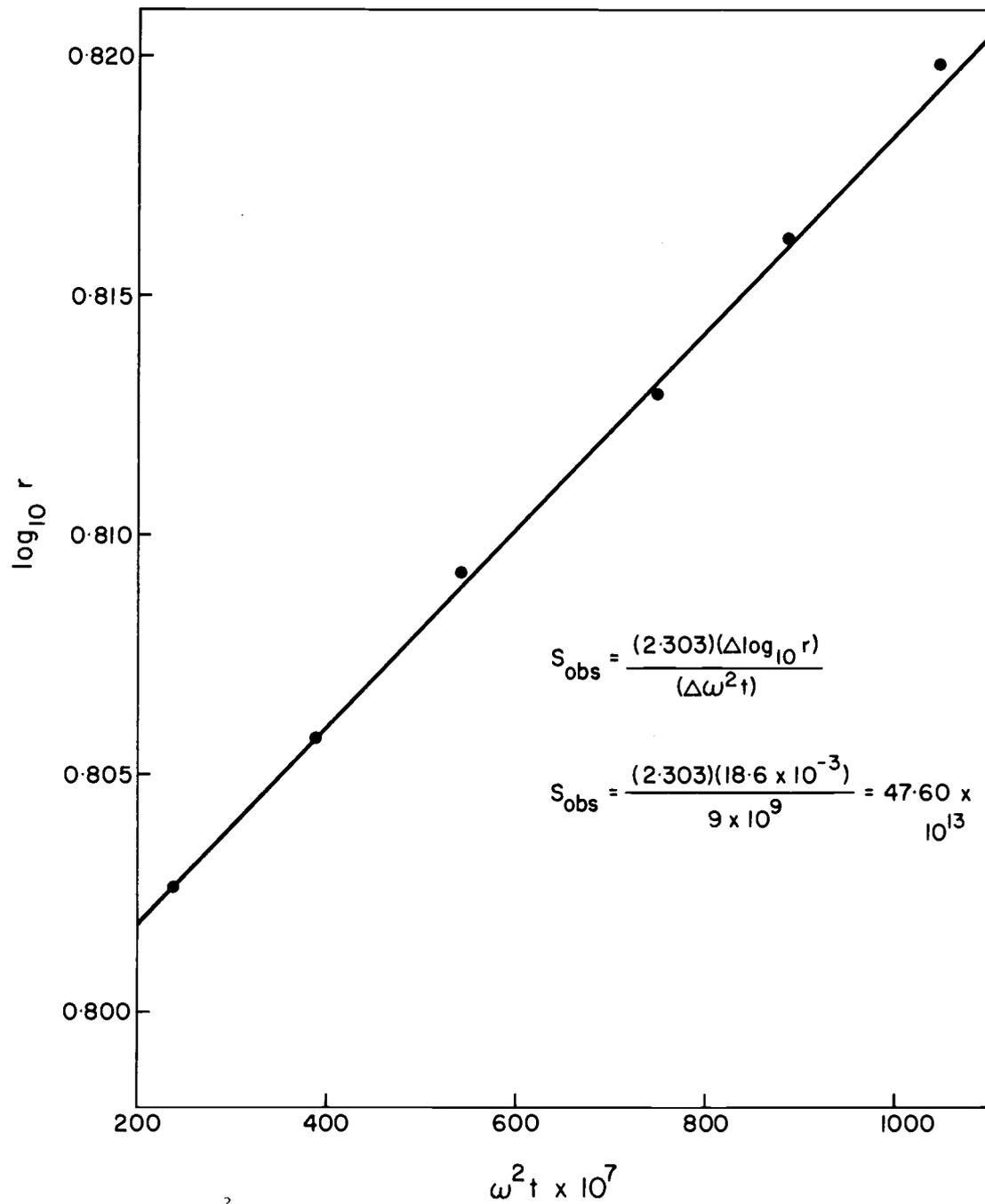
Figure 17. Plot of $\omega^2 t$ versus $\log_{10} r$, for the sedimentation run shown in Figure 14. ω is the velocity in radians per second, t is time in seconds, and r is radius. Points represent the radius from the center of rotation of the An-D rotor to the boundary of the sedimenting DNA at a given $\omega^2 t$. Also shown in the determination of observed sedimentation coefficient (S_{obs}) as calculated from the data plotted in this figure.

η_t is the viscosity of water at temperature t (temperature at which that run was made), η_{20} is the viscosity of water at $20^{\circ}C$, η/η_0 is the viscosity of the solvent relative to that of water, $\rho_{20,w}$ and ρ_t are the densities of water at $20^{\circ}C$ and of the solvent at temperature t, and $\bar{v}$ is the partial specific volume of the DNA molecule. The following values were used:

S_{obs} and η_t/η_{20} values varied with each run

$$\eta/\eta_0 = 1.0267$$

$$\rho_{20,w} = 0.9982 \quad \text{(Svedburg and Pedersen, 1940)}$$

$$\rho_t = 1.00674$$

$$\bar{v} = 0.55 \quad \text{(Mandelkern and Flory, 1952)}$$

Equation 2 then reduces to

$$S_{20,w} = (S_{obs})(\eta_t/\eta_{20})(1.0288) \tag{3}$$

For the run shown in Figure 16, the $S_{20,w}$ value became

$$S_{20,w} = (48.65)(0.9082)(1.0288) = 45.45 \text{ at } t = 24^{\circ}C.$$

The $S_{20,w}$ value was determined on two individual DNA preparations with a total of eight runs made at concentrations ranging from 1.5 to 16.5 g/ml.

The A_{266} of the DNA in each run was measured directly from the X-Y charts (Figure 16, for example). The Model E optical system was calibrated at 266 mμ for a path length in the cell of 1.2 cm. The absorbance was corrected to A_{266} at 1.0 cm and using the factor

of 24.45 A_{266}/mg at 1.0 cm path length, the actual concentration of the viral DNA solution was found.

The $S_{20,w}$ and concentration were then plotted as shown in Figure 18. The values fall on a straight line which, when extrapolated to zero concentration, gives an $S_{20,w}$ value of 57 S. The sedimentation value at the theoretical point of infinite dilution is used because this is the concentration at which no interaction would occur between DNA molecules.

The molecular weight must be calculated from a theoretical equation which has been shown to fit the data tested (Opschoor et al., 1968). Equation 4 is the equation used to determine the molecular weight of linear, double-stranded DNA.

$$0.445 \log M = 1.819 + \log (S_{20,w} - 2.7) \tag{4}$$

$$0.445 \log M = 1.754 + \log (S_{20,w} - 2.9) \tag{5}$$

Equation 5 is used for circular molecules and varies slightly from equation 4.

Figure 15 shows an ethidium bromide dye-CsCl gradient of the DNA used in the determination of S_{obs}. As can be seen the band appears at 1.565 gm/ml which is the position of linear dye-bound DNA. Therefore, equation 4 was used to calculate the molecular weight of the viral DNA which was found to be 9.56×10^7 daltons.
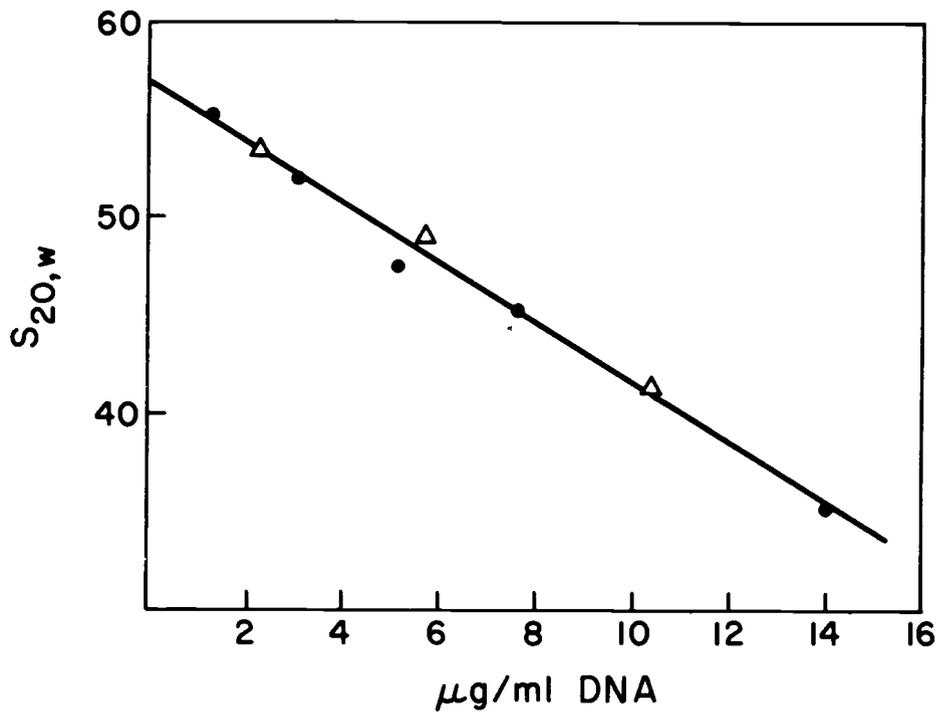
Figure 18. Plot of sedimentation coefficient of NPV DNA, $(S_{20, w})$ versus concentration of NPV-DNA. Solid circles (●) and open triangles (Δ) represent two independent NPV DNA preparations. Each point was obtained by correcting the observed sedimentation coefficient (S_{obs}), as determined in Figure 15 from a set of scans (Figure 14), to $S_{20, w}$ and measuring the $\mu g/ml$ concentration of the DNA in the sample being sedimented.

Ultraviolet Spectrum

Figure 19 shows a typical ultraviolet spectrum of the isolated viral DNA. The maximum absorption is at or near 258 mμ and a minimum is at 235 mμ. Below 235 mμ the absorption increases rapidly. The 260/280 mμ ratio is 1.44. The shape of the spectrum in general is typical of DNA preparations.

Melting Curve

The isolated material was tested for its hyperchromatic proper-ties when subjected to slow heat denaturation. Figure 20 shows the change in absorbance of the material as it was slowly heated from 45°C to 105°C. The technique used to determine the melting curve was first tested using $\underline{E}$. $\underline{coli}$ DNA. The temperature of melting (T_m) for $\underline{E}$. $\underline{coli}$, defined as the temperature at which half the DNA molecules have melted, was found to be 88°C which is in close agree-ment with published data (Marmur and Doty, 1959).

The melting curve for the viral material as determined by this method is characteristic of a DNA melting curve, although it is not ideal. The hyperchromicity is approximately 40% which is in the range for DNA and the temperature of melting (T_m) falls in a range characteristic of DNA. The T_m of 92°C is higher than would be ex-pected by its guanine + cytosine content as determined in the CsCl
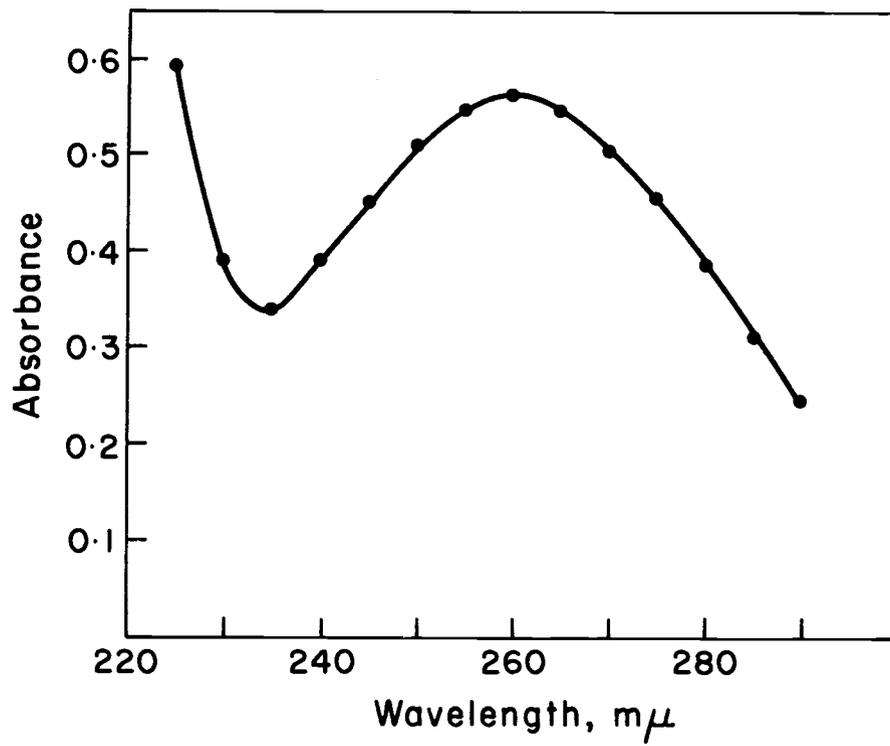
Figure 19. Ultraviolet spectrum of NPV DNA.
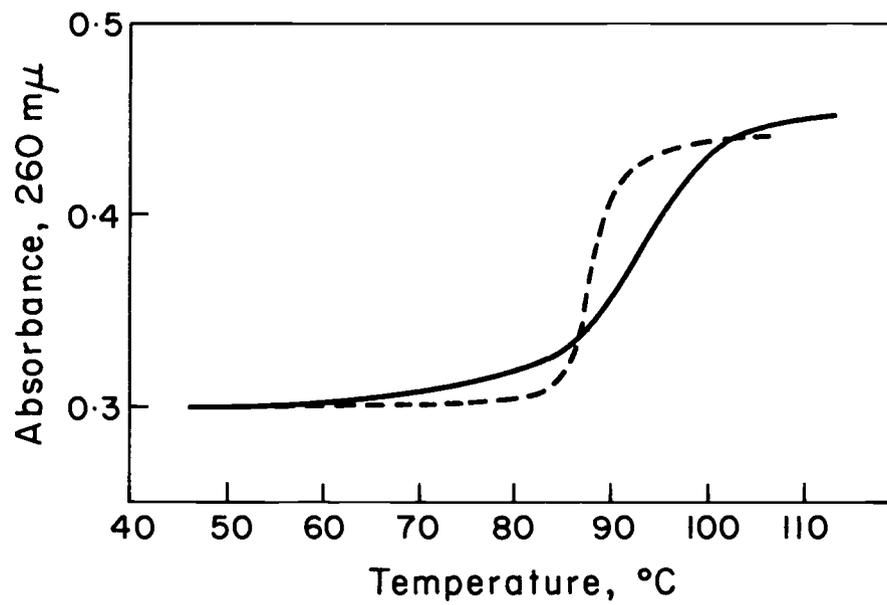
Figure 20. Plot of absorbance of NPV DNA (——) and E. coli (– – –) at 260 mμ as a function of temperature change.

gradient (Sueoka, Marmur and Doty, 1959). This relationship would

estimate the T_m to be near $86^{\circ}C$. The absorbance does not change

as abruptly as might be expected. The effect is to broaden the range

of absorbance increase and shift the curve toward higher tempera-

tures. These deviations from the usual DNA melting curve pattern

could be due to the presence of protein bound to the DNA molecule.

Such protein contamination would make the molecule more stable to

heat, shifting the T_m to a higher value. A rather non-specific,

heterogenous protein-DNA complex would also smooth out the curve

by increasing the range over which the melting or dissociation took

place.

Biological Activity Studies

The 50 S viral DNA was tested for biological activity by its

ability to cause nuclear polyhedrosis when injected into test larvae

as described in Methods. Table 2 shows the results of infectivity

tests on two different viral DNA preparations.

The tests were carried out as described in Methods. The tests

were normally concluded at 13 days post-inoculation. In one test a

positive polyhedrosis was diagnosed at 21 days post-inoculation (Table

2). The total number of insects tested was a sum of the number that

survived and the number that died from nuclear polyhedrosis. Insects

which died of bacterial septicemia or unknown causes were not

considered in this total.

Table 2. Infectivity of Isolated NPV DNA.

Inoculum*	DNA prep. no. **	No. insects tested	No. insects survived	Insects dead from polyhedrosis	
				no.	%
Viral DNA	21	5	2	3	60
	30	7	3	4***	58
Viral DNA treated with DNase	21	4	4	0	0
	30	6	6	0	0
Virus		5	0	5	100
Virus treated with DNase		5	0	5	100
Control (carrier only)		4	4	0	0

*Carrier in each case was 100 $\mu g/ml$ methylated albumin, 0.012 units penicillin and 0.012 μg streptomycin.

**The DNA in all cases was at a concentration of 25 $m\mu g/larva$ or 2.5 x 10^4 DNA molecules/larva.

***One insect died of polyhedrosis at 21 days postinoculation.

Several carrier materials, including streptomycin sulfate, protamine sulfate, E. coli DNA, polyhedra protein, as well as, methylated albumin were tested. The methylated albumin was the only one that gave positive results in infectivity studies. The antibiotics, penicillin and streptomycin, were added to lessen the chance of bacterial contamination in the inoculum. Inocula, from two individual DNA preparations, when injected directly into the hemocoel, caused the insect larvae to die from nuclear polyhedrosis. Inocula treated with deoxyribonuclease (DNase) were inactivated. However, DNase

treatment of virus did not affect its infective activity, as shown in rows 3 and 4, Table 2. The DNase tests on the virus and DNA preparations eliminated the possibility that virus rods contaminated the DNA inoculum of row 1, Table 2, since the DNase was incapable of destroying the infectivity of viral particles (rows 5 and 6, Table 2). It follows that the active infective agent in the DNA preparations was DNA.

The 14.5 S material from sucrose density gradients (Figure 2) was tested for infectivity. Although material from several preparations was tested, no infectivity was found in this lower molecular weigh peak. This was not in agreement with the results reported by Onodera et al. (1965) for the B. mori NPV DNA.

DISCUSSION

Molecular Conformation

The possibility of the H. pseudotsugata NPV DNA being circular was investigated using the intercalating dye, ethidium bromide, which binds differently to circular and to linear molecules, and centrifuging the dye-bound DNA to equilibrium in a CsCl density gradient. Using this technique, circular DNA molecules were found to be present in large amounts (nearly 100%) immediately after lysis of the virus particles. However, after additional purification steps, most of the circularity was lost. This suggests that the DNA molecule was circular in vivo, but is quite fragile and can be easily transformed to the linear form upon physical manipulation in the purified form.

Shvedchikova et al. (1968) have shown, by electronmicrographs, that the genome of the bombyx granulosis virus has a circular structure. Kozlov and Alexeenko (1967) studying the structure of the B. mori NPV with the electron microscope, suggest that the DNA might be circular and exist inside the viral particle as a tightly twisted "super-coil".

The data obtained in the present study are consistent with these reports. The significance of having a circular DNA molecule is not known. It is possible to speculate that the circularity may be

important in the DNA replication process as it is in the $\underline{E}$. $\underline{coli}$

genome (Davern, 1966) and λ bacteriophage (Gellert, 1967). One

might expect only circular molecules to be biologically active. Most

of the DNA molecules in the inocula which were infective for the $\underline{H}$.

pseudotsugata NPV system, however, were linear according to

ethidium bromide-CsCl density gradient analysis. The implications

of this will be discussed in more detail in a following section.

Molecular Weight

The isolated viral DNA genome was shown to have a sedimenta-

tion value $(S_{20,w})$ of 57 S.. This is much greater than the value of

13.1 S reported by Onodera $\underline{et}$ $\underline{al}$. (1965) and of 17 S reported by

Chistyakova (1967) for the silkworm ($\underline{B}$. $\underline{mori}$) NPV DNA. Luria and

Darnell (1967) suggested that the molecular weight should be about

10^8 daltons, based on quantitative measurements of the amount of

DNA per virus particle. Allison and Burke (1962) report a molecu-

lar weight of 76 x 10^6 daltons also based on quantitative analysis of

the DNA in $\underline{B}$. $\underline{mori}$ NPV. Shvedchikova et al. (1968) reported,

based on electronmicroscopic measurements, a molecular weight of

80 x 10^6 daltons also for $\underline{B}$. $\underline{mori}$ NPV. The similarities of the $\underline{H}$.

pseudotsugata virus and the $\underline{B}$. $\underline{mori}$ virus would lead one to expect

nearly identical genome molecular weights. The value of 57 S deter-

mined in this study, which corresponds to 95.6 x 10^6 daltons (from

the linear DNA model), is in agreement with the latter three re-ports.

It is possible, based on the early attempts to isolate the $\underline{H}$. pseudotsugata NPV DNA, to speculate on the error involved in the first two papers mentioned. During the sucrose density gradient work, we observed a rather homogeneous DNA peak in the range of 14.5 S. This peak amounted to only 10% of the material placed on the gradient, but nevertheless might have been interpreted as the viral genome. Upon further investigation of the remaining 90% which pelleted in these early sucrose gradients, the peak at about 50 S was observed.

The viral DNA was analyzed on the Spinco Model E analytical ultracentrifuge. The sedimentation coefficient, S_{obs}, of DNA is very concentration dependent (Tanford, 1961). Therefore, the sedimentation coefficient at infinite dilution is considered the true coefficient since this is the concentration at which there is no inter-action between DNA molecules. The sedimentation coefficient at infinite dilution is found by determining the S_{obs} at several very low DNA concentrations and extrapolating to zero concentration. S_{obs} values at concentrations of viral DNA from 16 μg/ml down to 1.5 μg/ml were determined on the Model E at 12,000 rpm (Figure 18). These points fell on a straight line which extrapolated to an S_{obs} of 57 S.

The sharpness of the sedimenting DNA boundary (Figure 16) indicated that the viral DNA preparation was made up of a homogeneous population of molecules. This is evidence that the DNA preparation is not made up of aggregates. If aggregates were to occur between DNA molecules, one would expect different size aggregates to be present. The preparation, however, was not heterogeneous. If reversible aggregation were occurring as the DNA concentration was decreased, a non-linear curve would result in the S_{obs} versus concentration plot.

Several facts cast suspicion on the centrifugation technique of Onodera et al. (1965). First, these workers centrifuged the viral DNA preparation in the Model E at 59,780 rpm. This speed would be sufficiently high to pellet 50 S material in dilute buffer almost immediately. Thus, the genome may have been sedimented to the bottom of the cell even before the first scans were recorded. Secondly, no mention was made in their publication (Onodera et al., 1965) as to the concentration of the DNA in their preparation and apparently the S_{obs} was determined for only one concentration. Even if the DNA concentration was quite low, not knowing the concentration dependence of the viral DNA molecule during sedimentation would lead to erroneous, low results. And finally, the sedimentation boundary suggests by its broadness, that the viral DNA preparation may be heterogeneous. It seems quite possible,

therefore, that these investigators were analyzing a heterogeneous fraction of the DNA genome.

The molecular weight was calculated using a linear DNA model. Although the molecule can exist as a circular structure, the material used in the sedimentation studies was shown to be linear by ethidium bromide-CsCl density gradient analysis.

Biological Activity

The isolated NPV DNA was shown to be biologically active by application of Koch's postulates. That is, when H. pseudotsugata larvae were injected with a solution of viral DNA isolated from H. pseudotsugata NPV, the insects developed symptoms of the disease, nuclear polyhedrosis, and polyhedra could be identified in the tissue of the test insects. The DNA in the active DNA preparations was shown to be predominantly a linear molecule. However, the ethidium bromide-CsCl density gradient used to analyze the material was not capable of detecting a very low concentration of circular molecules mixed in with the predominantly linear molecules. Therefore, it is impossible to conclude from these data which one of the forms of the molecule, if not both, is the biologically active form.

Several questions are raised by the fact that the NPV DNA can exist as a circular molecule. Does the viral genome become an

episome of the host cell? If so, does the viral genome replicate as

an integrated part of the host DNA or does it do so autonomously?

Does the viral DNA replication involve the existing DNA replicating

mechanisms or are new, viral specific elements (viral specific

DNA polymerase, for example) responsible for replication of the

virus genome?

The viral DNA was injected at a concentration of 25 mμg

DNA/larva. This amount of DNA corresponds to 2.5×10^7 DNA

strands/larva based on a molecular weight of about 10^8 daltons. The

inoculations resulted in about 60% mortality from polyhedrosis. No

attempts were made to determine the LD_{50} of the inocula. The

efficiency of infection, 2.5×10^7 DNA strands/larva, is quite high

when the fact that only a few polyhedra are required to produce

polyhedrosis is considered. However, the efficiency of infection

for pure DNA is always much less than for pure virus. This is no

doubt due at least partially to degradative action of enzymes in the

test insect. However, the low efficiency in this case may also be

due to the problem of circularity. If the circular molecules are the

only infective structural form, they would be present in very low

quantities, at least too low to be detected on the ethidium bromide-

CsCl density gradients.

Onodera et al. (1965) and Chistyakova (1967) have reported

isolating infectious DNA from the NPV of B. mori. Onodera et al.

(1965) reported 63% infection using viral DNA at a concentration of

0.4 µg/pupa, which is 16 times more concentrated than the inocula

used in this study. Onodera's infective viral DNA was reported to

be about 2×10^6 daltons molecular weight as determined by sedi-

mentation studies. However, as discussed earlier, close examina-

tion of his sedimentation patterns shows a large amount of material

already pelleted. He does not use the 2×10^6 dalton fraction for his

infection studies. Apparently, the total viral DNA is used for infec-

tivity after elution from the MAK column, which would include the

pelleted material of his sedimentation studies, as well as the

2×10^6 dalton fraction. This would explain why he reported having

an infective preparation when he was apparently working with only a

fraction of the genome. Both infectivity rates of the DNA from B.

mori NPV and H. pseudotsugata NPV are within the range of reported

infection rates for other deoxyriboviruses (Fenner, 1968).

The lethal infection period for the H. pseudotsugata NPV DNA

infectivity was nearly twice the usual six days at 30°C. No explana-

tion can be given for this phenomenon. It can be speculated, how-

ever, that the DNA concentration was not large enough to cause

death after the initial cells were infected. Death of the insect may

have resulted only after the initially infected cells were completely

destroyed and the virus "burst" dispersed to infect a larger number

of cells, sufficient to cause death.

Infectivity was observed for the H. pseudotsugata NPV DNA only when methylated albumin was used as carrier. Preparations of viral DNA alone and viral DNA with the other carriers mentioned in Results above, were not infective. Onodera et al. (1965) found that methylated albumin was a good carrier for B. mori NPV DNA and preserved infectivity. The protective mechanism of the protein is not known.

DNA Synthesis in Infected Larvae

The synthesis of DNA in the H. pseudotsugata larvae after inoculation with NPV was studied using CsCl density gradients. It was found that the viral DNA and the cellular DNA could be separated on CsCl density gradients. The NPV DNA banded at a density of 1.710 gm/ml and the cellular DNA banded at 1.695 gm/ml. This fact allowed us to observe the changes in the rate of synthesis of both types of DNA after the host larvae were injected with NPV.

Studies on DNA synthesis in NPV infected insects have been carried out by several investigators (Watanabe, 1967; Morris, 1968a, 1968b; Onodera et al., 1968; Shigematsu and Noguchi, 1969).

Watanabe (1967) and Morris (1968a, 1968b) observed an increase in DNA synthesis in infected cells up to a point just prior to polyhedra development. Onodera et al. (1968), using isolated in vitro DNA polymerase activity as an indicator of DNA synthesis,

reported that DNA synthesis began to increase logarithmically at about 24 hours after infection and continued to increase until 60 hours after infection, at which time the activity was 20 times that of uninfected cells. The synthesis then decreased as polyhedra began to appear. Shigematsu and Noguchi (1969) report two periods of high DNA synthesis in infected cells at 3-5 hours and 25 hours. None of these workers were able to satisfactorily differentiate between viral and cellular DNA, however. Apparently there are several periods of increased DNA synthesis in infected cells. A phase pattern of DNA synthesis is apparently present, with the magnitude of the phases increasing with time after infection. The last period, just prior to polyhedra development apparently has the highest rate of DNA synthesis.

In this study the increase in viral DNA can be observed separately from whatever may be occurring with cellular DNA because of the difference in the CsCl buoyant densities. A large increase in viral DNA synthesis was observed somewhere between 72 and 96 hours after infection of larvae with NPV. This data is consistent with the final period of DNA synthesis observed by other workers. The analysis, as performed in this study, was apparently not sufficient to detect the earlier but lower rates of viral DNA synthesis.

Comparison of the NPV to Other Deoxyriboviruses

Table 3 summarizes the properties of the DNA of B. mori and H. pseudotsugata NPV as reported by several investigators and as determined in this study. Table 4 shows the properties of the DNA of some insect polyhedra viruses.

The NPV of the tussock moth (order Lymentriidae) fits near the top of Table 4, nearer the gypsy moth than the silkworm, on the basis of the percent guanine + cytosine. As can be seen from Table 4, however, the NPV's have a broad percentage range of guanine + cytosine composition.

On the basis of the properties of the viral DNA, the H. pseudotsugata NPV does not fit nicely into any of the major groups of deoxyriboviruses. Table 5 shows the major groups of deoxyriboviruses and summarizes the properties of their DNA's.

On the basis of the molecular weight of the DNA the NPV would fit best with the herpesvirus group and the T_2, T_5 and Subtilis phage SP8 bacteriophages. On the basis of circularity and buoyant density of the DNA in CsCl, the NPV would fit well with the bacteriophage. On the basis of the buoyant density of the DNA in CsCl, the melting temperature, and the percent guanine + cytosine, the NPV would fit into the papovavirus group (Bellett, 1967). In addition, the DNA from the papovaviruses, as with the NPV, has been shown to have

Table 3. Properties of B. mori and H. pseudotsugata NPV DNA.

Host insect	Molecular weight x 10^6	Molecular weight determination method	Buoyant density on CsCl, gm/ml	Melting temperature, $^\circ$C	Percent guanine + cytosine	Reference
B. mori	1.8-2.2	sedimentation	---	86-68	40.7-43.2	Onodera et al. (1965).
"	80	electron microscope	---	---	---	Shvedchikova et al. (1968)
"	4.6	sedimentation	---	87-88	43-44	Chistyakova (1967)
"	76	quantiative analysis	---	---	---	Allison & Burke (1962)
H. pseudotsugata	96	sedimentation	1.710	92	47	this study

Table 4. Properties of the DNA of Insect NPV.*

Host species	Host family and order	Percent guanine + cytosine	Melting temperature, $^\circ$C**	Buoyant density on CsCl, gm/ml***
Porthetria dispar, gypsy moth	Lepidoptera, Lymantriidae	58.7	93.0	1.722
Choristoneura fumiferana, spruce budworm	Lepidoptera, Tortriadae	52.2	90.5	1.715
Bombyx mori, silkworm	Lepidoptera, Bombycidae	47.2	86.5	1.7055
Malacosoma americanum, Eastern tent caterpillar	Lepidoptera, Lasiocampidae	42.2	86.3	1.705
Neodiprion sertifer, pine sawfly	Hymenoptera, Tenthredinidae	37.4	84.4	1.700

*Table from Wyatt (1952).

**Melting temperatures predicted from percent guanine + cytosine (Marmur and Doty, 1959).

***Buoyant density on CsCl gradient predicted from percent guanine + cytosine (Sueoka et al., 1959).

Table 5. Physical Properties of DNA's From Several Deoxyriboviruses. *

Groups	Virus	Molecular weight x 10^6	Buoyant density in CsCl, gm/ml	Melting temperature, $^\circ$C	Percent guanine + cytosine
Parvovirus	Minute virus of mice	1.8	1.7222	---	39
Papovavirus	Polyoma	3.4	1.709	89.0	48
	SV 40	3.2	---	---	41
	Rabbit papilloma	5.0	1.711	89.5	49
Adenovirus	type 2	23	1.716	92.5	56
	type 3	23	1.714	90.3	51
	type 12	21	1.708	89.0	48
Herpesvirus	Herpes simplex	68	1.727	97	74
	Equine abortion	92	1.716	---	57
Poxvirus	Cowpox	160	1.694	84	36
	Fowl pox	240	1.694	---	35
Bacteriophage	T_2	120	1.697**	83.5***	35
	T_5	77	1.702	85.0	39
	λ	33	1.712	89.0	49
	P22	28	1.713	89.5	50
	ϕX 174	1.7	1.705	86.0	42
	Subtilis phage SP 8	110	1.706	86.5	43

*Animal virus data from Fenner (1968). Bacteriophage data in part from Luria and Darnell (1967).

**Bacteriophage DNA buoyant densities in CsCl gradients were predicted from the percent guanine + cytosine (Sueoka et al., 1959).

***Bacteriophage DNA melting temperatures were predicted from the percent guanine + cytosine (Marmur and Doty, 1959).

an infective capacity.

The papovaviruses are small, non-enveloped icosahedral DNA viruses and possess a relatively small genome. The NPV is more complex having the crystalline polyhedral inclusion body. Phylogenetically the genome of the papovaviruses and NPV might have been closely related, but the additional complexity of the NPV would necessitate the increase in genome size. On the other hand, it must be remembered that the polyhedrosis and granulosis viruses are unique and they may very well not fit into any of the major deoxyribovirus groups.

It has been shown (Reichmann et al., 1966) that single-stranded DNA of a molecular weight of 4×10^5 daltons carries the genetic code for a 4×10^4 dalton protein. The H. pseudotsugata NPV DNA is 90×10^6 daltons double-stranded and 45×10^6 daltons single-stranded. This means that there is enough genetic information for a 45×10^5 dalton protein or 150 different proteins of 30,000 daltons. This is a relatively large number of proteins for a virus. However, the number seems more reasonable if the possibility of redundancy in the genome is considered. For example, it would be of great advantage to the virus to be able to produce many copies of the polyhedron protein subunits at the same time. It would be possible to imagine 90% of the genome being used for this purpose alone.

The fact that the DNA of the H. pseudotsugata NPV, and in all
probability of the B. mori NPV, is of a high molecular weight dis-
tracts somewhat from its desirability as a model system with which
to study DNA replication. The task of isolating an intact genome
would be much harder than with a smaller molecular weight DNA.
The other advantages of the system, the availability of material and
biological assay potential in particular, still make study of this
virus attractive. Investigation of transcription and translation are
not seriously affected by the size of the genome and these processes
still could be studied quite easily.

The information presented in this paper should help clarify
the disagreement over the molecular weight of the NPV DNA and set
the stage for further study of the relationship between this animal
virus and its host.

SUMMARY

A method was developed to isolate the DNA from the NPV

which infects the tussock moth larvae (Hemerocampa pseudotsugata).

The method is not complex and involves a minimum number of isola-

tion steps, thus providing a procedure which reduces the danger that

the DNA molecule would be physically degraded (Davison, 1966).

The polyhedra were collected as described by Martignoni et al.

(1968). The crystalline polyhedron, which contains the bundles of

virus rods, was dissolved in bicarbonate buffer at pH 11.6. The

virus rods are not destroyed by this treatment. The bundles of

rods, as well as the protein coat of the virus itself, are then dis-

rupted by the detergent, SDS. This treatment releases the viral

DNA molecules. The DNA is separated from the protein on a CsCl

density gradient, the peak collected and dialyzed against a desirable

buffer.

This procedure was used to isolate DNA from the NPV. The

DNA was identified and characterized by a series of studies. These

studies also yielded information about the purity of the isolated

DNA, about the chemical and physical characteristics, and about the

infective properties of the viral DNA molecule.

The viral DNA molecule was degraded by the DNA specific en-

zyme, pancreatic endonuclease (DNase). This study was done using

a sucrose density gradient to observe the size of the molecule after

DNase treatment. The treated molecule was observed to move into

the 3 S region after centrifugation on the sucrose gradient. No other

size of DNA was observed in the gradient which indicated that all of

the ^{32}P-labeled material in the preparation was DNase sensitive.

This is evidence that the isolated material was DNA and that there

were no major contaminants in the preparation other than what might

be bound to or part of the DNA structure.

The isolated viral genome was chromatographed on a MAK

column to compare its chromatographic characteristics to E. coli

DNA. The isolated viral DNA genome eluted at about the same salt

concentration as E. coli DNA and identical to the salt concentration

eluting DNA from the B. mori NPV DNA (Onodera et al., 1965).

This further suggests that the isolated viral genome was DNA.

The isolated viral genome banded at a density of 1.710 gm/ml

in a CsCl density gradient, which is characteristic of DNA. This

density corresponds to a guanine + cytosine content of 47% on a molar

percent basis (Sueoka et al., 1959). The results of CsCl gradient

centrifugation also agreed with the above mentioned DNase treatment

results in that no RNA was shown to be present in the preparation.

However, a second peak was observed at a density slightly less than

that of the main DNA peak. It was determined by ultraviolet absorp-

tion studies to be composed of nucleoprotein material and could be

essentially eliminated from the preparation by pooling the more dense tubes from the DNA peak on the preparative CsCl gradients.

The ultraviolet spectrum of the DNA was typical of nucleic acid. The 260/280 mµ ratio of 1.44 was consistent with the ratio predicted from the guanine + cytosine content (Fredericq et al., 1961).

The melting temperature of the DNA molecule was also typical of double-stranded DNA, but was a little higher than would have been predicted from the guanine + cytosine content of 47% (Marmur and Doty, 1959). The elevated T_m and the shape of the melting profile may indicate the presence of a small amount of protein still bound to the DNA molecule.

The conversion factor of 24.45 absorbance units at 266 mµ/mg DNA, determined by the diphenylamine reaction, also suggested the presence of a small amount of protein in some preparations.

The molecular weight of the viral genome was found to be 97 x 10^6 daltons by calculation from sedimentation data as determined on the analytical ultracentrifuge.

The viral genome, as analyzed in this study, was in the linear form. However, the DNA was demonstrated to have a very fragile circularity immediately after release from the virus particle which was transformed to the linear form upon further purification.

The isolated viral DNA was found to be biologically active. When the DNA was injected into test larvae at a concentration of

25 mμg/larva, 63% of the test insects died of nuclear polyhedrosis.

Based on the physical characteristics of the viral DNA, the NPV does not fit well into any major group of deoxyriboviruses and the insect inclusion viruses should probably be considered a separate group.

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